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Article

Pelargonidin protects retinal ganglion cells in streptozotocin-induced diabetic rat model by reducing intraocular pressure, suppressing TGF-β and activating JAK2/STAT3 signaling pathway

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ABSTRACT

Diabetic retinopathy (DR) is one of the primary causes of vision impairment, affecting individuals with diabetes, and is marked by the neurodegeneration of the retina along with increased intraocular pressure (IOP). This study sought to determine the effects of pelargonidin on extracellular matrix (ECM) modulation and the inhibition of transforming growth factor-β (TGF-β) and Janus Kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3) pathway in retinal ganglion cells of streptozotocin-induced diabetic rats. Male Sprague-Dawley rats (180–200g) were rendered diabetic by intraperitoneal administration of streptozotocin (STZ). The rats were divided into 5 groups: control, diabetic model (STZ), STZ + low dose pelargonidin (12.5 mg kg^{-1} per day), STZ + medium dose pelargonidin (25 mg kg^{-1} per day) and STZ + high dose pelargonidin (50 mg kg^{-1} per day). IOP was monitored using tonometer. Whole-mount retinal immunofluorescence staining using RNA-binding protein

with multiple splicing (RBPMS) was performed to assess retinal ganglion cell (RGC) density. Protein expression levels of apoptotic markers, ECM components, and the transforming growth factor-β (TGF-β) and Janus Kinase 2/Signal Transducer and Activator of Transcription 3 pathway (JAK2/STAT3) signaling pathways were evaluated by western blotting. Pelargonidin treatment dose-dependently reduced the elevated IOP. Importantly, immunofluorescence analysis revealed a marked dose-dependent preservation of retinal ganglion cell (RGC) density: STZ-induced RGC loss was significantly reversed by pelargonidin, with the highest dose restoring RGC density to near-control or higher levels in both the central and peripheral retina. This was achieved via modulation of apoptosis-related proteins through the upregulation of Bcl-xL, Bcl-2, and downregulation of Bad, Bax and cleaved caspase-3. Furthermore, pelargonidin modulated ECM remodeling protein expression in the RGC layer. In particular, TGF-β2/Smad2/3 signaling was downregulated and the JAK2/STAT3 pathway was upregulated. By reducing IOP, preserving RGC density, modulating ECM deposition, inhibiting TGF-β and upregulating the JAK2/STAT3 pathway, pelargonidin exerts protective effects against diabetic retinal injury. The results of this study further confirm the pharmacological potential of pelargonidin as a therapeutic agent for diabetic retinopathy.

Keywords: diabetic retinopathy, pelargonidin, intraocular pressure, retinal ganglion cells, TGF-β

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INTRODUCTION

Worldwide, diabetic retinopathy (DR) is the foremost reason for vision impairment amongst adults of working age, and it poses growing challenges to public health systems (1). Effective treatment and prevention measures are urgently needed to address the risk of diabetes-related sight-threatening visual impairment which, according to estimates, may grow twofold within the next two and a half decades (2). Traditionally, DR has been viewed as a microvascular disease with a hallmark injury to the retinal microvascular system (3). Known clinical indicators such as microaneurysms, hemorrhages, and new blood vessels have long been associated with the pathogenesis of visual disability in diabetics (4).

Increasing data, however, suggest that DR also involves some degree of neurodegeneration as well (5). It is both interesting and concerning that the loss of RGCs is also a prominent feature of DR – an abnormal condition commonly associated with high IOP (6, 7). Furthermore, an association has been established between diabetes and elevated IOP, indicating that there may be common pathological pathways between DR and glaucoma (8). This investigation seeks to determine whether diabetes has an effect on IOP by assessing diabetic rats in comparison to age-matched controls, with the intention of clarifying how metabolic illness interconnects with damage caused by pressure on the retina.

Among the inventory of experimental models, the STZ-induced diabetic rats are arguably the most frequently used for the study of DR (9). Its early pathological stage can be observed alongside human-like DR features, especially in terms of vascular and neuronal complications (10). While rats are well documented in DR research, there is a gap where rats fulfill various experimental roles (11). Evidence provided by multiple studies supports that diabetic rats indeed suffer from the loss of retinal neurons, making them appropriate subjects for research aimed at neurodegeneration brought about by diabetes (5). Regardless, outcomes from diabetic murine models do not appear to be consistent. Some studies report significant RGC loss, whereas others show no marked neuronal degeneration. Such discrepancies require further research to determine the degree and timing of neurodegeneration in diabetic rats.

Newer studies have noted that the TGF- β signaling pathway is vital for maintaining retinal vasculature and represents a prospective site of intervention for DR (12). TGF-β is crucial for the regulation of vascular homeostasis, including endothelial barrier maintenance and pericyte maturation. Pericyte communication is necessary for vascular integrity and many of these interactions are facilitated by members of the TGF-β family (13). A breach in this signaling pathway gives rise to retinal pathology mimicking DR. For instance, mice having a conditional TGF-β knockout in retinal tissues form microaneurysms, hemorrhages and cotton wool spots which are characteristic of diabetic patients (14). The role that TGF-β plays in the pathogenesis of DR is dualistic. Initially, it seems to offer protective effects by stabilizing the vascular system. Yet, chronic or excessive TGF-β activation or dysregulation can lead to pathological features, such as excessive endothelial proliferation, pericyte loss, and fibrosis. Thus, TGF-β dysregulation is increasingly regarded as central to microvascular dysfunctions that accompany diabetes, and as a potential therapeutic target (15). Alongside TGF-β, the Janus kinase/signal transducer activator of transcription 3 (JAK/STAT3) pathway also regulates retinal neuroprotection (16). This pathway is known for its stress response activity, promoting

neuronal survival during heightened IOP. JAK/STAT3 activation has been associated with increased survival of RGCs in optic nerve injury (17).

Also, there is increasing evidence regarding the protective effects of pelargonidin, an anthocyanidin found in berries and other pigmented fruits (18). These compounds serve as potent antioxidants and have been shown to alleviate oxidative damage, ischemic brain injury, and age-associated decline in cognitive functions (19). In this light, we propose that pelargonidin, a bioactive agent with anti-inflammatory and neuroprotective activity, may have the ability to modulate TGF-β2 and JAK/STAT3 signaling to control IOP elevation.

EXPERIMENTAL

Chemicals and antibodies

Santa Cruz Biotechnology (Texas, USA) provided primary antibodies against cleaved caspase-3, TGF-β, TGF-β2, JAK2, STAT3, p-JAK2, p-STAT3, Bcl-2, Bcl-xL, Bad, β-actin, along with HRP-conjugated IgG secondary antibodies. Cell Signaling Technology (Danvers, MA, USA) provided antibodies for Smad2, p-Smad2, Smad3, p-Smad3, as well as elastin, fibronectin, collagen types I and IV, laminin, MMP-1, MMP-9, and TIMP1. Pelargonidin (purity ≥ 90 % by HPLC) and other reagents and buffer constituents used in expression and biochemical analyses were purchased from Sigma-Aldrich (USA). All other chemicals not mentioned here were of analytical grade.

Experimental animals

The institutional animal facility provided a total of fifty male Sprague Dawley rats (180–200 g). The methodological procedures in this study concerning animal care were approved by the Institutional Animal Ethics Committee of Xi 'an Trade Union Hospital, Xi 'an, and was carried out in compliance with national guidelines for the care and use of laboratory animals, in accordance with international standards. The rats were housed in sterile polypropylene cages (three animals per cage) under a controlled environment with a 12-hour light/dark cycle, and the temperature was maintained at 22–23 °C, while the relative humidity was set at 55–60 %. The animals were allowed to acclimatize to the laboratory environment for five days prior to the experiment.

Study design

The rats were randomly divided into five experimental groups (n = 10 per group). For two consecutive days, intraperitoneal injections of 100 mg kg^{-1} streptozotocin (STZ, Sigma, USA)

were administered. Prior to injection, the STZ was dissolved in a sodium citrate buffer (0.1 mol L^{-1} pH 4.5). Control group rats received an equal amount of sodium citrate buffer, however, without the STZ. Intraocular pressure (IOP) was assessed immediately after induction.

Group I (Control) rats received only sodium citrate buffer without STZ for the duration of the five-week experimental period. Group II (Model) rats received STZ injections to induce diabetes and were maintained without further treatment for five weeks. Group III rats received STZ injections followed by oral administration of pelargonidin at a dose of 12.5 mg kg^{-1} body weight on alternate days for five weeks. Group IV rats received STZ injections followed by oral administration of pelargonidin at a dose of 25 mg kg^{-1} body weight on alternate days for five weeks. Group V rats received STZ injections followed by oral administration of pelargonidin at a dose of 50 mg kg^{-1} body weight on alternate days for five weeks.

The doses of pelargonidin (12.5, 25, and 50 mg kg^{-1}) were selected based on previous pharmacological studies demonstrating the safety and biological activity of pelargonidin within this dose range in rodent models (20, 21). These doses were chosen to evaluate a dose-dependent response while remaining within a range reported to exert antioxidant, anti-inflammatory, and neuroprotective effects without producing toxicity.

Measurement of intraocular pressure (IOP)

Procedures for measurement of IOP were performed while the animals were maintained under inhalation anesthesia with isoflurane. The IOP was assessed weekly for five weeks using a calibrated TonoLab rebound tonometer (Colonial Medical Supply Co., USA) according to standard methods (22). All measurements were done at the same time each day to eliminate the effects of circadian rhythm changes.

Although isoflurane anesthesia has been reported to influence ocular physiological parameters, including transient changes in IOP, the same anesthetic protocol was applied consistently across all experimental groups to minimize variability in the measurements.

Immunofluorescence staining of RGCs in flat-mount retinas

Rats were sacrificed at the end of the treatment period by cervical dislocation, and the eyeballs were immediately enucleated. Six retinas per experimental group were immunostained. Eye globes were fixed in 4 % paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4) at room temperature for 2 h, and washed with PBS and kept in 30 % sucrose at 4 ^{o}C. The retinas were then isolated, rinsed in PBS, and incubated in blocking/permeabilization buffer (PBS with 2 % Triton X-100 and 5 % FBS).

To selectively label the retinal ganglion cells, the retinas were incubated with RBPMS primary antibody (Phosphosolutions #1832; 1:500) for 48 h at 4 ^{o}C. Following PBS washes, tissues were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200, Invitrogen) for 48 h at 4 ^{o}C. Again, retinas were washed and flat-mounted on glass slides, and coverslipped with DAPI-containing mounting medium (Vector Labs H-1200).

Images were obtained using a standard fluorescence microscope. Four radial and opposite fields were recorded on each retina in the central part (around 500 µm from the optic nerve head) and the peripheral part (around 500 µm from the retinal edge). ImageJ was used to process all the images. The RGC density was computed as the number of $RBPMS^{+}$ cells per scanned field. For quantification, images were analyzed using ImageJ software (NIH, USA) under identical acquisition and threshold settings for all groups. $RBPMS^{+}$ cells were manually counted within defined fields of equal area, and cell counts were converted to RGC density (cells/mm^2). All analyses were performed in a blinded manner to minimize observer bias.

Western blot analysis

The levels of expressed proteins in retinal tissues were assessed by western blotting. RIPA buffer that was supplemented with a protease inhibitor cocktail (Sigma-Aldrich) was used to lyse tissues, and total protein concentrations were determined by a BCA protein assay kit (Thermo Fisher Scientific, USA). Equal amounts of protein (60 µg) were separated by 10–12 % SDS-PAGE, and the proteins were subsequently transferred to PVDF membranes (Invitrogen). Membranes were blocked with 5 % non-fat dry milk in TBST (137 mM NaCl, 20 mM Tris, 0.1 % Tween-20, pH 7.6) for 1 h at 37 °C and then incubated with primary antibodies at a dilution of 1:1000 overnight at 4 °C. Afterward, membranes were washed and incubated with HRP-conjugated secondary antibodies (1:2000 dilution) at 37 °C for 1 h. Enhanced chemiluminescence (Millipore, USA) was used for western blotting detection, and images acquired from ChemiDoc XRS system (Bio-Rad, USA) were analyzed.

For each experimental group, retinal protein extracts from independent animals (n = 10 per group) were used as biological replicates, and western blot analysis was performed in triplicate to ensure reproducibility. Band intensities were quantified using ImageJ software, and the expression levels of target proteins were normalized to β-actin, which served as the internal loading control. The normalized values were then expressed relative to the control group, and data were presented as mean ± SEM to account for biological variability.

Statistical analysis

SPSS version 21.0 (SPSS Inc., USA) was the tool utilized for statistical analysis. One-way ANOVA was performed to assess the differences among groups, followed by Duncan's Multiple Range Test (DMRT) post hoc analysis. A threshold of $p < 0.05$ was used to define statistical significance.

RESULTS AND DISCUSSION

Pelargonidin significantly reduces intraocular pressure in diabetes induced rats

Diabetes induced by STZ resulted in a sustained and progressive rise in the IOP of rats over the 5-week experimental period (Fig. 1). The baseline values of IOP were similar in all the groups (16.5–17.8 mmHg). Nevertheless, IOP showed a marked increase in the diabetic model group (Group II) beginning at Week 1, and continued to rise over time. Week 3 showed an increase in IOP beyond baseline (~34–35 mmHg) and this further increased to about 38 mmHg in Weeks 4 and 5, indicating the successful development of diabetes-related ocular hypertension. Pelargonidin systemic administration significantly inhibited the STZ-induced increase in IOP in a dose-dependent manner. Group III (12.5 mg kg^{-1}) recorded a moderate decrease in IOP with the values of 28–31 mmHg in Week 3, 29–30 mmHg in Week 4 and 26–28 mmHg in Week 5. In Group IV (25 mg kg^{-1}), a more significant drop was observed and IOP reached about 26–29 mmHg during Week 3, 26–27 mmHg during Week 4 and approached near-normal levels (approximately 22–23 mmHg) during Week 5. The greatest effect was observed at the highest dose (50 mg kg^{-1}, Group V), where IOP values decreased to 24–26 mmHg at Week 3, 21–23 mmHg at Week 4, and 17–19 mmHg at Week 5, which were similar to the level of control levels.

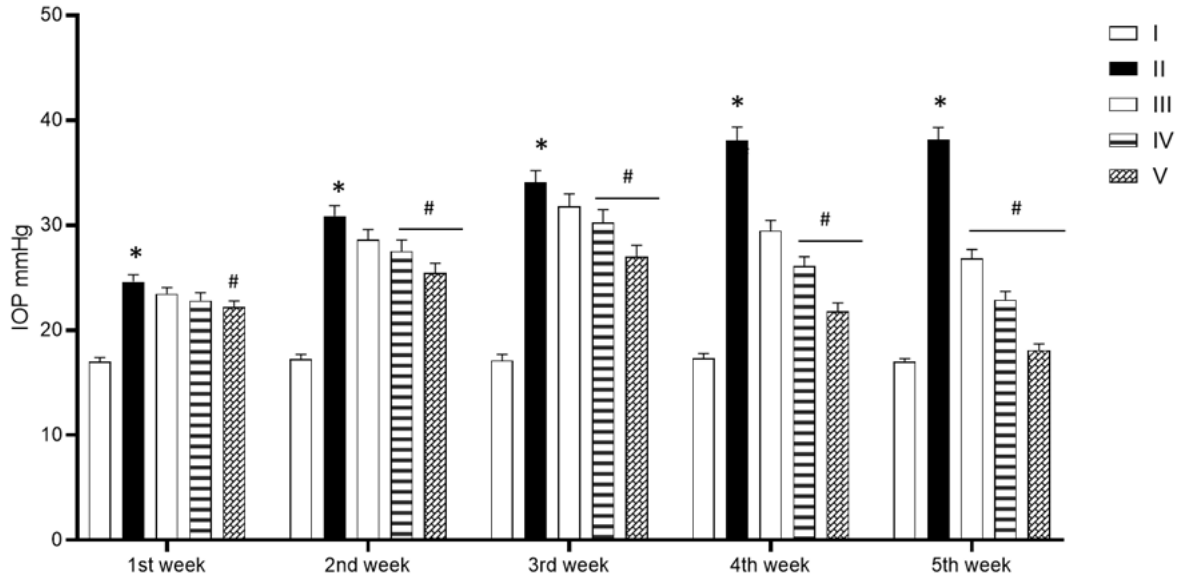

Fig. 1. Pelargonidin treatment reduces IOP in STZ-induced diabetic rats. IOP was determined over a period of 5 weeks. Pelargonidin significantly reduced IOP in STZ-induced diabetic rats in a dose-dependent manner. Groups: I (Control), II (STZ), III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), IV (STZ + Pelargonidin @ 25 mg kg^{-1}) and V (STZ + Pelargonidin @ 50 mg kg^{-1}). Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

Diabetic Retinopathy (DR) is an advanced neurovascular consequence of diabetes and is still one of the most important causes of irreversible loss of sight globally (23). There is an ongoing concept of nerve cell damage taking place at an early stage, including RGC degeneration prior to significant microvascular insults developing (24). It is in this light that we documented the action of pelargonidin, a natural anthocyanidin, in a streptozotocin-induced diabetic rat model. An important finding from the current study is that pelargonidin is able to significantly reduce IOP in diabetic rats. Increased IOP is not only a feature of glaucoma, but has also been increasingly noted in diabetic states, thereby adding to the stress and degeneration of RGCs (25). Restoring IOP is consistent with previous research reporting ocular hypotension induced by flavonoids and propounds that pelargonidin can mitigate the impact of RGC damage in diabetic retinopathy, potentially *via* alteration of aqueous humor circulation or trabecular meshwork homeostasis (26).

Pelargonidin effect on RGC density

Retinal immunofluorescence staining of whole-mount retina with an RGC-specific marker, RBPMS, showed that the distribution of RGCs among the five experimental groups differed significantly (Fig. 2). Group I rats (control group) exhibited a homogenous high cell density in both the central and peripheral parts of the retina, indicating normal retinal architecture. Conversely, the retinal density of RGCs in Group II diabetic rats (STZ-induced) showed a significant reduction throughout the retina, indicating diabetes-induced neurodegeneration, with greater loss in the peripheral region. Pelargonidin treatment resulted in dose-dependent preservation of RGC density. Group III (12.5 mg kg^{-1}) showed a slight but significant improvement in the density of RGCs relative to the diabetic group, indicating partial neuroprotection. The Group IV (25 mg kg^{-1}) showed a more pronounced recovery of RGC density in both central and peripheral retina, restoring a significant proportion of lost cells. Interestingly, Group V (50 mg kg^{-1}) had comparable or higher RGC densities in both regions compared to the control group, indicating a strong protective effect.

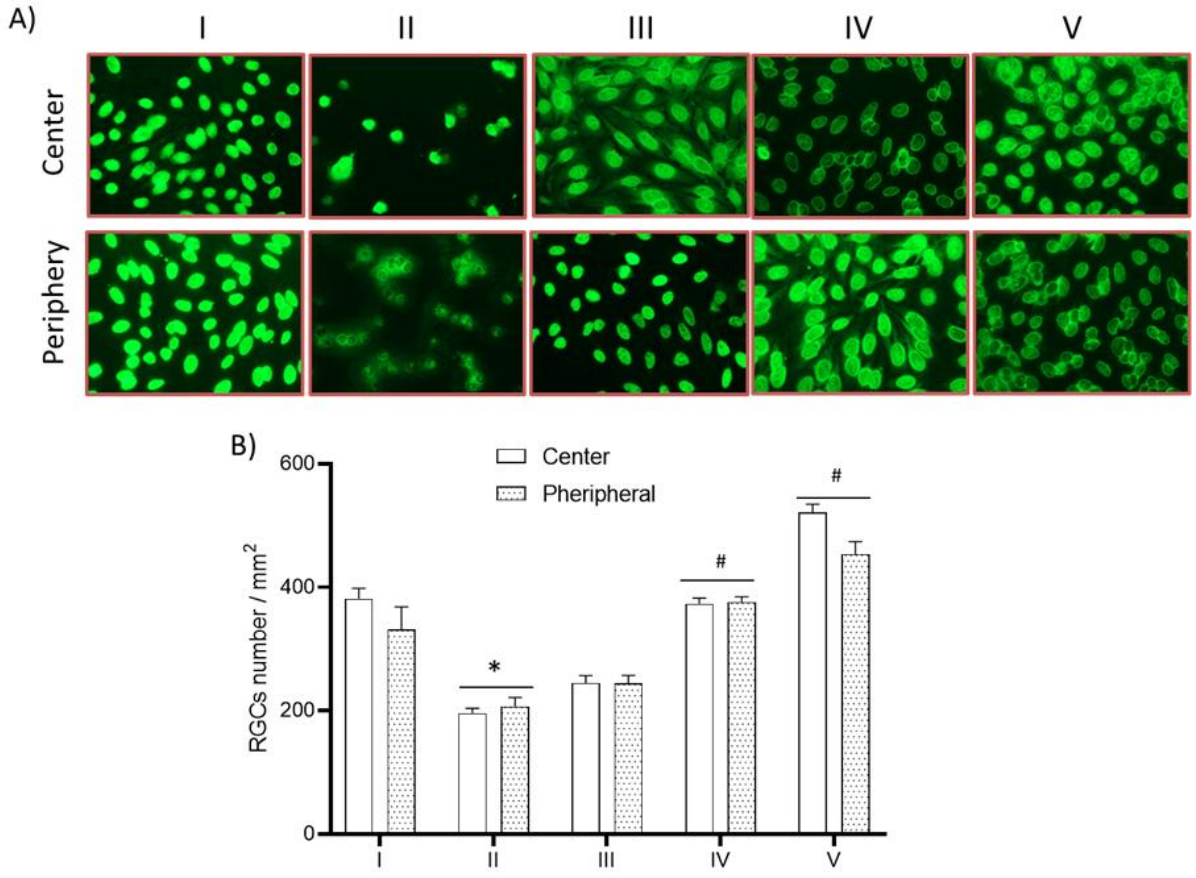

Fig. 2. Flat-mount retina staining of RGC. (A) Representative images of $RBPMS^{+}$ RGCs in whole-mount retinas from the five experimental groups, I (Control), II (STZ), III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), IV (STZ + Pelargonidin @ 25 mg kg^{-1}) and V (STZ +

Pelargonidin @ 50 mg kg^{-1}). STZ-induced diabetes caused a significant decrease in RGC density, whereas pelargonidin treatment preserved RGCs in a dose-dependent manner. (B) RGC density was quantified in the central and peripheral retina. Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

Diabetic retinopathy is marked by apoptosis of RGCs which contributes significantly to the emergence of neurodegenerative alterations. The pronounced reduction in RGC density in the diabetic condition demonstrates the vulnerability of these neurons to the effects of hyperglycemia-induced oxidative stress and inflammatory damage. The dose-dependent preservation of RGCs following treatment with pelargonidin indicates its strong neuroprotective effect. This protective effect can be attributed to its antioxidant and anti-inflammatory properties, which minimize retinal damage and preserve neuronal survival. The considerable restoration of RGC density with increasing doses of pelargonidin further suggests that it can reverse diabetes-induced retinal neurodegenerative changes and preserve retinal integrity.

Pelargonidin alters apoptosis by modulating cleaved caspase-3 and bcl-2 family protein expression

Western blot analysis showed that STZ-induced diabetes resulted in a significant increase in apoptotic signaling in the retina (Fig. 3). Particularly, the cleaved caspase-3 expression was significantly increased, indicating enhanced apoptosis. Simultaneously, pro-apoptotic proteins Bax and Bad were greatly increased whereas anti-apoptotic proteins Bcl-2 and Bcl-xL were greatly reduced in the diabetic group, as compared to control. These changes were reversed successfully using a dose-dependent pelargonidin treatment. Pelargonidin administration reduced cleaved caspase-3, Bax, and Bad and increased Bcl-2 and Bcl-xL expression in all groups of treatment. The strongest effect was shown in the highest dose (50 mg kg^{-1}), where the level of expression of the apoptotic proteins returned close to that of the control group.

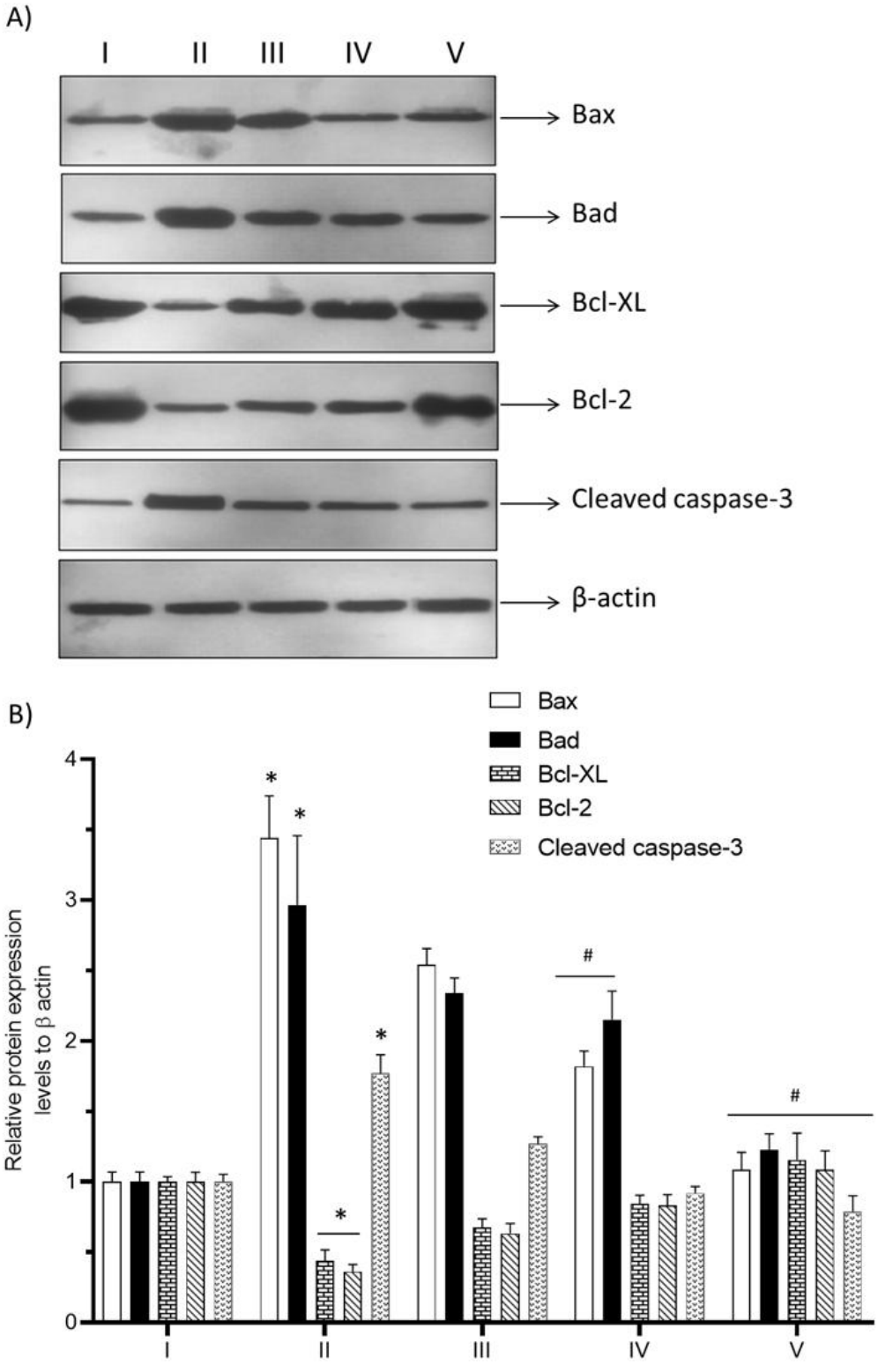

Fig. 3. Influence of pelargonidin on cleaved caspase-3 and other apoptosis-related proteins. (A) Representative Immunoblots showing cleaved caspase 3 and other apoptosis-related proteins. Lane I (Control), Lane II (STZ), Lane III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), Lane IV (STZ

+ Pelargonidin @ 25 mg kg^{-1}) and Lane V (STZ + Pelargonidin @ 50 mg kg^{-1}). (B) Densitometry analysis of the target proteins. Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

The apoptosis of RGCs is one of the major pathological changes observed in DR which is also associated with oxidative stress, inflammation, and deficiency of neurotrophic factors (27). The observed alterations in apoptotic markers suggest activation of the mitochondrial apoptotic pathway in the diabetic retina, characterized by an imbalance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. The ability of pelargonidin to restore this balance indicates its protective role against diabetes-induced retinal damage. By enhancing anti-apoptotic signaling and suppressing pro-apoptotic mediators, pelargonidin may help preserve RGC integrity under stress conditions. These findings suggest that pelargonidin exerts its neuroprotective effects, at least in part, through modulation of mitochondrial apoptosis pathways and regulation of caspase-3 activity.

Pelargonidin suppresses TGF-β2/p-Smad2/3 signaling pathway

A Western blot analysis revealed that STZ-induced diabetes significantly activated the TGF-β2/Smad signaling pathway in retinal tissue significantly (Fig. 4). TGF-b2 expression was significantly increased, and phosphorylation of Smad2 and Smad3 was also increased, indicated activation of the canonical fibrotic signaling pathway. It is worth noting that the overall expression levels of Smad2 and Smad3 did not differ among groups. This pathway was suppressed by pelargonidin treatment in a dose-dependent manner. Pelargonidin administration produced a high degree of downregulation of TGF-β2 expression and decreased the phosphorylation of Smad2 and Smad3. The total Smad 2/3 was found to be close to normal, especially at the highest dose (50 mg kg^{-1}), which implies that it effectively inhibited fibrotic signaling through TGF-β2.

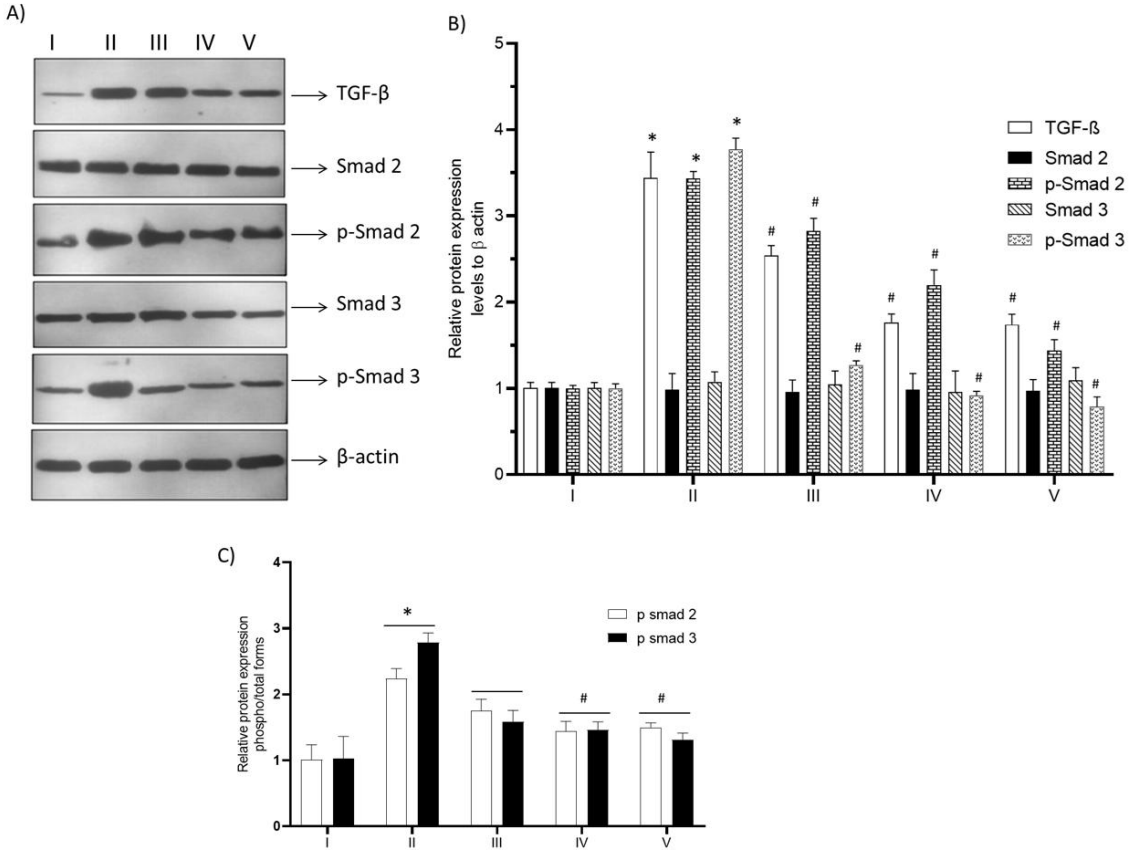

Fig. 4. Impact of pelargonidin on the TGF-β2/Smad signaling pathway. (A) Representative immunoblots showing proteins associated with the TGF-β2/Smad signaling pathway associated proteins. Lane I (Control), Lane II (STZ), Lane III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), Lane IV (STZ + Pelargonidin @ 25 mg kg^{-1}) and Lane V (STZ + Pelargonidin @ 50 mg kg^{-1}). (B and C) Densitometry analysis of the target proteins. Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

TGF-β pathway is a central signaling cascade involved in retinal remodeling in diabetes and DR and it could lead to hypertensive complications (28). These conditions can lead to TGF-β2-induced fibrosis that increases ECM protein deposition through the activation of canonical Smad2/3 signaling. Such mechanisms may be aggravated by cellular stress and were observed in our study. In diabetic rats, we noted a remarkable increase in TGF-β2 along with p-Smad2 and p-Smad3, indicating activation of the fibrotic signaling pathway. Treatment with pelargonidin resulted in a marked reduction in Smad2/3 phosphorylation along with a significant dose-dependent downregulation in TGF-β2 expression. This indicates that pelargonidin may attenuate the TGF-β signaling pathway, which otherwise contributes to

pathological ECM remodeling in diabetic retinal tissue. As much as there is an association of inflammation with STAT3, its identification in neuronal tissues during diabetic stress has drawn attention as a compensatory survival response. Hence for our intention, the results showing that pelargonidin augments the said pathway without worsening the state of the inflammatory cytokines – at least – indicate a shift towards neuroprotective STAT3 signaling rather than a pro-inflammatory one. Indeed, this aligns with the growing perspective of context-dependent activation of STAT3, emphasizing the complexities in the treatment of retinal pathologies.

Pelargonidin inhibits ECM remodelling in retinal tissue

The ECM profiling showed that there was a major remodeling of the retinal tissue in STZ-induced diabetic rats compared to the control group. Namely, the levels of collagen I, fibronectin, and elastin were significantly higher than in the control group, which suggests increased deposition of the extra-cellular matrix and fibrosis associated proteins (Fig. 5). Treatment with pelargonidin greatly suppressed these changes in a dose-dependent manner. The collagen I, fibronectin, and elastin expression were progressively reduced across the treatment groups, with the most significant decrease observed at the highest dose (50 mg kg^{-1}), and they were nearly restored to control levels.

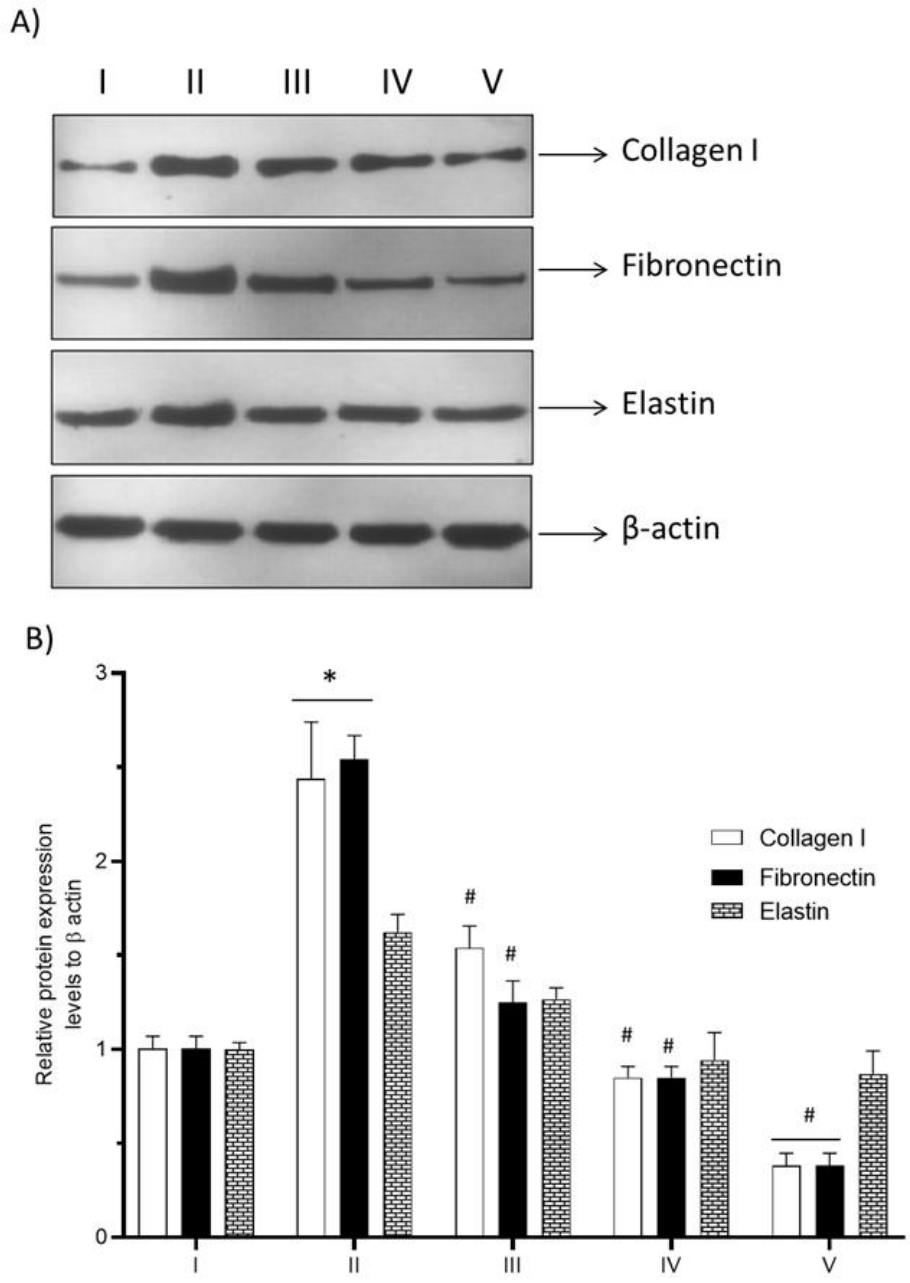

Fig. 5. Effect of pelargonidin on the expression of ECM proteins in retinal tissue. (A) Representative immunoblots showing collagen I, fibronectin, and elastin proteins. Lane I (Control), Lane II (STZ), Lane III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), Lane IV (STZ + Pelargonidin @ 25 mg kg^{-1}), and Lane V (STZ + Pelargonidin @ 50 mg kg^{-1}). (B) Densitometry analysis of the target proteins. Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

TGF-β signaling not only leads to structural alterations of the ECM, but also actively suppresses debris-clearing neuroprotective activity. To support this, our ECM profiling showed

obvious remodeling in the retinal tissue of diabetic rats which was marked by increased expression of collagen I, fibronectin, and elastin. Pelargonidin treatment markedly reduced the expression of these ECM markers, particularly at higher doses.

Pelargonidin activates phosphorylation of JAK2/STAT3 signaling pathway

The analysis of protein expression indicated that, under STZ-induced diabetes, the level of phosphorylation of JAK2 and STAT3 was significantly reduced compared to the control group, but the total protein levels of JAK2 and STAT3 changed minimally (Fig. 6). This signifies inhibition of JAK2/STAT3 signaling activity in diabetic conditions. The activation of this pathway was depended on the pelargonidin treatment dose. In particular, the phosphorylation of JAK2 and STAT3 (p-JAK2 and p-STAT3) was significantly enhanced after the administration of pelargonidin, and the overall protein concentration was not dramatically altered. The highest dose (50 mg kg^{-1}) had the strongest activation implying the restoration of JAK2/STAT3 signaling under diabetic stress conditions.

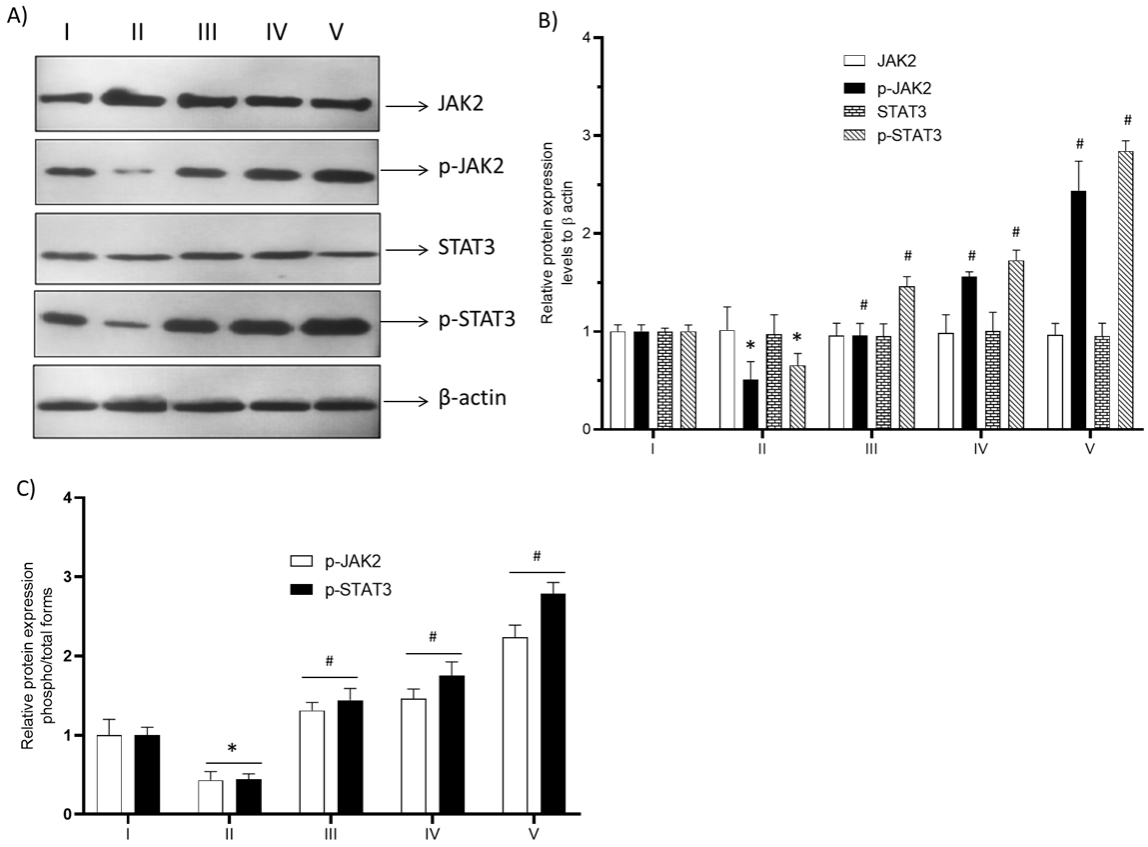

Fig. 6. Impact of pelargonidin on JAK2/STAT3 signaling pathway. (A) Representative immunoblots showing proteins associated with the JAK2/STAT3 signalling pathway. Lane I

(Control), Lane II (STZ), Lane III (STZ + Pelargonidin @ 12.5 mg kg^{-1}), Lane IV (STZ + Pelargonidin @ 25 mg kg^{-1}), and Lane V (STZ + Pelargonidin @ 50 mg kg^{-1}). (B and C) Densitometry analysis of the target proteins. Data are expressed as mean ± SEM (n = 10 rats per group). * $p < 0.05$ *vs.* control; $^{\#}$ $p < 0.05$ *vs.* STZ control.

The most interesting element of our study is the dual regulation of the JAK2/STAT3 signaling pathway. In circumstances of diabetes-related stress, this pathway tends to mediate both pro-inflammatory and neuroprotective responses, depending on the context of activation as well as the cellular microenvironment (29). In our study, pelargonidin increased the phosphorylation of JAK2 and STAT3 in STZ-induced diabetic rats, while the total protein levels of these proteins were unchanged, as they were already present. As it is widely accepted, activation of STAT3 can lead to increased cell survival, which support our data on apoptosis and strengthens the statement that pelargonidin augments intrinsic neuroprotection in RGCs through JAK2/STAT3 signaling.

However, it should be noted that the present analysis was performed using whole retinal tissue and therefore does not provide cell-type–specific information. Considering the well-documented dual role of STAT3 signaling in both neuronal survival and inflammatory responses in glial and immune cells, the precise cellular source of JAK2/STAT3 activation cannot be definitively determined in this study. Thus, the interpretation of STAT3 activation as primarily neuroprotective should be considered with caution, and future studies employing cell-type-specific approaches will be required to clarify the underlying mechanisms.

Study limitations

Despite the promising findings, several limitations of the present study should be acknowledged. First, the experiments were conducted in an STZ-induced diabetic rat model, and therefore the results may not fully reflect the complexity of human diabetic retinopathy. Second, the molecular analyses were performed using whole retinal tissue, which does not allow identification of the specific retinal cell types responsible for the observed signaling changes. Third, although the study demonstrates neuroprotective and anti-fibrotic effects of pelargonidin, pharmacokinetic properties, long-term safety, and optimal dosing were not investigated. Therefore, while our results suggest a potential protective role of pelargonidin in diabetic retinal injury, these findings should be interpreted cautiously and require further validation in additional experimental models and well-designed clinical studies before considering therapeutic application in humans.

CONCLUSIONS

To summarize, we show that pelargonidin provides significant neuroprotection in a diabetic model induced by streptozotocin through reduction in IOP, fibrosis, and apoptosis, along with enhancement of survival pathways. Importantly, pelargonidin preserved retinal ganglion cell (RGC) density in a clear dose-dependent manner, with the highest dose restoring RGC levels close to normal. These findings suggest that pelargonidin may represent a promising candidate for further investigation as a potential therapeutic strategy for diabetic retinopathy. However, additional studies, including further preclinical validation and clinical evaluation, are required to confirm its therapeutic applicability.

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Conflict of interest. – The authors declare that they have no known conflicts of interest.

Author's contribution. – Conceptualization, Y.L. and A.A; investigation, H.Y. and Y.L; writing, original draft preparation: A.A.; writing, review and editing: E.A.W.; funding acquisition, A.A. All authors have read and approved the final version of the manuscript.

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