Protective effects of a composition of Chinese herbs-Gurigumu-13 on retinal ganglion cell apoptosis in DBA/2J glaucoma mouse model

Qiu-Li Zhang¹, Wei Wang², Yan Jiang³, Tian-Zi Zhang⁴, Zhan-Jun Lu¹, Ao Jiang⁴

¹Department of Ophthalmology, Affiliated Hospital of Inner Mongolia University for the Nationalities, Tongliao 028000, the Inner Mongolia Autonomous Region, China
²Department of Ophthalmology, Tongliao Hospital, Tongliao 028000, the Inner Mongolia Autonomous Region, China
³Department of Neurosurgery, Affiliated Hospital of Inner Mongolia University for the Nationalities, Tongliao 028000, the Inner Mongolia Autonomous Region, China
⁴China-Japan Union Hospital of Jilin University, Changchun 130000, Jilin Province, China

Correspondence to: Zhan-Jun Lu. Department of Ophthalmology, Affiliated Hospital of Inner Mongolia University for the Nationalities, Tongliao 028000, the Inner Mongolia Autonomous Region, China. Ao Jiang. China-Japan Union Hospital of Jilin University, Changchun, Economic Development Zone, Changchun 130000, Jilin Province, China. jiangao0211@163.com

Received: 2017-08-04        Accepted: 2018-01-15

Abstract
● AIM: To explore the concrete mechanism of a Mongolian compound medicine-Gurigumu-13 (GRGM) for glaucoma treatment.
● METHODS: DBA/2J mice, as glaucoma models, were intragastric administrated with GRGM to study the effect of GRGM on retinal ganglion cells (RGCs). The loss of RGCs was evaluated with the number of RGCs and axons. The expression of the target protein of RGCs or mouse retinas was determined by Western blot. The relative content of malondialdehyde (MDA) was examined by ELISA assay.
● RESULTS: GRGM distinctly improved retina damage via increasing the number of neurons, RGCs and axons in a concentration dependent manner. Meanwhile, GRGM obviously decreased the high level of MDA and the expression of oxidative stress-related proteins in retinas of DBA/2J mice, but promoted the expression of antioxidant proteins. Additionally, GRGM also significantly inhibited the protein expression of Bip and Chop, which were markers of endoplasmic reticulum stress-induced apoptosis.
● CONCLUSION: GRGM have obvious protective effects on RGCs and axons via inhibiting oxidative stress and endoplasmic reticulum stress.
● KEYWORDS: glaucoma; retinal ganglion cells; oxidative stress; endoplasmic reticulum stress

DOI:10.18240/ijo.2018.03.03


INTRODUCTION

Glucoma is an ocular neurodegenerative disease that usually accompanied by raised intraocular pressure (IOP), which characterized by optic atrophy and visual field defect. The primary pathological basic is selective apoptosis of retinal ganglion cells (RGCs), which results in irreversible damage of vision and even permanent blindness. At present, glaucoma usually resorts to surgery or control of IOP with drugs, but they do not work for everyone. Lowering IOP alone will not completely inhibit RGCs apoptosis and axon neuropathy. Therefore, investigating the molecular mechanisms underlying RGCs apoptosis to prevent RGCs apoptosis is the nowadays treatment direction.

Oxidative stress injury is considered as a major mechanism of RGCs apoptosis in the development of glaucoma, which reflects the imbalance of the oxidative system and antioxidative system in certain pathological changes of human body. In vivo, studies showed that glaucoma and other retinopathy diseases were accompanied by ischemic and hypoxic and made direct damage to the retina, causing oxidative stress that involved in RGCs. Ischemia-reperfusion injury in retina could aggravate oxidative stress in retina, which led to excessive producing of free radicals and cellular energy metabolism disturbance, furthermore this induced DNA damage to promote RGCs apoptosis. Additionally, it also found that the process of oxidative stress-induced RGCs apoptosis in vitro relied on caspase to some extent. Inhibition of RGCs apoptosis induced by oxidative stress injury is of a very vital significance to glaucoma therapy and causes concerns from more and more researchers. Currently, the
studies of inhibitory effect of anti-oxidative stress drugs on RGCs apoptosis and its mechanisms have been carried out to look for an effective therapeutic targets, which has important value in the treatment of glaucoma. It is accepted that endoplasmic reticulum stress (ERS) is a critical aspect in the response mechanism of cells and it may trigger ERS mediated apoptosis signals in certain conditions\(^{(16-19)}\). Recent studies found that ERS played an important role in retinopathy of experimental animal models, such as diabetic retinopathy, retinitis pigmentosa, glaucoma\(^{(20-21)}\). ERS inducer tunicamycin was injected into the vitreous and then significantly promoted RGCs death and thinning of the retina\(^{(12)}\), suggesting that ERS could cause degenerative nerve of the retina. Cauteronization of episcleral vein is a new method to create an animal model of chronic glaucoma\(^{(22)}\). In this animal model, the protein expression of Chop, ERS related protein, in the retina was gradually increased with prolonged time, accompanied with the lose of RGCs\(^{(23)}\). Intravitreal injection of NMDA could induce Chop over-expression in NMDA-induced neurodegeneration in mice, and the mice with Chop gene knocked out were able to resist neurological damage induced by NMDA\(^{(24)}\). These findings indicate that ERS exerts very important role in neuronal damage and thus involves in the occurrence and development of glaucoma. Gurigumiu-13 (GRGM), a mongolian medicine, has been used in ophthalmology clinical treatment for many years and achieve significant efficacy. It is a complex compound which is constituted with 13 kinds of Chinese herbs, Carthamus tinctorius L. (Xinjiang, China), Syzygium aromaticum (Yunnan, China), Semen Nelumbinis (Hunan, China), Ophiopogon japonicas (Thunb.) Ker-Gawl (Sichuan, China), Radix Aucklandiae (Yunnan, China), Fructus Chebulae (Guangxi, China), Fructus Toosendan (Sichuan, China), Gardenia jasminoides Ellis (Jiangxi, China), Pterocarpus santalinus (Hainan, China), Moschus (Beijing, China), Pulvis Cornus Bubali Concentratus (Hebei, China), Calculus Bovis (Hunan, China), Cinnabar (Hunan, China). Mongolian medicine believes that the liver tends to be over-active, resulting in glaucoma. Although GRGM is used in the clinical treatment of glaucoma to have the full pharmacology theory basis, the underlying mechanism remains to be fully elucidated. In the present study, we evaluated the protective effects of GRGM on RGCs in DBA/2J mice and further investigated its mechanism. The study provides experimental bases for the further application of GRGM in the treatment of glaucoma.

**MATERIALS AND METHODS**

**Animal Experiments** The animal experiments were approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia University for the Nationalities. All procedures for animals were conducted in accordance with the ARVO Guidelines for the Use of Animals in Research. DBA/2J and C57BL/6J mice were obtained from the Laboratorial Animal Center at Affiliated Hospital of Inner Mongolia University for the Nationalities. DBA/2J mice (3mo) were randomly divided into four groups, and 10 mice per group, and mice were given different doses of GRGM (0, 0.4, 0.8, 1.6 g/kg·d) once a day lasting 7mo. The control group has 10 C57BL/6J mice. All mice were in a standardized light-dark cycle with food and water freely.

**Intraocular Pressure Measurements in Mice** The IOP of all mice was measured by a rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH, USA) with intraperitoneal injection of 10% chloral hydrate. The probe tip of tonometer was placed at the site of 2-3 mm away from the surface of mouse cornea, then started the tonometer to make the probe tip hit the center of cornea surface. Three consecutive sets of six measurements of IOP were averaged as the IOP of mice. All IOP measurements were performed by the same researcher.

**Tissue Preparation** After 7mo perfusion, retinas and optic nerves were quickly isolated from the eyes of mice. Retinas and optic nerves were rinsed and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde respectively for RGCs, neurons and axon counting. For Western blot and PCR, retinal tissues were quickly dissected, stored in liquid nitrogen.

**Axon Counting** The glutaraldehyde-fixed optic nerves were cut into small pieces, and then stained by p-phenylenediamine according to the instruction. Number of axon was calculated on 18 visual fields chosen from each image of optic nerve under a inverted microscope, including center, mid-periphery and peripheral margin of optic nerve. The axon number of 18 visual fields were counted manually and then normalized to the total area of the optic nerve.

**Retinal Ganglion Cells and Neurons Counting** RGCs and neurons were counted as the previous study with small modification. Briefly, DAPI was used to label RGCs, and the whole retina was divided into central, middle and peripheral regions. RGCs number per retina was calculated with 10 visual fields through DAPI-labeled RGCs counting under a inverted microscope.

**Western Blot** Total protein was extracted from retinas or RGCs using RIPA buffer (1% NP-40, 0.25% Na-deoxycholate, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL Aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L Na3VO4, 1 mmol/L NaF). The concentration of total protein was measured with BCA Protein Assay Kit (Thermo Fisher Scientific). Total protein (25 μg per sample) was diluted in Tris-HCl (pH 7.4) and separated by SDS-PAGE. The gel-separated proteins were transferred to the PVDF membrane, and then the PVDF membrane was immersed in 5% skim milk for 30min. The primary antibodies (Abcam) were added and incubated with the target proteins on PVDF membrane at 4°C overnight. Proteins were then
incubated with corresponding HRP-conjugated secondary antibodies (Abcam). The target proteins were observed by the ECL development solution (Thermo Fisher Scientific). Relative expression level was measured with an ImageMaster® VDS and normalized to β-actin. Data were mean±standard deviation (SD).

**Detection of Malondialdehyde** Malondialdehyde (MDA) in retinas of mice was measured with commercial kit (Beyotime Biotechnology) according to the kit instructions. The relative contents of MDA were expressed as nmol/mg protein.

**Statistical Analysis** All statistical analysis were performed with SPSS 17.0 software (IBM Corporation). The numbers of cells, protein expression, IOP and relative content of MDA were expressed as the mean±SD. A $P$ value of $<0.05$ was considered as statistically significant. The Student’s $t$-test was used to compare paired groups.

**RESULTS**

**Effect of GRGM on the Damage of Retinal Ganglion Cells in DBA/2J Mice** After continuously treated with 0, 0.4, 0.8 and 1.6 g/kg·d GRGM for 7mo, the numbers of total neurons, RGCs and RGC axons in retinas were counted and IOP levels of experimental mice were detected. We found that total neurons, RGCs and RGC axons in retinas of DBA/2J mice were obviously lower than those in C57BL/6J mice; GRGM significantly increased the number of total neurons, RGCs and RGC axons in retinas of DBA/2J mice with the dependence of concentration (Figure 1A, 1B). However, IOP levels of DBA/2J mice were much higher than those of C57BL/6J mice, and GRGM had no effect on the IOP levels of DBA/2J mice (Figure 1C).

**Effect of GRGM on the Expression of Apoptosis Pathway Related Molecules in Retinal Ganglion Cells** To investigate the protective mechanisms of GRGM in retinas of DBA/2J mice, the expressions of apoptosis pathway related proteins in RGCs were detected in this part. Western blot assays showed that GRGM could dramatically promote the expression of Bcl-2 protein, while the expression of Bax and cleaved caspase-3 in the levels of protein were markedly decreased with the increase of GRGM concentration (Figure 2).

**Inhibition of GRGM on the Retinal Lipid Peroxidation in DBA/2J Mice** MDA content reflects the degree of lipid peroxidation, so we further examined the relative content of MDA of mouse retinas. The relative content of MDA in retinas of DBA/2J mice was significantly higher than that of C57BL/6J mice. Furthermore, the relative content of MDA was the highest in retinas of DBA/2J mice at 7 months old and then decreased. Importantly, GRGM could obviously decrease MDA content in retinas of DBA/2J mice at 7, 10 months old, while the content of MDA in retinas of DBA/2J mice did not significant change with different concentrations of GRGM at 3 months old (Figure 3).

**Inhibition of GRGM on the Oxidative Stress in Retinas of DBA/2J Mice** Oxidative stress involved in multiple pathophysiological processes through induced oxidative
Protective mechanism of GRGM for retina

**DISCUSSION**

Herein, the study demonstrated that mongolian medicine GRGM could possess significantly protective effect on RGCs loss of DBA/2J mice. In recent studies, DBA/2J mice were used extensively for antiglaucoma research because of their pigment dispersion, iris atrophy and RGCs loss, which were similar to human glaucoma. In our study, GRGM were used extensively for antiglaucoma research because of their pigment dispersion, iris atrophy and RGCs loss, which were similar to human glaucoma. In our study, GRGM were administered to DBA/2J mice from 3 to 10 months old. First, we examined the effects of GRGM on the number of neurons, RGCs and RGC axons and IOP in the retinas. We found that GRGM distinctly improved retina damage via increasing the number of neurons, RGCs and axons in a concentration dependent manner. However, GRGM had no significant effect on IOP of DBA/2J mice at the concentration range. Numerous studies show that apoptosis is the main way of the loss of RGCs, and RGCs apoptosis is the final common pathway in many diseases with optic nerve damage including glaucoma[16-17]. Hence, we believe that GRGM is able to inhibit RGCs apoptosis that prevents RGCs loss in retinas. To further verify this conclusion, we evaluated the effects of GRGM on the expression of apoptosis-related proteins. Western blot results supported that GRGM could promote Bcl-2 expression while Bax and cleaved caspase-3 expression were reduced by GRGM.

More significantly, the study also found that GRGM could obviously down-regulate the high level of MDA in retinas of DBA/2J mice since they were 7 months old with the dependence of concentration. MDA is one of the most important products of the lipid peroxidation and also the very important detection target for oxidative cell damage. Oxidative stress injury is considered as a major mechanism during RGCs apoptosis of glaucoma, and the lipid peroxidation is a part of oxidative stress. So we speculated that GRGM protected RGCs through regulating oxidative stress. Further study showed that GRGM significantly inhibited the expression of oxidative stress-related proteins including Gfap, Cp, HO-1, Nos-2, but promoted the expression of antioxidant proteins. The imbalance of the oxidative system and antioxidative system in certain pathological changes of human body causes oxidative stress[18-19]. Corporately, these results demonstrated that GRGM could suppress oxidative stress and subsequent RGCs apoptosis.

In recent years, many researches have confirmed that ERS could contribute to the occurrence and development of glaucoma via inducing RGCs apoptosis[20-21]. A small number of primary angle-closure glaucoma could be caused by muscle fiber protein mutation in trabecular meshwork cells[22]. When the mutant protein highly expressed in trabecular meshwork cells, ERS activation and a series of glaucoma phenotypes were observed in mice[23]. In our study, GRGM treatment evidently reduced the protein expression of Bip and Chop, ERS related proteins. Bip, a chaperone protein, can bind the transmembrane proteins located in endoplasmic reticulum to regulate ERS-induced apoptosis. Increase in Bip expression is considered to be one of the important indicators for the initiation of ERS-induced apoptosis[11,24]. Increase in Bip expression is considered to be one of the important indicators for the initiation of ERS-induced apoptosis[11,24]. Increase in Bip expression is considered to be one of the important indicators for the initiation of ERS-induced apoptosis[11,24].

**Effect of GRGM on Antioxidative Ability of Retina in DBA/2J Mice**

The antioxidative ability of retina is an important parameter of maintaining the function of the retina. Our findings showed that the expression of antioxidant protein Nrf2, GST, GPx4, Prdx2 in retinas of DBA/2J mice were markedly lower than that of C57BL/6J mice. However, GRGM could significantly promote antioxidative proteins expression in retinas of DBA/2J mice (Figure 5).

**Inhibition of GRGM on Endoplasmic Reticulum Stress in Retinal Ganglion Cells of DBA/2J Mice**

ERS-mediated apoptosis was closely related to retinal damage, so we further detected the expression of ERS related protein Bip and Chop in RGCs. The results showed that both Bip and Chop highly expressed in RGCs of DBA/2J mice, but their protein expression levels were significantly decreased in RGCs after DBA/2J mice treated with GRGM (Figure 6).

![Figure 3](image_url)

**Figure 3** Effect of GRGM on lipid peroxidation of DBA/2J mouse retinas. The relative content of MDA of mouse retinas was evaluated at 3, 7, 10mo respectively. β-actin acted as the internal reference. *P<0.01 vs C57BL/6J; †P<0.05, ‡P<0.01 vs DBA/2J without GRGM treatment.

**Effect of GRGM on lipid peroxidation of DBA/2J mouse retinas.** The relative content of MDA of mouse retinas was evaluated at 3, 7, 10mo respectively. β-actin acted as the internal reference. *P<0.01 vs C57BL/6J; †P<0.05, ‡P<0.01 vs DBA/2J without GRGM treatment.

**DISCUSSION**

Herein, the study demonstrated that mongolian medicine GRGM could possess significantly protective effect on RGCs loss of DBA/2J mice. In recent studies, DBA/2J mice were used extensively for antiglaucoma research because of their pigment dispersion, iris atrophy and RGCs loss, which were similar to human glaucoma. In our study, GRGM were administered to DBA/2J mice from 3 to 10 months old. First, we examined the effects of GRGM on the number of neurons, RGCs and RGC axons and IOP in the retinas. We found that
Our results indicated that GRGM had significant inhibitory effect on ERS in retinas, and then demonstrated the antiapoptosis effects. In summary, our results provided strong evidence that GRGM had obvious protective effects on RGCs in DBA/2J mice via inhibiting oxidative stress and ERS as shown by increased the number of RGCs and axons. The study provides experimental basis for the further application of GRGM in the treatment of glaucoma.

ACKNOWLEDGEMENTS
Foundation: Supported by the National Natural Science Foundation of China (No.81660734).

Conflicts of Interest: Zhang QL, None; Wang W, None; Jiang Y, None; Zhang TZ, None; Lu ZJ, None; Jiang A, None.
Protective mechanism of GRGM for retina

REFERENCES


3 Lee KY, Nakayama M, Aihara M, Chen YN, Araie M. Brimonidine is neuroprotective against glutamate-induced neurotoxicity, oxidative stress, and hypoxia in purified rat retinal ganglion cells. Mol Vis 2010;16(31-32):246-251.


