Experimental Studies

Retinal apoptosis and the effect of tyrosine kinase inhibition in experimental diabetes

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Background: Chronic hyperglycemia in diabetes results in the activation of pathochemical pathways, causes glial, nervous and microvascular dysfunction, and may trigger retinal apoptosis. Apoptosis in diabetic retinopathy (DR) and better understanding opportunities for the control of apoptosis are a promising area of research for the management of DR.

Purpose: To investigate apoptosis and assess caspase-3 expression, Bax and Bcl-xl protein levels in the retina and the effect of imatinib, a tyrosine kinase inhibitor, in experimental diabetes.

Material and Methods: Experimental diabetes was induced in 45 three-month Wistar rats by a single 50 mg/kg intraperitoneal streptozotocin injection (Sigma-Aldrich, Shanghai, China). The rats with sustained hyperglycemia were randomly divided into three groups: group 1 (untreated diabetic controls), group 2 (rats treated with insulin only), and group 3 (rats treated with insulin plus imatinib from Grindeks, Latvia). Retinal caspase-3 expression was determined using immunohistochemical staining with monoclonal antibodies (ThermoFisher Scientific, USA) whereas Bax and Bcl-xl levels, Western immunoblotting.

Results: Sustained hyperglycemia developed after streptozotocin injection and was accompanied by the development of early morphological signs of DR such as degenerative changes in retinal neural cells and their processes, retinal edema and ischemia. Insulin only injection inhibited the development of DR, whereas insulin plus imatinib injections prevented the development of DR. Caspase-3 expression in retinal cells (especially ganglion and Muller cells) increased with an increase in time points, was inhibited by insulin only, and disappeared after insulin plus imatinib injections. Western immunoblotting found that retinal Bax expression increased many times in untreated diabetic rats, whereas insulin only and, especially, insulin plus imatinib injections inhibited an increase in retinal Bax expression. Imatinib induced inhibition of the accumulation of Bax proteins in the retina.

Conclusion: The retina in rats that received insulin plus imatinib, a tyrosine kinase inhibitor, exhibited a more profound inhibition of both the development of DR and apoptosis activation compared to those that received insulin only.

Keywords: diabetic retinopathy, immunohistochemistry, immunoblotting, streptozotocin, imatinib, caspase-3, Bax, Bcl-xl

Introduction

Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus (DM) and a cause of blindness in working-age adults, affecting almost one third of patients with diabetes over 40 years of age [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].

Sustained hyperglycemia causes overproduction of reactive oxygen species by the mitochondria of pancreatic cells and activation of the polyol pathway, advanced glycation end products accumulation, the protein kinase C pathway and the hexosamine pathway, which induces inflammation and stimulates increased production of transcription factors and abnormal gene expression [3]. A cascade of pathological responses causes glial, nervous and microvascular dysfunctions, hypertrophy, proliferation, remodeling and apoptosis [4].

Caspases (cysteine-aspartate-specific proteases) are key molecules in apoptosis [5]. The caspase family mediates cellular processes such as cell death, cell differentiation, immune response, axonal transport and proliferation. During apoptosis, activated caspase-3 cleaves a wide variety of downstream substrates that lead to typical morphological changes in apoptotic cells [6]. The detection of activated caspase-3 is a very reliable way to identify cells destined to die by apoptosis, even before many of the
morphologic characteristics (e.g., DNA fragmentation) are present [7]. Several studies demonstrated that caspase-3 is involved in the apoptotic death of retinal ganglion cells induced by ischemia, excitotoxicity, axotomy, and chronic ocular hypertension. Inhibition of caspase-3 activity reduced apoptotic cell death induced in retinal cells by excitotoxicity and ischemia [8].

The intrinsic apoptotic pathway (but not the extrinsic apoptotic pathway) is triggered through the activation of pro-apoptotic BAX and BAK, and, after the activation of BAX-BAK dependent mitochondrial outer membrane permeabilisation (MOMP), cytochrome c is released from the mitochondria, stimulating the activation of caspase 9 and its downstream effector caspases 3 and 7 to initiate apoptosis [7, 9]. Unlike proapoptotic Bax proteins, BclxL protein is a factor preventing apoptosis [5]. A reduction in BclxL, an antiapoptotic member of the Bcl2 family, was found in diabetic retinas in comparison with control retinas [10].

Therefore, apoptosis in DR and better understanding opportunities for the control of apoptosis are a promising area of research for the management of cellular damage. Inhibition of receptor tyrosine kinases, a class of membrane receptors that phosphorylate the hydroxyl groups of protein tyrosine resi¬dues, seems promising in this regard [11, 12]. Research in this field is aimed at inhibiting malignant cell transformation, since many oncogenes encode tyrosine kinases.

A diabetes study in rats demonstrated that Mitogen-activated Protein Kinase (MAPK)/ Extracellular Signal-regulated Kinase (ERK) pathway, a major tyrosine kinase pathway, was significantly expressed in vascular endothelial cells [13, 14]. The inhibition of this pathway by antioxidants was found to prevent the development of phenotypic symptoms of diabetes including DR [15, 16]. These findings substantiate the possibility that the MAPK pathway may be directly inhibited by conformation-specific kinase inhibitors [17].

Imatinib Mesylate, an effective small-molecule inhibitor of certain tyrosine kinases, significantly inhibits directly the tyrosine kinase activity as well as the cellular processes mediated by this activity [18]. According to a bioinformatical drug repurposing analysis and an comprehensive analysis of the mechanisms of neovascularization in DR by Boneva and colleagues [19], imatinib emerged as a potential immunomodulatory drug option for future treatment of PDR.

The purpose of this study was to investigate apoptosis and assess caspase-3 expression, Bax and Bcl-xl levels in the retina and the effect of imatinib, a tyrosine kinase inhibitor, in experimental diabetes.

**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 (meeting minutes no. 165 of 05.12.2022). Animals were maintained in vivarium conditions and fed conventionally.

Forty-five Wistar male rats (age, 3 months; weight, 140-160 g) were used in experiments. Experimental diabetes was induced by a single 50 mg/kg intraperitoneal streptozotocin injection (Sigma-Aldrich, Shanghai, China). 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. Five control rats were injected with citrate buffer only. Three days after injection, the blood glucose level in rats that received streptozotocin was 17 mMol/l or higher. No control rat showed a blood glucose level above 5.7 mMol/l throughout the observation period. Four (8.9%) streptozotocin-treated animals showing a blood glucose level ≤ 6.7 mMol/l were withdrawn from the experiment. The mortality rate over a 30-day observation period was 13.3%.

Urine glucose and ketone levels were assessed using CITOLAB test strips (Farmasco LLC, Vyshgorod, Ukraine).

On day 7, the rats with sustained hyperglycemia were randomly divided in three groups. Group 1 (controls) was composed of 10 non-treated hyperglycemic animals. Of these, 4 rats were euthanized with sodium thiopental (75 µg/kg intraperitoneally) and decapitated on day 7; 3 rats, on day 14, and 3 rats, on day 28. In addition, the control group included 5 animals which were euthanized on the day of streptozotocin injection (day 0). Group 2 was composed of 7 animals which were treated intraperitoneally with short-acting 30 U insulin (Actrapid HM Penfill, Novo Nordisk A/S, Bagsvaerd, Denmark) every other day. Group 3 was composed of 8 animals which were treated not only with short-acting intraperitoneal 30 U insulin every other day, but also with Imatinib (Imatinib Grindeks, 100 mg, Grindeks, Riga, Latvia) per os at a dose of 20 µg/kg bwt. Rats in groups 2 and 3 were euthanized on day 28.

After sodium thiopental injection and decapitation, both eyes were enucleated and fixed in 10% buffered neutral formalin (DIAPATH S.p.A., Martinengo, Italy), 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 visualized by light microscopy.

Immunohistochemical examination (IHC) was performed using monoclonal antibodies against caspase-3 (Caspase 3 Monoclonal Antibody, clone74T2; ThermoFisher Scientific, USA). The Master Polymer Plus Detection ( Peroxidase, DAB chromogen) detection system (Master Diagnostica, Granada, Spain) was utilized. Sections were additionally stained with hematoxylin. Light microscopes (ZEISS, Köln, Germany) and Axio Imager. A2 processing system (ZEISS) was utilized for microscopic studies and...
digital image archiving. A semiquantitative score system was used in scoring IHC. IHC immunoreactivity was graded as follows: 0 indicates no staining; 1 (+) indicates mild staining; 2 (++) indicates moderate staining; and 3 (+++) indicates strong staining [20].

Expression levels of Bax and Bcl-xl proteins in retinal cells lysates were determined by Western immunoblotting with specific antibodies. Tissue samples were kept in liquid nitrogen, ground and homogenized. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel in a Bio-Rad vertical electrophoresis system and transferred onto nitrocellulose membranes by electroblotting. Membranes were probed with anti-Bax (Sigma Aldrich, USA, no. B3428, rabbit, 1:2,000 diluted) and anti-Bcl-xl (Sigma Aldrich, USA, SAB4502623, rabbit, 1:1,000 diluted) monoclonal antibodies. Anti-β-actin antibodies (Catalog No, MA5-15,739 mouse, 1:3,000, Invitrogen, USA) were used for detection of β-actin. After primary incubation, membranes were washed prior to incubation with horseradish peroxidase-conjugated specific secondary antibodies (goat anti-rabbit or anti-mouse IgG, Invitrogen, USA, cat. nos. G-21234 and 31430, respectively, 1:8,000 diluted). Blots were scanned, and quantitation of the signals was performed by densitometry using scanning analysis software (TotalLab TL120; Nonlinear Inc, Durham NC, USA). The results of Western immunoblot analysis of VEGF and HIF-1α were calculated as relative optical density normalized to actin controls for each particular polypeptide (VEGF/actin and HIF-1α/actin).

Statistical analyses were conducted using Statistica 10.0 (StatSoft, Tulsa, OK, USA) software. For descriptive statistics, data are presented as mean and standard deviation (SD). Analysis of variance (ANOVA) was used for comparison of means across analysis groups. The level of significance p ≤ 0.05 was assumed.

Results

Sustained hyperglycemia developed in rats after streptozotocin injection, and, on day 28, the mean plus or minus SD blood glucose level was 27.6 ± 1.0 mMol/l in rats in group 1 (controls). All time point measurements in control rats demonstrated high blood glucose level values (≥ 17 mMol/l). In addition, sustained hyperglycemia was accompanied by increased daily fluid consumption (310 ± 16 ml/kg compared to a norm of 111 ± 8 ml/kg), increased daily diuresis (274 ± 14 ml/kg compared to a norm of 84 ± 5 ml/kg), glycosuria (≥ 14 mMol/l) and ketonuria (≥ 3.9 mMol/l). Moreover, the mean plus or minus SD body weight decreased from 145.1 ± 2.5 g at baseline to 115.3 ± 1.6 g on day 28 (p < 0.05). Six animals (13.3%) in this group died over the observation period. The aforementioned data indicate that our rat model of sustained hyperglycemia with ketosis was adequate.

In groups 2 and 3, correction of hyperglycemia resulted in decreased blood glucose level values on day 28 (16.8 ± 0.7 mMol/l and 11.1 ± 0.9 mMol/l, respectively) compared to group 1, and this difference was significant (p < 0.05). Correspondingly, other phenotypic manifestations of diabetes were less severe in groups 2 and 3 than in group 1.

Morphological examination found initial signs of DR in group 1 (controls) on day 28 (Fig. 1A). 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), vascular dilation with signs of microthrombosis, and areas of diffused ischemic damage with vacuolated cytoplasm of neural cells, intracellular edema and pyknotic nuclei. In addition, retinal ganglion cells were decreased in number and showing either vacuolation or hyperchromic neurons with pyknotosis. These changes indicated degenerative alterations in neuronal cells in the presence of inadequate microcirculation and abnormal metabolism.

In group 2, diabetic retinal changes were less severe (Fig. 1B) than in the control group, although the nuclear layers exhibited a low cell density and were edematous, with ischemic regions, and with some cells showing hypochromia and cytoplasmic vacuolation. In group 3, retinal damage was minimal (Fig. 1C). There was mild edema of all retinal layers as well as hyperemic vascular bed.

Therefore, over a 28-day period of sustained hyperglycemia, initial DR developed with microcirculatory abnormalities.
abnormalities like retinal edema and ischemia, which was accompanied by the degeneration of retinal neural cells. A combination of insulin with imatinib resulted in improved prevention of DR and protection of retinal neural cells from degeneration compared to insulin only.

IHC (caspase-3 staining) was used to evaluate the development of retinal apoptosis in controls over the observation period (Fig. 2). At baseline (Fig. 2A), mild expression of caspase-3 was seen in radial processes of neural retinal cells (the cells morphologically similar to Muller cells) and small cells (possibly, astrocytes) in the retinal ganglion cells layer, with a staining score of ≤ 1 [20].

On day 7, caspase-3 expression significantly increased (Fig. 2B). Some cells exhibited immune positive staining along an entire fiber, with long radial cell processes crossing the retina from the outer limiting membrane to the inner limiting membrane, and with this cell structure corresponding to the typical structure for Muller cells. The number of small cells exhibiting immune positive staining in the retinal ganglion layer was increased. In addition, ganglion cells had immune positive inclusions along their peripheral cytoplasm in the form of pits, and horizontal axons of these cells also exhibited immune positive staining. These retinal cells had a staining score of 2 to 3. At this time point, inner segment photoreceptors showed mild staining.

On day 14 (Fig. 2C), the inner plexiform layer exhibited immune positive staining, and the inner photoreceptor layer, more intensive immune positive staining, in addition to the aforementioned phenomena. Moreover, moderate immune positive staining was seen in numerous outer plexiform layer cells (likely, the horizontal cells of the outer plexiform layer). At the border of the inner nuclear layer and inner plexiform layer, there were solitary dendritic cells with their dendrites extending into the inner nuclear layer, with the structure of these cells corresponding to that of amacrine cells. These retinal cells had a staining score of 2 to 3.

On day 28, the above features were present in the presence of manifestations of developed DR (Fig. 2D). Round cells in the walls of the vessels of retinal microvasculature (endothelial cells) exhibited clear positive staining. Interestingly, the neurons of the outer and inner nuclear layers did not demonstrate positive staining for caspase 3.

On day 28, in group 2, immune positive staining in the retina was less intense than seen in group 1 (Fig. 2E). It was preserved in Muller cells, within the retinal ganglion layer, and in solitary small dendritic cells of the retinal nuclear layers. At this time point, these retinal cells had a staining score of 1 to 2.

In group 3, practically no immune positive staining was seen in the retina (Fig. 2E). There was a trace positive staining in the ganglion layer in the presence of mild morphological symptoms of DR. Solitary ganglion cells, however, showed apoptosis (it is likely that these were the cells that had been apototic before the rats of this group received insulin and imatinib injections).

To assess the apoptosis intensity in ganglion cells, we calculated the numbers of these cells depending on the intensity of staining among the groups of animals (Fig. 3).
In animals that received insulin only, there was a significant increase in the percentage of non-stained and slightly stained cells (having a staining score of 0 to 1), with a decrease in the percentage of more intensely stained cells (having a staining score of 2 to 3). In animals that received insulin plus imatinib, an absolute majority of cells (93%) had no specific staining, whereas the rest displayed a highly intense staining. We believe that, in the latter cells, apoptosis developed before the rats received the injections, and, therefore, imatinib prevented but did not stop apoptosis in ganglion cells in DR. These findings confirmed the inhibiting effect of insulin and the preventive effect of imatinib, for apoptosis activation in retinal ganglion cells in experimental diabetes.

Therefore, immunohistochemical studies showed a certain increase in the expression of caspase-3, a proapoptotic protein, in the retina. Apoptosis was initially triggered in Muller cells, and, in some time, spread to almost all types of retinal cells, including ganglion cells, glial cells and vascular endothelial cells. Although neurons of the outer nuclear layer (rod and cone neurons) and inner nuclear layer (bipolar cells) exhibited morphological signs of injury, they showed no immune positive staining at any time point.

Proapoptotic Bax proteins have a constitutive expression of monomers which, in the presence of accumulated pericellular damage, become incorporated into the mitochondrial membrane with formation of dimmers [21]. This promotes the release of cytochrome c from the mitochondria to the cytoplasm and triggering the mitochondrial apoptotic pathway. We determined the level of Bax expression in the rat retina (Fig. 4).

In group 1 (controls), retinal Bax expression increased twenty-eight-fold from baseline to day 28 (Fig. 4B). The expression of Bax dimer was maximal on day 7, which could indicate a significant accumulation of products of pathological metabolism as early as this time point. It is noteworthy that at baseline, the retinal expression of Bax dimer as assessed by the optical density ratio for Bax / actin dimer was almost negligible (0.001 unit). On day 7, in group 1, the retinal expression of Bax dimer increased to 3.38 ± 0.02 units on day 7, and 1.30 ± 0.01 units on day 28, i.e., 3,380-fold and 1,300-fold, respectively, compared to baseline.

These results allow us to state that, under conditions of sustained hyperglycemia, the expression of Bax monomer significantly increased, and the conditions developed for monomer polymerization and the formation of Bax dimer. This could explain a significant activation of apoptosis in group 1 animals with sustained and marked hyperglycemia, which was demonstrated immunohistochemically through the activation of effector caspase-3. Insulin only and, especially, insulin plus imatinib injections resulted in a decreased total Bax monomer expression and a decreased Bax dimer expression (Fig. 4B), which was accompanied by apoptosis inhibition.

Discussion

Bcl-xL protein has antiapoptotic properties due to the induction of the dissociation of Bax oligomers [21]. In groups 1 and 2, antiapoptotic protein Bcl-xL expression in the retina was found by our immunoblotting study neither at baseline nor at day 28 (Fig. 4A). In group 3, however, there was an insignificant accumulation of this protein in the retina on day 28, which suggested activation of antiapoptotic mechanisms after imatinib injection, and explained the practical absence of apoptosis in the retina of these animals. Therefore, we may hypothesize that the induction of antiapoptotic Bcl-xL protein expression in the retina is another protective mechanism of the effect of tyrosine kinase inhibition.

There have been reports on increased levels of pro-inflammatory markers, proapoptotic caspase-3, Fas and Bax in the ganglia and retina in humans and experimental rats with diabetes. In addition, at present, it is well known that expressions of proteins such as Bax, Fas, and Fas/ Fasl, and active caspases-3, -8 and -9 are upregulated in the diabetic retina of humans and rodents, which is in agreement with our findings [8, 22].

Our finding of high susceptibility of the inner retinal layers to apoptosis may be explained by their vulnerability to hyperglycemia [23]. The inner retinal cells (ganglion and amacrine cells) exhibited more severe degeneration compared to the neuronal cells located closer to the outer retina [24]. Retinal ganglion cells and amacrine cells are the first neurons in which diabetes-induced apoptosis is detected, but photoreceptors also have an increased apoptotic rate. The structural consequence of this apoptotic death is a reduced thickness of inner retinal layers and the nerve fiber layer, which can be detected by optical coherence tomography [25]. This is in agreement with our findings of the activation of caspase-3 in the inner photoreceptor layer. The pathological pathways of DR include metabolic abnormalities, oxidative stress, endoplasmic reticulum stress, and raised growth factor levels. In addition, apoptosis has an important role in the pathology and is a factor which is accompanied by mild retinal

![Fig. 3. Overall caspase-3 staining score distribution among ganglion cells of animal groups 1, 2 and 3 on day 28. Note: *, P < 0.05 compared to group 1](Image 305x116 to 541x256)
inflammation resulting in increased vascular permeability, neovascularization and neuronal injury [26]. In diabetes, retinal neurons and vascular cells die by apoptosis, a final common pathway for retinal neurodegeneration. Whether the triggers are hyperglycemia, glutamate excitotoxicity or neurotrophin deficiency, apoptosis links these potential mechanisms of neuronal injury and the ultimate death of cells. Therefore, inhibiting apoptosis could be a potential means of preventing neurodegeneration in DR [27]. Thus, calpain inhibitor injection prevented ganglion cell death in diabetic mice [28], whereas constant intake of a lutein-supplemented diet prevented reactive oxygen species generation and suppressed the apoptosis induced in the diabetic mouse retina [29]. The inhibition of the intracellular signal pathways associated with protein tyrosine kinases also inhibits the development of DR. Thus, Raf-1 kinase inhibitory protein prevented diabetic retinal neurodegeneration and resulted in the enhancement in the protein expression of caspase-3 in a rat model of diabetes presumably by inhibiting p38-MAPK pathway [30].

Therefore, our finding of a gradual increase in caspase-3 expression and Bax protein accumulation in retinal cells of rats with streptozotocin-induced diabetes is in agreement with the literature. In addition, our finding of insulin-induced apoptosis inhibition and reduced increase in retinal Bax level was expected and could be explained by reduced glycaemia. However, our finding of an almost complete inhibition of retinal caspase-3 expression and reduction in retinal Bax level in diabetic rats treated with insulin plus imatinib was rather unexpected. To the best of our knowledge, there are no reports on such studies. Moreover, the induction of antiapoptotic Bcl-xL protein expression in the retina in diabetic rats treated with insulin plus imatinib was another unexpected finding, and supposes another effect of tyrosine kinase inhibition with imatinib.

**Conclusion**

Sustained hyperglycemia over 7-28 days resulted in the development of progressive phenotypic symptoms of DR, activation of caspase-3 in retinal cells (especially, internal retinal layer cells) and a significant accumulation of proapoptotic Bax proteins in the retina. A combination of insulin with imatinib resulted in improved inhibition of the development of DR and prevention of an increase in accumulation of Bax proteins in the retina. The tyrosine kinase inhibition with imatinib induced accumulation of antiapoptotic Bcl-xL protein in the retina. Findings of this study create the prerequisites for applying tyrosine kinase inhibitors (particularly, imatinib) for the prevention of DR development through the inhibition of excessive apoptosis of retinal neural cells.

**References**


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