

Original Research Article

Amygdalin protects apoptosis of retinal ganglionic cells in glaucoma rats by regulating the expressions of anti- and pro-apoptotic proteins

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Abstract

Purpose: To evaluate the protective effect of amygdalin against glaucoma.

Methods: Glaucoma was induced in rats via ischemia/reperfusion. The rats were treated with amygdalin (1 mg/kg) intraperitoneally (ip) for 5 weeks. Intra-ocular pressure (IOP), viability of retinal ganglion cell (RGCs) and histopathological changes in the retinal tissue of the glaucoma rats were determined. The expression levels of inflammatory cytokines and expressions of apoptotic factors were assessed in retinal tissues of all groups.

Results: Intra-ocular pressure was reduced in amygdalin-treated group, when compared with the glaucoma group ($p < 0.05$). Moreover, the viability and thickness of RGCs in the amygdalin-treated group were enhanced, relative to untreated glaucoma rats. There was decrease in retinal cytokine levels in amygdalin-treated group, when compared with untreated glaucoma rats. Amygdalin treatment ameliorated altered expressions of apoptosis proteins in the retinal tissue of ischemia/reperfusion-induced glaucoma rats.

Conclusion: Amygdalin has a protective effect against ischemia/reperfusion-induced glaucoma in rats. Thus, it has a potential for use in the clinical management of glaucoma.

Keywords: Glaucoma, Amygdalin, Cytokines, Ischemia/reperfusion, Apoptosis

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INTRODUCTION

Glaucoma is a severe ocular disorder which results in irreversible loss of vision due to degeneration of retinal ganglion cells [1]. A report has revealed that the number of glaucoma patients may increase to about 112 million in 2040 [2]. Intraocular pressure (IOP) is elevated in glaucoma patients, leading to apoptosis of retinal ganglion cells [3]. Studies have revealed that

ischemia/reperfusion in retinal tissue contributes to changes in IOP. The pathogenesis of apoptosis of retinal ganglion cells involves several factors, including neuro-inflammation and oxidative stress [4]. Conventional drugs available for the treatment of glaucoma are not able to prevent damage to retinal ganglion cells. Thus, there is need for development of newer and more effective drugs for the treatment of glaucoma.

Over the years, alternative medicine has shown beneficial effect in the management of several disorders, amongst which is glaucoma. Amygdalin is an aromatic cyanogenic glycoside present in different plants such as plum, cherry, peach and apricot [5]. The potential of amygdalin as an anti-histaminic, anti-tussive, anti-ulcer and anti-inflammatory agent has been demonstrated in many studies [6-9]. Moreover, amygdalin protects the lung from hypoxia-induced injury, and it exerts beneficial effect against atherosclerosis [10, 11]. In addition, amygdalin shows protective effect against several types of cancers such as rectal, colon, lung and prostate cancers, and it mitigates cancer-induced pain [12]. The present investigation was carried out to determine the effect of amygdalin on ischemia/reperfusion-induced glaucoma in rats.

EXPERIMENTAL

Animals

Sprague-Dawley rats (200-230 g) of either sex were kept under 12-h light/dark cycle under controlled conditions of 60 ± 5 % relative humidity and 24 ± 3 °C. Experiments on animals performed in the report as per the directions given in Association for the Assessment and Accreditation of Laboratory Animal Care International guideline [13] and approved by the Institutional Animal Ethical Committee of The Affiliated Hospital of Southwest Medical University, China (no. IAEC/AH-SMU/2017/12).

Animal grouping and treatment

Intraocular hypertension was produced by ischemia/reperfusion as per studies reported previously [14]. The rats were anesthetized using pentobarbitone (100 mg/kg, *ip*). Topical application 0.5 % alcaine eye drops was used for anesthetizing the corneas. Tropicamide (1%) was used to dilate the pupils. Thereafter, cannulation was done using 30-gauge needle to the right eye. This procedure was performed by maintaining an IOP of 70 mmHg for 1 h. Later withdrawing needle was used to normalize the IOP. The rats were divided into three different groups: normal group, glaucoma group which received saline solution, and amygdalin-treated group which was given amygdalin at a dose of 1 mg/kg, *ip* for 5 weeks. Intraocular pressure (IOP) was estimated after cauterization of vein using Tonopen XL tonometer.

RGC labeling and quantitation of survival

The rats were placed on a stereotactic apparatus after anesthetizing them with pentobarbital (100

mg/kg, *ip*). Povidone-iodine (PVP-I) was used to clean the exposed skull. Bilateral holes were drilled in the superior colliculi, into which 1 μ L of flurogold (FG; 4%) was injected. Then, tobramycin was applied on the dissected-out scalp.

Histopathological examination

Histopathological changes were determined as per previously reported studies. Davidson's solution containing acetic acid (12.5%), paraformaldehyde (9.3%) and ethanol (37.5%) was used to fix the enucleated eyes at room temperature for 24 h. The isolated eyes were seeded in molten paraffin after removing the lenses. Retinal sections of 5-mm thickness were sliced using a microtome and subjected to hematoxylin and eosin (H&E) staining. The stained slices were examined under a light microscope and photographed, followed by estimation of counts of retinal ganglion cell layer (GCL).

Determination of cytokines

Cytokines level was observed in the retinal tissue of I/R induced glaucoma rat model by using ELISA as per the guidelines suggested by the manufacturer of kits.

Determination of mRNA expression of apoptosis factors

In the retinal tissue, mRNA expression of factors contributes in the apoptosis were determined by qRT-PCR. Trizol Reagent was used to isolate the RNA from the separated gingival tissues and further Revert Aid First Strand cDNA Synthesis Kit was used to reversely transcribed the isolated RNA to cDNA. Quantitative SYBR Green PCR assay was used to estimate the expressions of gene by mixing the RT 2 SYBR Green Master with the used primers. All the samples were maintained at the series of programmed temperature and relative standard curve was used to estimate the level of mRNA expressions.

Western blot assay

NP40 protein lysis buffer was used to extract the protein from the isolated retinal tissues and concentration of protein was estimated by DC protein assay. Isolation of protein was done by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10%) and further filter the collected protein with membrane. Further membrane was treated with blocking agent to block the activity of membrane and separated membrane was incubated at 4 °C with primary

antibodies for Akt, NF- κ B, Bcl-2, Bax, caspase-3 and β -actin for the period of overnight. Later secondary antibodies was used to further incubate the membrane for 60 min at room temperature. ImageLab software was used to determine the density of the blot, which was enhanced by chemiluminescence.

Statistical analysis

Result of the study expressed as mean \pm SEM (n = 8). One-way ANOVA was used for statistical analysis with post hoc test (Graph pad Prism 6.1, CA, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Effect of amygdalin IOP

The effect of amygdalin on the IOP in ischemia/reperfusion induced glaucoma rats is shown in Figure 1. The IOP was estimated after each week of amygdalin treatment in ischemia/reperfusion-induced glaucoma rats. There was ($p < 0.01$) increase in IOP in glaucoma group, when compared to the normal group. It was observed that IOP was reduced ($p < 0.01$) in amygdalin group, relative to the glaucoma group.

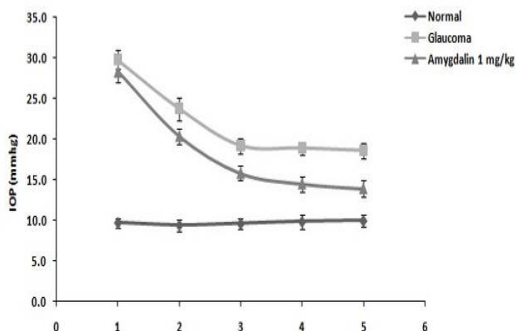


Figure 1: Effect of amygdalin on IOP in ischemia/reperfusion-induced glaucoma rats. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; $**p < 0.01$ vs. glaucoma group

Effect of amygdalin on survival of RGCs

The effect of amygdalin on the survival of RGCs in ischemia/reperfusion-induced glaucoma rats is shown using FG-labeled RGCs in Figure 2 A. A weak gold fluorescence was observed in the glaucoma group. However, gold fluorescence in the FG-labeled RGCs was diffuse and bright in the normal and in amygdalin-treated group of ischemia/reperfusion-induced glaucoma rats. Moreover, survival of RGCs was decreased ($p < 0.01$) in glaucoma group on the 2nd and 4th

weeks, when compared with the normal group. There were significant increases in the survival of RGCs on 2nd and 4th weeks in amygdalin-treated group of rats, relative to the untreated glaucoma group (Figure 2 B).

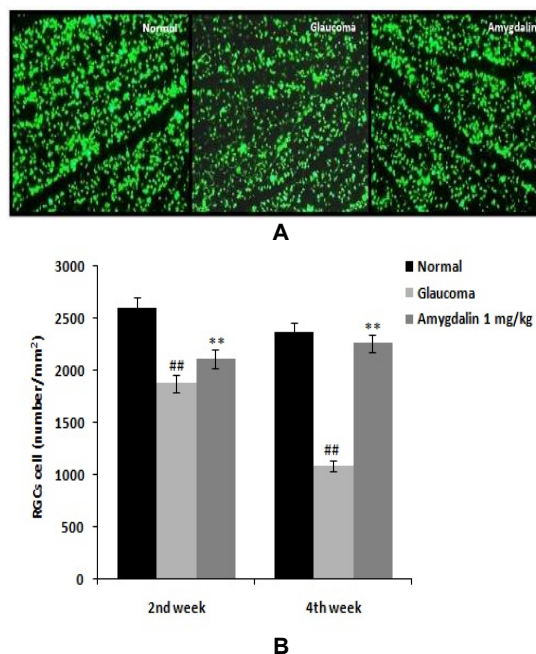


Figure 2: Effect of amygdalin on the survival of RGCs in ischemia/reperfusion-induced glaucoma in rats. A: Images of FG-labeled RGCs in the retinal tissue; B: number of surviving RGCs in the retinal tissue. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; $**p < 0.01$ vs. glaucoma group

Effect of amygdalin on histopathology of retina

Histopathological changes in the retinal tissue of ischemia/reperfusion-induced glaucoma in rats using H & E staining are shown in Figure 3 A&B. There were normal structure and normal thickness of retinal tissue, and the number of cells was also high in the retina of normal group. In contrast, transverse sections of retinal tissue in the glaucoma rats showed few cells with reduced layer thickness. However, treatment with amygdalin ameliorated the changes in structure, thickness and number of cells in the retinal tissue of ischemia/reperfusion-induced glaucoma rats (Figure 3A). In glaucoma group, number of cells in the retinal tissue was reduced than in the rats of normal group. Moreover, there was increase in the number of cells in the retinal tissues of amygdalin treatment ameliorates the cells number, relative to the glaucoma group of rats (Figure 3 B).

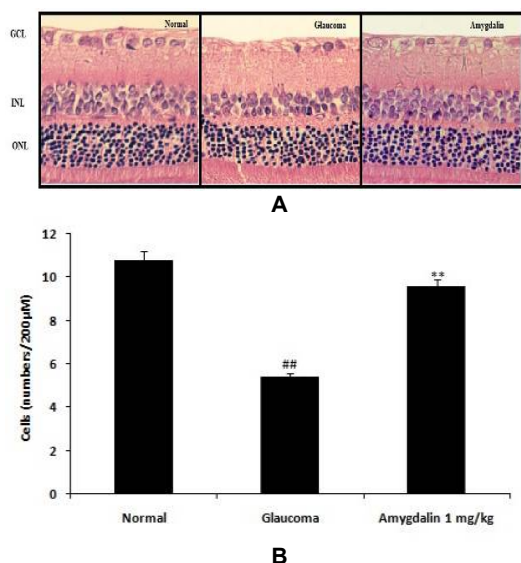


Figure 3: Effect of amygdalin on histopathology of retinal tissue in ischemia/reperfusion-induced glaucoma rats. A: H&E stained retinal tissue; B: number of cells present in the retina. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; ** $p < 0.01$ vs. glaucoma group

Effect of amygdalin on cytokines

There were decreases in the levels of cytokines in the retinal tissue of glaucoma group, when compared to the normal group of rats. However, treatment with amygdalin enhanced the levels of these cytokines in the retinal tissue, when compared to glaucoma group of rats (Figure 4).

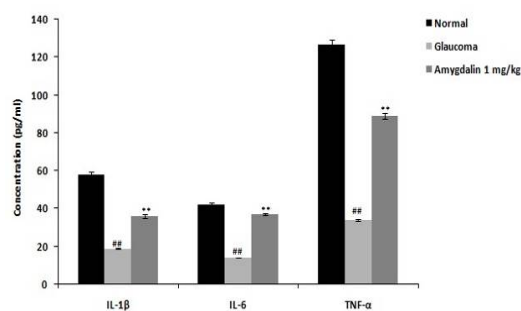


Figure 4: Effect of amygdalin on levels of cytokines in the retinal tissue of ischemia/reperfusion-induced glaucoma rats. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; ** $p < 0.01$ vs. glaucoma group

Amygdalin ameliorates the mRNA expressions of apoptotic factors

The mRNA expressions of apoptotic factors in the retinal tissue of ischemia/reperfusion-induced glaucoma rats are shown in Figure 5. There were significant increases in the mRNA expressions of caspase-3, caspase-8, Akt and Bax, while the

mRNA expression of Bcl-2 was decreased in the retinal tissue of glaucoma group, when compared with the normal group of rats. However, mRNA expressions of caspase 3 and 8, Akt and Bax were downregulated, while the expression of Bcl-2 was increased in the retinal tissue of amygdalin-treated group, when compared with the glaucoma group.

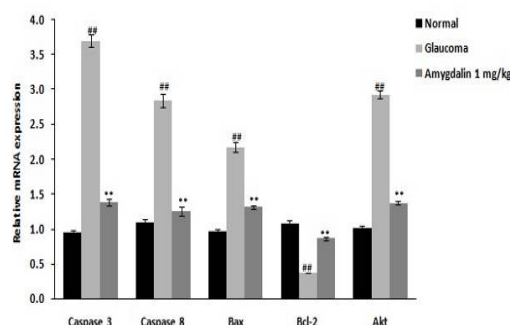


Figure 5: Effect of amygdalin on mRNA expression of apoptotic factors in the retinal tissue of ischemia/reperfusion induced glaucoma rats. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; ** $p < 0.01$ vs. glaucoma group

Amygdalin ameliorates the expressions of apoptotic and NF- κ B protein

The protein expressions of apoptotic and NF- κ B were enhanced, while that of Bcl-2 was reduced in the retinal tissue of glaucoma group, when compared to normal group. However, treatment with amygdalin attenuated the altered protein expressions of caspase-3, Bax, Bcl-2, NF- κ B and Akt in the retinal tissue of ischemia/reperfusion-induced glaucoma rats (Figure 6).

DISCUSSION

Glaucoma is an ocular disease in which IOP and degeneration of retinal ganglion cells are increased, leading to loss of vision [15]. Conventional drugs used for the treatment glaucoma are not focused on the degeneration of retinal ganglion cells. The present study evaluated the protective effect of amygdalin against glaucoma which was induced in rats through ischemia/reperfusion. The rats were then treated with amygdalin at a dose of 1 mg/kg for the period of five weeks. The effect of amygdalin was determined via estimation of IOP, survival of RGCs and histopathological changes in the retinal tissue of the glaucoma rats. Moreover, levels of inflammatory cytokines, and protein and mRNA expressions of anti-apoptotic and pro-apoptotic factors were estimated in the retinal tissues.

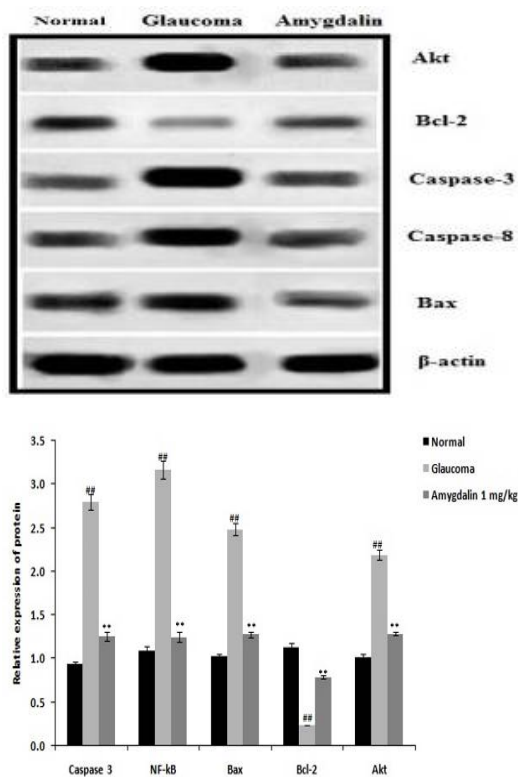


Figure 6: Effect of amygdalin on the protein expressions of apoptotic factors and NF- κ B in ischemia/reperfusion-induced glaucoma rats. Data are mean \pm SEM (n = 8); $p < 0.01$ vs. normal group; $**p < 0.01$ vs. glaucoma group

Glaucoma causes irreversible loss of vision. Drugs with antioxidant and anti-neuroinflammatory properties protect RGCs and thereby reduce IOP. Studies have shown that apoptosis of RGCs contributes to retinal damage in glaucoma due to increase in IOP [16]. The results from the present investigation reveal that treatment with amygdalin reduced IOP in glaucoma rats. The loss of RGCs is attributable to increased IOP in glaucoma. It has been reported that amygdalin protects against neuroinflammation by attenuating altered levels of inflammatory cytokines and NF- κ B [17]. Moreover, it has been suggested that inflammatory cytokines are upregulated in glaucoma [18]. In the present study, the amygdalin-treated group had reduced cytokine levels in the retinal tissue, when compared with the glaucoma group of rats.

Several factors are involved in cellular apoptosis, especially pro-apoptotic and anti-apoptotic proteins. Expressions of caspase-3, caspase-8, Bax and Akt (pro apoptotic proteins) are enhanced in apoptosis of cells [19]. The mitochondrial pathway of apoptosis occurs

through activation of caspase enzymes [20]. The activation of the caspase cascade contributes to apoptosis of cells. Moreover, upregulation of Bax and downregulation Bcl-2 protein take part in mitochondrial dysfunction and activate cellular apoptosis. The results of the present study suggest that treatment with amygdalin alleviates the altered levels of pro-apoptotic and anti-apoptotic factors in the retinal tissues of ischemia/reperfusion-induced glaucoma rats.

CONCLUSION

The findings of this study demonstrate the protective effect of amygdalin against ischemia/reperfusion-induced glaucoma in rats by regulating the apoptotic factors. These data indicate that amygdalin may be developed for the treatment of glaucoma.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We confirm that the mentioned authors in the manuscript performed the presented work and all the data shown in the manuscript generated the author of it. Xiaoli Zeng performed the methodology and literature review, Hongbin Lv supervised and prepared the manuscript and Xuewen Huang performed histopathology study.

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