Neuroprotective Effects of Methyl 3,4-dihydroxybenzoate on NMDA-induced Injury in Rat Retina

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Abstract

The purpose of present study is to evaluate the neuroprotective effect of methyl 3, 4-dihydroxybenzoate (MDHB) on the injury caused by N-methyl-D-aspartate (NMDA). NMDA is an excitatory amino acid, when it overdosage exists in retina, it can cause neuron excitatory toxicity, eventually leading RGCs to death. Under chloral hydrate anesthesia, SD rats are subjected to intravitreal injection of NMDA (40mM) at 1μl/eye. Each animal is given intraperitoneal injection of MDHB or normal saline after the intravitreal NMDA injections. MDHB is a small chemical compound extract from Malan it has the molecular weight of 168.15. .MDHB can rapidly penetrate the blood eye barrier and reach the retina, after about 2minutes it can be detected in the retina via HPLC. The tests results suggest that MDHB can prolong the duration of animals staying in a dark zone. Besides, it can significantly increase the number of survival RGCs after NMDA injection, enhancing the amplitude of photopic negative responses to flash. MDHB treatment can attenuate the activation of apoptotic process as indicated by the elevated ratios of Cleaved Caspase3/ Caspase-3 and Bax/Bcl-2, promoting RGCs survival by activating Akt. These data indicate that MDHB have potential clinical application prospects in retinal diseases.

Keywords

Methyl 3,4-dihydroxybenzoate, N-methyl-D-aspartic, excitotoxicity, retinal ganglion cells, neuroprotective effects.

1. Introduction

Optical nerve degeneration disease is a kind of optical diseases, which companied with the pathological features of retinal ganglion cells (RGCs) apoptosis and optic nerve atrophy. Delaying optic ganglion cell apoptosis and promote the survival of ganglion cells is an important aspect of the protecting the optic nerve [1]. There are several cellular layers in retina, of which the retina ganglion cells (RGCs) are neurons located in the inner layer of the retina that transmit visual signals from the retina to optic chiasm, then transmit signal to the visual cortex, this is the key step in the formation of vision. RGCs are mainly expressing the N-methyl-D-aspartate receptors (NMDARs) in the retinal neuron, whilst these cells are susceptible to excess glutamate which would cause excitotoxicity[2]. RGCs dysfunction has much connection with neuropathies. Excess excitatory amino acid can induce optic neuropathies associated with degeneration of RGCs and respective axons, leading to visual field loss such as glaucoma[3]. Overwhelming evidence supports the notion that excitotoxicity plays an important role in the pathogenesis of neurodenerative diseases.

NMDARs receptors are regarded as the main receptors involved in retinal cell death. Activating the NMDA receptors will be followed by massive Ca2+ influx via NMDA receptor-operated channels [4]. This superfluous intracellular Ca2+ is involved in neuronal excitatory process and is perceived as one of the pathogenesis of glaucoma-induced neuronal cell death[5]. Considering the limitation and multifactorial etiology, novel agents and treatment strategies are in urgent need. Neuroprotection and axonal regeneration provide a novel direction in the treatment. Neurotrophin can prolong the survival of the damaged RGCs to some extent[6]. One of the mechanisms of glutamate-induced neurotoxicity in retina can be explained that glutamate stimulates non-NMDA receptors and opens the cation channels, then depolarizes the retinal neurons. Membrane depolarization releases Mg2+, which blocks NMDA receptors, thereby activating NMDA receptor-gated cation channel which results in an increase in Ca2+. The overdose Ca2+ is considered to trigger the delayed death of retina neurons, while the inhibition of Ca2+ entry by NMDA receptor antagonist through NMDA receptor associated channel attenuates effects of glutamate-induced neurotoxicity, and calcium channel antagonists can also attenuate NMDA induced neurotoxicity in a certain way[7]. The NMDA-induced damage model provides marketable drugs screening for the retinal generation diseases. Excitotoxicity caused by the elevated glutamate in retinal extracellular can activate the caspase and apoptosis in the isolated RGCs from rats[8], thinning the inner plexiform layer. This model helps to examine the RGCs structure and functions progress in the development of molecular diagnostic [9]. This method also favors to explore potential neuroprotective drug, which promotes the progression of new therapies to cure vision loss. It is reported that many drugs and biological active factors, such as ciliary neurotrophic factor (CNTF) and brain derived neurotrophic factor (BDNF), have neuroprotective effects against NMDA-induced neuronal damage. While substantial agents is restricted because they cannot enter the blood ocular barrier [10].

Memantine is a representative drug in NMDA receptor blockers. It can activate the nervous system, in the synaptic gap with a large number of glutamate, given a certain concentration of memantine, with the glutamate concentration increased, the blocking effect is also increasing [11]. According to the theory of excitatory amino acid (EAA) poisoning, EAA in vivo can selectively activate NMDA receptors in physiological conditions, make calcium channels open, large amounts of calcium influx, regulate excitatory synaptic transmission and synaptic plasticity, but when the EAA transition activates NMDA, can cause intracellular calcium overload and then cause neuronal apoptosis, memantine is a rapid ion channel receptor blocker, blocking the cell membrane surface depends on the membrane voltage, these The characterization of memantine as a NMDA receptor blocker in the central nervous system in the special role of the application, the clinical use of memantine very good treatment. Recent studies have shown that glutamate excitotoxicity may be one of the mechanisms of RGCs injury in glaucoma patients [12]. The concentration of glutamate in the vitreous was generally elevated in patients with primary glaucoma and monkey and dog glaucoma models. When the concentration of glutamate in the vitreous remains at a high level, a large number of RGCs are subsequently apoptotic, but this effect is improved after administration of memantine[13]. Previous studies have shown that memantine has a good effect on RGCs damage and retinal ischemia and has a significant effect on optic nerve injury, suggesting that memantine is a safe and effective NMDA-type glutamate receptor blocker for RGCs [14]. In addition, the clinical trials of memantine showed a significant improvement in AD and vascular dementia in patients with cognitive dysfunction, lack of neurological exercise, depression and so on, AD and vascular dementia have a significant effect. Currently, the only drug approved by the FDA is for the treatment of moderate to severe Alzheimer's disease. In animal experiments, the treatment of monkey glaucoma with memantine[15], the visual evoked potential proved that memantine has a significant protective effect on RGCs, Vorwerk and memantine and glutamate into the rat vitreous at the same time, Amino acid group, the number of RGCs increased significantly. It is thought that memantine is a potent and side-effect NMDA receptor blocker. But did not show a better effect than placebo in the three trials of glaucoma [16].

In recent years it has been reported that some traditional Chinese medicine or natural extract can improve eye blood flow and promote axonal regeneration [17,18]. Methyl3,4-dihydroxybenzoate

(MDHB) is a chemical compound that extracted from Malan. It has the moclcular weight of 168.15. The chemical formula is C8H8O4, with long bonds and attached single and double chain. The chemical structure is as Fig.1.

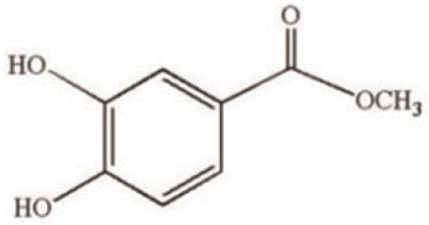


Fig. 1 The chemical structural formula of MDHB

Previous studies have shown that MDHB can protect against cortical neurons in vitro against against β-amyloid protein (Aβ), There is no report that MDHB has a protective effect on RGCs in vivo.hile promote neurite outgrowth of cortical neurons cultured in vitro[9]. MDHB promotes neurite outgrowth of cortical neurons cultured in vitro[19]. Besides MDHB extends the lifespan of Caenorhabditis elegans, partly via W06A7.4 gene[20]. The mechanism is probably that act on the pathway of apoptosis, inhibiting the expression of apoptotic protein[21]. The previous results imply that MDHB might be a potential medicine for neuronal regeneration neuropathy[22,23]. Neuroprotective therapies focus on RGCs regeneration factors the survival of RGCs. MDHB is structurally similar to catecholamine. while testing the influence of MDHB administration. The model was made by intravitreal NMDA injection bilaterally in the subject rats' eyes. The purpose of this study is to verify the effects and possible mechanisms of MDHB on NMDA-induced injury in rat retina. Considering that too many agents cannot transmit the brain blood barrier (BBB), while this experiment provides a new view that an agent with small molecular weight which can transmit BBB quickly that treat neurodegeneration diseases. The focus of this test is that if administration of MDHB exerts neuroprotective effect just as neurotrophin in retina diseases.

2. Materials and Methods

2.1 Animals

Animals We use SPF grade Sprague-Dawley rats as experimental subjects, half of the animals are males and the rest are females, weighing 200 ± 20g, purchased from Guangdong Medical Laboratory Animal Center (certificate number: SCXK Guangdong 20008-0002). Animals were housed at room temperature 25 ± 2°C and a relative humidity of 60 ± 10%. With 12/12 day and night cycle light patterns, all animals were kept with adequate food and water supply. The experimental animals meet the experimental standards of the Guangdong Provincial Ophthalmology Research Association, and follow the "Jinan University Medical College Animal Use Ordinance" and the requirements of ethics, with minimal animal and minimize the principle of animal suffering.

2.2 Methods

The tested animals were anesthetized with intraperitoneal injection of chloral hydrate (380mg/kg body weight), and dilated with normal saline. Intravitreal injections were performed on both eyes, and tropicamide was employed to dilate pupils. Using a needle connected to a 50μl micro-syringe approximately 1mm behind the dorsal corneal limbus, then utilizing 1μl of 50mM. The dosage was equal to the 40nmol NMDA, which was purchased from Sigma Shanghai, China. It would be injected into the vitreous, the glass electrode was kept in the vitreous for 10 seconds, and then the glass electrode was pulled out mildly, followed by a drop of levofloxacin after NMDA injection to each eye, every SD rat that developed cataract was rejected from this study. All the animal experiments were conducted on the base of the experiment protocol. After administrated 380mg/kg chloral hydrate to the rats, the rats' retina were taken from the eye. Then the rats were sacrificed by cervical dislocation under the anesthesia of choral hydrate. All the procedure of this experiment obey the rule of human endpoint and insure animals' welfare.The experiment was divided into 6 groups: the blank group was

not treated, and the other groups were injected intraperitoneally with 1μl of 40mM NMDA. Each group of SD rats 12, weight 200 ± 20g, for 5 days of administration. The blank control group was given saline (NS);model group given NS; positive drug given Memantine 10mg/kg; MDHB low dose group: given MDHB 25mg/kg; MDHB medium dose group: given MDHB 50mg/kg; MDHB high dose group: given MDHB 100mg/kg; The whole experiment procedures were approved by competent ethics committees in Jinan University, and performed in rigid accordance with the recommendations in the Guidance for the Care and Use of Laboratory Animals of the National Institutes of Health. Every effort was taken to relieve the animals' suffering. Sprague-Dawley rats, weighing 200±20g, half males and half females were purchased from Experimental Animal Center of Guangdong Province, China. All animals were kept under standard laboratory conditions with 12h/12h light/dark cycles and were supplied with regular and adequate food and water. Rats were randomly divided into six groups and each group contains 12 rats.

2.2.1 MDHB Traverse Bbb Test Via HPLC-MS

The HPLC system was equipped with UV detector (Aglient Technology USA). Chromatogrphic separation was performed on a Phenonex C 18 column. The mobile phase consisted of methanol and water and was delivered at a flow rate of 1.0ml/min. Chromatograms were monitored at a wavelength of 330nm. The temperature of column was kept 35 °C.

Visual function behavior test

2.2.2 Visual Function Behavior Test

The visual behavior test was performed on the 4th day after NMDA injection in both eyes. The black-white box is a practical instrument to survey visual recognition ability. The box consists of a dark chamber, (30cm×50cm×50cm) and a larger white chamber (50cm×50cm×50cm, illuminated with bright white light). An aperture (30cm×50cm) is situated between the black and white chamber. The black chamber was illuminated with no light, while the white chamber was illuminated by a bright light. Two cameras were installed in the two chambers respectively, monitoring the rats' activities, and the system was linked to Noduls Ethovision XT 8.0 software. The rats were placed in the middle of the white box, the duration is 5 minutes. This test index was contained the number of intercompartmental crosses, the mean velocity, the time in the black zone.Fig.2.

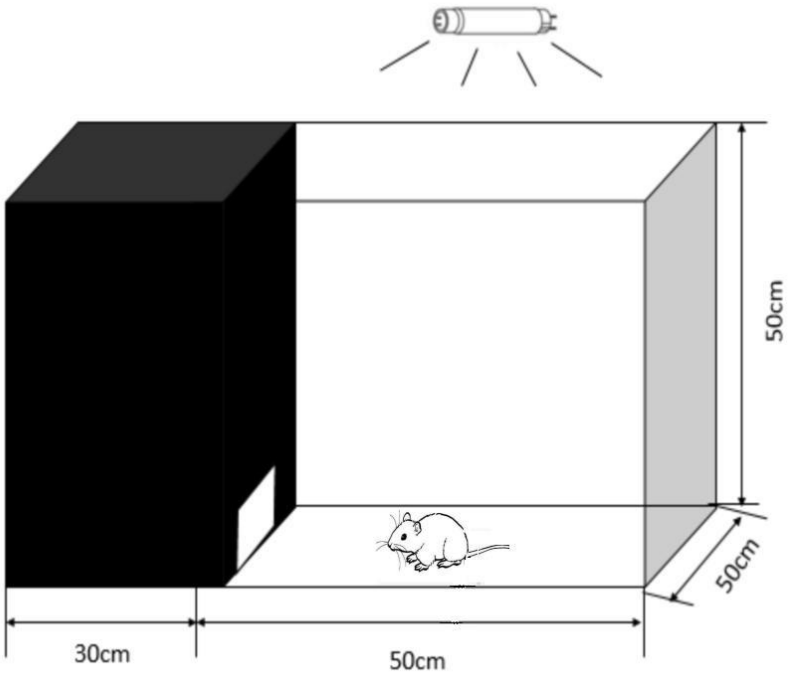


Fig. 2 The sketch drawing of White and Black Box

2.2.3 Morphological Analysis

In this experiment the RGCs munber were counted by Immunohistochemistry. After SD rats were intraperitoneally injected with NMDA for 5 days, then SD rats were intraperitoneal injected with chloral hydrate, the eyeballs were removed and placed in precooled PBS. The retina was stripped under the stereomicroscope and the retina was covered with four leaf Smooth filter paper, placed in 24-well plate, adding 4% paraformaldehyde fixed at room temperature for 2h. 4% paraformaldehyde was aspirated with plastic tube, rinsed with PBS buffer 6 times, PBS was changed every 10min. After washing, 300μl of blocking solution was added to the well plate. The blocking solution was prepared by 5% BSA and 3% PBST solution, After blocking for 2 hours, add the blocking solution, add 300μl of anti-dilution (from the ratio of 1: 200 Brn-3a and pre-cold PBST mixed preparation), incubated for more than 36h, PBS washed 3 times, each time 10min. Add the ratio of 1: 1000 goat anti-mouse secondary antibody dilution, with tin foil 24-well plate wrapped tightly, placed at room temperature for 3h, and then washed with PBS secondary antibody 3 times, each 10min. Finally, the retina was tiled on a slide and sealed with a quencher. After dark treatment, place the refrigerator at 4 °C.

The immunohistochemical stained slides were placed under fluorescence microscope, and the focal length was adjusted. Excited light with excitation at 488 nm was observed. GFP channel was observed under microscope. The positive RGCs labeled with Brn-3a were marked with green. It will be adjusted to the upper left corner of the retina, with the first complete field of view as the origin of the right, down 1mm, select the field shot. The photos were taken in accordance with this method into the Image-J software. The number of positive cells per slice was calculated and the density of the nodal cells was calculated from the size of each slice. Finally, the cell density calculated by each group was statistically analyzed and the conclusion was drawn. The number and intensity of the surviving RGCs have been evaluated using a manual method. That is counting the whole-mount RGCs with 1mm interval lattice method. The rats were anesthetized by injection of 10% chloral hydrate after the ERG test. Animals were euthanized by anesthetic overdose and the eyes were enucleated. Whole mount retinas were collected and fixed with 4% paraformaldehyde in the 0.01M PBS for 2 hour, then the retinas were washed and placed by 0.01M PBS containing 5%normal donkey serum with 0.3%Trition-X-100 for 2h. After that the retinas were incubated in goat-anti-Brn-3a which marked the RGCs, antibody (1:200, Santa Cruz) for more than 36hours. Secondary antibody was applied after wash the retinas for 3 times with 0.01M PBS, and the wash duration was 10minutes each time. Thorough washes were performed and retina ganglion cells were sampled and the number of marked RGCs with Brn-3a was counted. Survival of RGC was assessed by counting the number of Brn-3a-positive cells in the flat whole mount retina under a fluorescent microscope(Leica 6000, Germany), The size of the image is 641μm479μm , The photos were taken by separating from each other for 1mm, cover the retina. The number of Brn-3a positive cells was numbered from all images adopting the cell counter of the image-J software program. Finally the average number of survival RGCs was calculated per square millimeter.

HE staining was used in to observe the morphacological changes.The eyes were immersed in 4% paraformaldehyde in 0.1M phosphate sodium buffer for overnight at 4°C. The embedded tissue was placed on a paraffin section and sliced 5μm in thickness. Baking sheet: The slices were placed on a baking machine, the temperature was 37°C , 2h; Dewaxing: The sections were placed in xylene I, xylene II, 95% ethanol, 90% ethanol, 85% ethanol, 75% ethanol solution for 5 min, washed for 1 min; Staining: hematoxylin staining 5min, washed 1min; with 1% hydrochloric acid alcohol differentiation 10 seconds, washed 10min; Iraq red staining 5min, washed 1min;Dehydration: the above sections were placed in 75% ethanol, 85 ethanol, 90 ethanol, anhydrous ethanol Ⅰ,anhydrous ethanol Ⅱ 2min, xylene Ⅰ, Ⅱ 5min; Seal: transparent with xylene, neutral resin seal. Dough: the sealed slide placed in the 37° C baking machine 2min, the film. The morphology of the layers of the retina was observed under the optical microscope and the film was taken. Then the eyes were dehydrated and embedded in paraffin. Transverse sections were stained with a thickness of 5μm were made through the optic disc. This section would be stained by hematoxylin and eosin. The injury caused by

NDMA in retina would be shown in the number of survival RGCs and the thickness of inner plexiform layer.

2.2.4 Electroretinogram Test

After the behavior test, rats were placed in dark place to dark adaptation for 12hours, and the ERG were recorded using protocols as described. The animals were anesthetized with chloral with hydrate 380mg/kg body weight. The pupils were dilated by topical application of tropicamide (0.5%) and the eyes were lubricated with 1% methylclleuose. Animals were placed on a warm platform that adopted a circulating pump-water bath to maintain a constant temperature. ERG test was recorded with gold-plated wire loop electrodes toughing the corneal surface as the active electrodes. Stainless steel needle electrodes were inserted into the supraorbital margin near the eye and into the tail, serving as reference and ground electrode, respectively. Tested animals were light adapted for 5min with bright green background. Photopic responses to green flashes of 10cd/m2, were recorded using a Ganzfeld of a Roland RETI-scan system (Roland Consult, Branednburg, Germany). ERG data was gathered through the amplifier of the RETI-sacn system at a sampling rate of 2kHz. ERG waveforms were analyzed with Clampfit 10.2 after applying 50 Hz low-pass filtering.

2.2.5 Western Blot Analysis

After the end of the electrophysiological experiment, take the blank EP tube weighing, rat intraperitoneal injection of 10% chloral hydrate anesthesia, in the ice quickly stripped of the retina, into the 1.5ml of the EP tube, weighing again, the difference between two value is the quality of the retina, and quickly frozen in the -80refrigerator, the entire process are running on the ice to prevent degradation of the protein in the retina.

Extraction of total protein and protein quantification. The lysate was prepared by adding RIPA and PMSF to 1:100, and the phosphorylated protease inhibitor was added. The lysate was added into the EG tube with the ratio of retinal mass: lysis solution = 1mg: 10μl. The cracked product was lysed on the ice on the ice, the cycle of 2 to 3 times until the tube without block after the organization, set the ice for 30min,then put the EP tube into the 4°C freeze centrifuge 12000 rpm rotation speed about 15min, suck the supernatant, placed on ice The Take a small amount of supernatant diluted 50 times with saline, 5mg/ml standard protein diluted 5 times, according to BCA protein quantitative kit instructions.

In this study, protein concentration was measured using bisquinoline formic acid (BCA) reagent For Western blot experiments, the residual rats were euthanized. Five days after initially being injected with NMDA retina from control treated eyes were homogenized and lysed for 30 minutes with PIRA and PMSF (50:1). After centrifugation, Immuno-positive 30 was determined using a bicinchoninic acid (BCA) assay kit (Beyotime). 40 μg of retinal protein per lane was loaded and separated electrophoretically on a 12%SDS-PAGE gel, and then was transferred to a poly of soluble retinal protein per lane was loaded and separated electrophoretically on a 12% SDS-PAGE gel, then was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Antibodies used were the following: primary antibodies raised in rabbits included anti-caspase-3, anti-cleaved caspase-3, anti-Bax, anti-Bcl-2 and anti-β-actin(all used at 1:1000). Secondary antibody was horseradish peroxidase (HRP)–conjugated goat-anti–rabbit antibody (1:5000). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, US). Immuno-positive bands were revealed with enhanced chemiluminescence (Millipore).Image processing and grey value were performed with Quantity one software (Bio-Rad). Measurements were repeated more than three times for each experiment.

3. Results

3.1 MDHB Can Traverse the Blood Retina Barrier in 2 Minutes

MDHB can traverse the blood retina barrier in 2minutes, after 30 minutes, MDHB decrease sharply. After intraperitoneal injection of MDHB, it can reach the retina through the blood-eye barrier soon. After intraperitoneal injection of MDHB for 2min, 30min, it can be detected by HPLC in the retina of

MDHB, indicating that MDHB can quickly through the blood eye barrier to reach the retina, 30min after the drug concentration rapidly reduced to 3%.Fig.3.

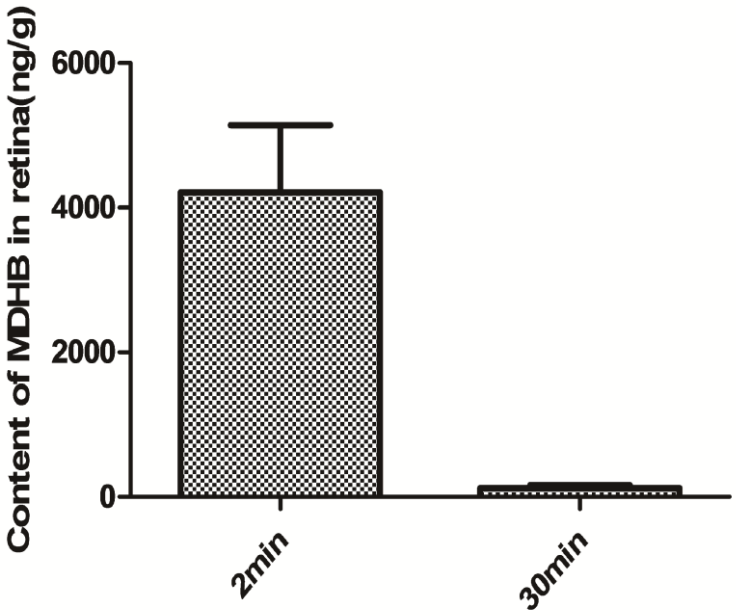
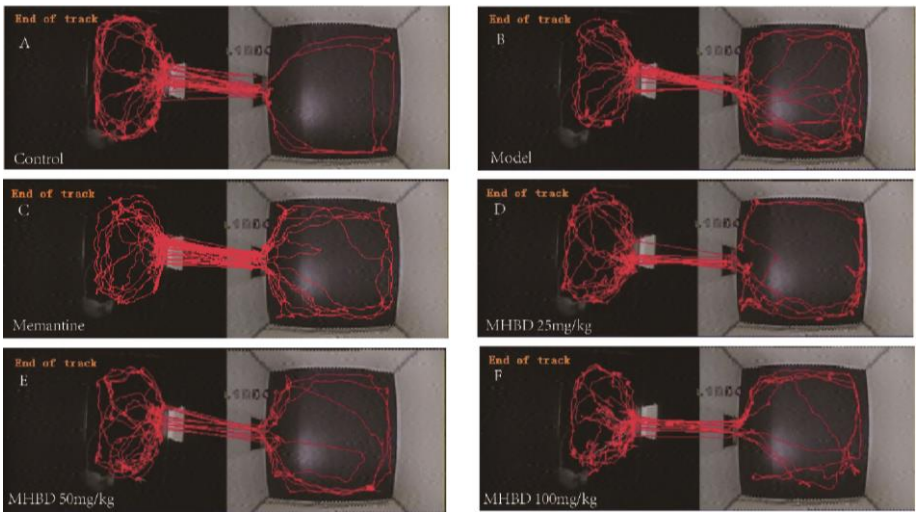


Fig. 3 MDHB can traverse the blood retina barrier in 2minutes, after 30 minutes, MDHB decrease sharply.

3.2 Black and White Box Test

According to the habits of rats in the dark and narrow space, the visual behavior of rats was measured to reflect their visual function. By examining the residence time of the rats in the black box to determine whether the rats after imposing MDHB can improve visual function. In the middle of the black and white box in a central room with a lighting, recording the rat into the black box frequency and stay time. Then the intravitreal injection of NMDA, and then observe the rats stay in the dark room time, found to 72% of the control group. After intraperitoneal injection of MDHB, the residence time of the rats in the black box was significantly prolonged. The tracks of SD rats in black-white box. Behavior condition of rats in the black and white box. The representative rats in the control group spent more time in the black box control group (B). Meanwhile (D,E,F) indicates that MDHB extend the time in the black chamber. The results are reported as the mean±SEM. #P<0.05 versus the control group. *P<0.05 versus the model group (n=13)(Fig.4).



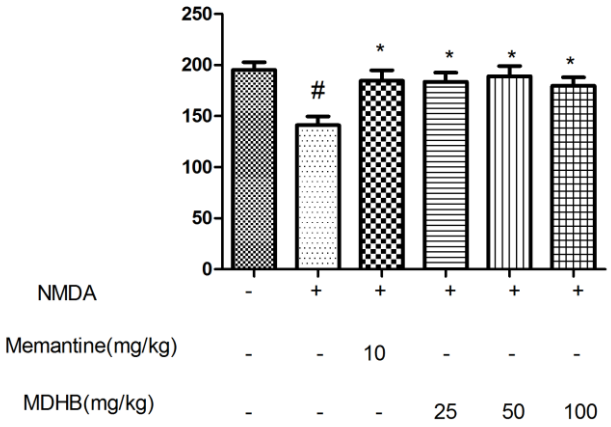
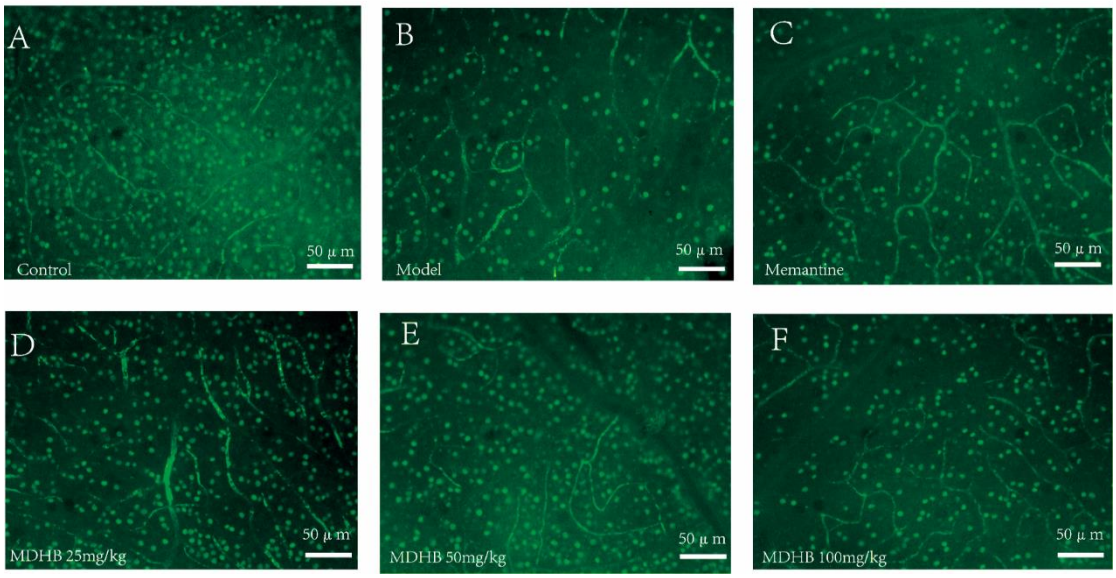


Fig. 4 Behavior condition of rats in the black and white box. The representative rats in the control group spent more time in the black box than in the white chamber(A). In the NDMA group, the rats spent less time than the control group (B). Meanwhile (D,E,F) indicates that MDHB extend the time in the black chamber. The results are reported as the mean±SEM. #P<0.05 versus the control group. *P<0.05 versus the model group **P<0.01 versus the model group(n=18).

3.3 Morphological Results

Effects of MDHB on the density of retinal ganglion cells after NMDA injury. To investigate the potential protective effect of MDHB on RGC, it was observed by Brn-3a (RGC-specific biomarker) staining. In the control group, the number of Brn-3a positive cells was significantly decreased after 5 days of model establishment, while the number of surviving RGCs increased significantly after MDHB administration, suggesting that MDHB had protective effect on RGC.

After the 6th day of intravitreal injection of NMDA, the cells in retina layer were shown above. The ganglion cell number of control group was more intensive than model group, while under the post-treatment of MDHB, the survival ganglion cell number was increased, the memantine group can also reverse the injury caused by NMDA. The effects of MDHB on NMDA-induced the change in the number of RGCs in SD rats. The number of RGCs in the NMDA group was significantly reduced compared with control (A) via the Brn-3a marked cells. MDHB increase the positive number of memantine group and MDHB group. The number of RGCs wad significantly reduced. The data represent the Mean±SEM. *P<0.05 versus control group; #P<0.05 versus NMDA group(n=6)(Fig.5).



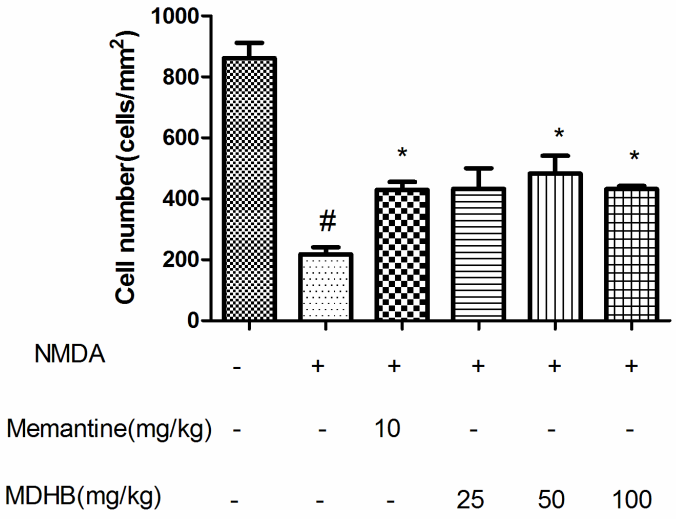


Fig. 5 The effects of MDHB on NMDA-induced the change in the number of RGCs in SD rats. The number of RGCs in the NDMA group was significantly reduced compared with control (A) via the Brn-3a marked cells. MDHB increased the positive number of memantine group and MDHB group. The number of RGCs was significantly reduced. The data represent the mean ±SEM. *P <0.05 versus control group; #P <0.05 versus NMDA group(n=6).

HE staining was used to observe the morphological changes of the cells in the retina after NMDA injury. The number of ganglion cells in the model group was significantly decreased, the number of ganglion cells in the nucleus layer, the positive drug and the MDHB group were increased. Data in H&E staining indicates that MDHB could cause neuroprotective effect in the RGCs. The survival RGCs number in MDHB 50mg/kg group increased significantly compared to the NMDA group(Fig.6).

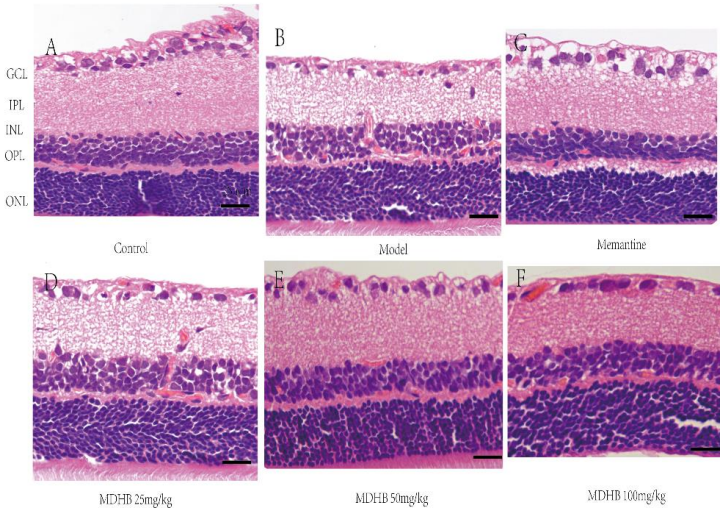


Fig. 6 After the 6th day of intravitreal injection of NDMA, the cells in retina layer were shown above. The ganglion cell number of control group was more intensive than model group. While under the post-treatment of MDHB, the survival ganglion cell number was increased, the memantine group could also reverse the injury caused by NDMA.

3.4 Western Blotting Results

Previous studies have shown that MDHB can improve the visual behavior of RGCs impaired animals and maintain more RGC survival. In order to elucidate the neuroprotective mechanism of MDHB, this study is intended to explore molecular and signal transduction pathways. NMDA is known to induce apoptosis, so this experiment first from the MDHB can affect the apoptosis to start. Changes of apoptosis - related proteins induced by intravitreal injection of NMDA vitreous. For example, the

expression of proapoptotic protein Bax and activated cysteine aspartate protease-3 (Cleaved Caspase-3) increased, while the expression of anti-apoptotic protein Bcl-2 was reduced. The expression levels of activated caspase-3 and Bax were significantly increased after NMDA injury, while the expression levels of Caspase-3 and anti-apoptotic protein Bcl-2 were very small. (P <0.05), it was inferred that the apoptotic process was activated by the ratio of activated type Cleaved Caspase-3/Caspase-3 and Bax/Bcl-2, respectively. These rates were significantly lower after administration of 50 mg / kg MDHB (P<0.05). Suggesting that MDHB has the effect of inhibiting NMDA-induced retinal ganglion cell apoptosis.

The expression of MDHB of Bax/Bcl-2 in RGCs. The expression of apoptotic protein Bax/Bcl-2 value in NMDA-induced retina, the bcl-2 was significantly increased in the NMDA group compared with the control group.Fig.6

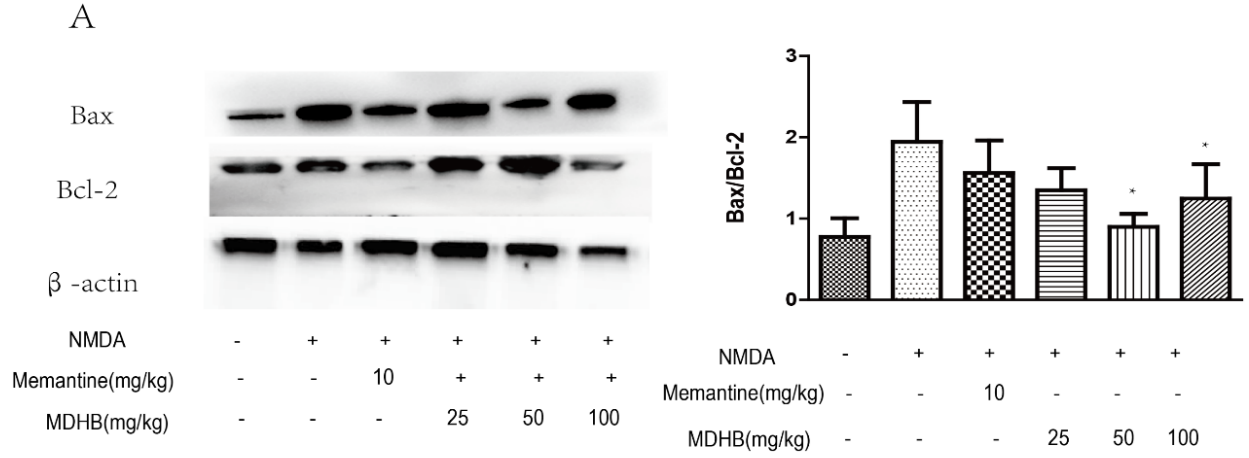


Fig. 7 The expression of apoptotic protein Bcl-2, Caspase3, Cleaved caspase3 protein expressed in NMDA-induced retina, the bcl-2 was significantly increased in the NMDA group compared with the control group. The ratio of cleaved caspase3/caspase3 was significantly elevated in the NMDA group, while it was reserved by MDHB, *P<0.05 versus the control group. (n=4). Western blot of phosphorylated Akt in NMDA-induced retina damage. The ratio could be reversed by the MDHB group. The results are reported as the mean±SEM. #P <0.05 versus the NMDA group (n=3).

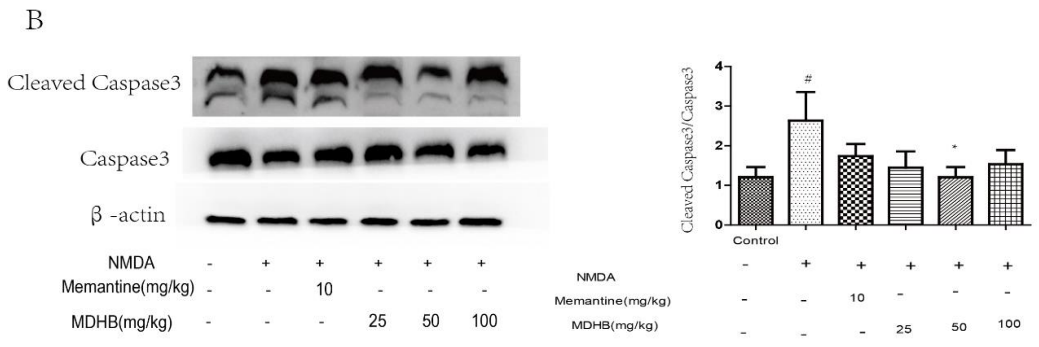


Fig. 8 The effects of MDHB on the expression of Cleaved Caspase-3/Caspase-3.The ratio of Cleaved Caspase-3/Caspase-3 was significantly elevated in the NMDA group, while it was reserved by MDHB, *P<0.05 versus the control group (n=4).

Effect of MDHB on phosphorylation of Akt. Phosphorylation of Akt in cell signaling plays an important role in the phosphorylation of Akt can activate the downstream pathway to promote cell

survival, the use of Western blot detection of phosphorylation of Akt expression, compared with the non-phosphorylation, found MDHB25mg / Kg group and MDHB50mg/kg group (P <0.05). The expression of Akt protein expressed in NMDA-induced retina.

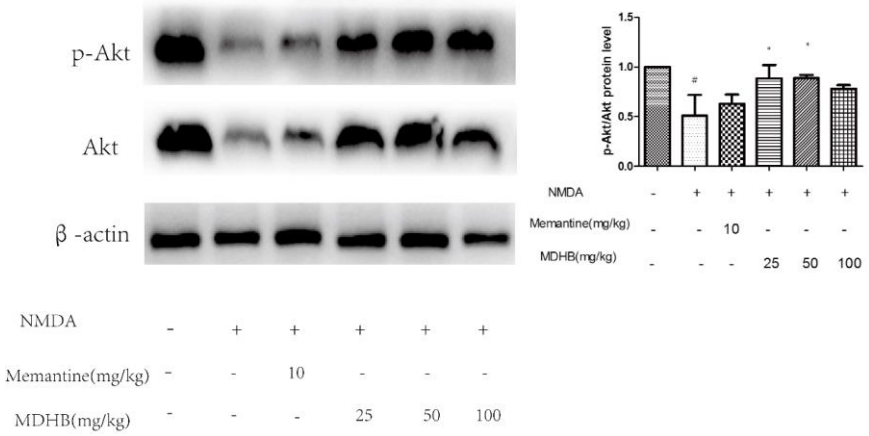


Fig. 9 Western blot of phosphorylated Akt in NMDA-induced retina damage. The ratio could be reversed by the MDHB group. The results are reported as the mean±SD. #P <0.05 versus the NMDA group (n=3)Fig.

3.5 Effects of MDHB on Photoreactivity of Retinal Ganglion Cells after NMDA Injury in Rats

In the ERG experiment, the responses of the rats to each light intensity were detected under dark adaptation and adaptation, respectively. There was no PhNR wave in the dark environment. Therefore, photopic analysis was performed under light adaptation. PhNR is the first trough position after b-wave (Figure 10). The amplitude of PhNR reflects the activity of ganglion cells. The amplitude of MDHB50mg/kg group is significantly increased under photopic10.0 condition (P<0.05).The change of MDHB on NMDA-induced changes in ERG in SD rats. Picture A represent the PhNR wave. Statistical analysis of mean amplitudes of PhNR waves of and photopic 10.0. The amplitude of NMDA group is significantly reduced, while administration of MDHB, it can slightly enhance the amplitude.

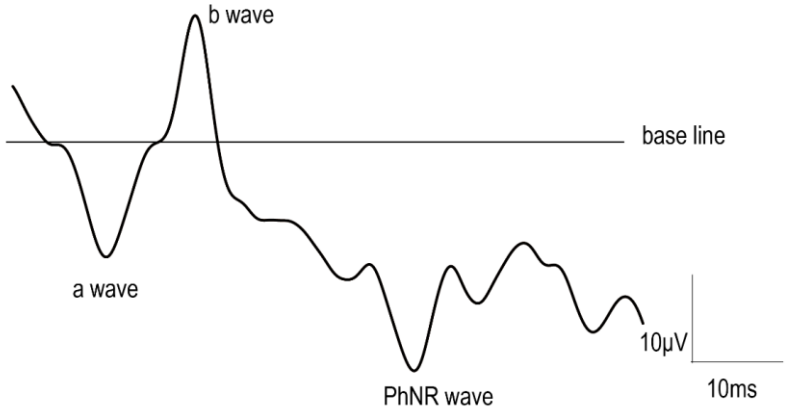


Fig. 10 The change of MDHB on NMDA-induced changes in ERG in SD rats. Picture A represents the PhNR wave. Statistical analysis of means amplitudes of PhNR waves of and photopic 10.0. The amplitude of NMDA group is significantly reduced, while administration of MDHB, it can slightly enhance the amplitude. The data in all bar graphs is shown relative to control. Data represents mean±SEM. #P<0.05versus control; *P<0.05 versus the NMDA group(n=6).

4. Disscussion

The purpose of this study is focus on the protection of the optic nerve and RGCs in order to find the neuroprotective drugs, meanwhile explore the optic nerve protection drug scientific research methods. The neuroprotective effect of MDHB on retinal ganglion cells in SD rats and its mechanism were studied by behavioral, morphological, molecular biology and electrophysiology. The results showed that MDHB had different protective effects on the morphology and visual function of rat retinal ganglion cells.

There are many kinds of retinal ganglion cell damage models [24], such as traumatic optic nerve injury, excitatory neurotoxicity injury, anterior chamber perfusion, laser damage and so on. RGCs injury model caused by NDMA has been reported for many years. Many research results have shown that many neurological diseases are manifested as endogenous glutamate and the activation of glutamate receptor, It is neurotoxic in the retina which can cause severe optic nerve injury [25]. The mechanism of NMDA injury is that NMDA is an excitatory amino acid, it can activate NMDA receptor, causing calcium influx. The intracellular calcium will be overload eventually leading RGCs to apoptosis; In addition, NMDA can also cause eye vasoconstriction, ischemia hypoxia and oxidative stress, calcium ions can also be activated after the activation of nitric oxide synthase, resulting in free radical damage (26). NMDA-induced excitotoxicity is an animal model commonly used to explore the pathogenesis of various degenerative diseases such as glaucoma, which can better predict the pathogenesis of optic nerve injury [27]. In this study, intravitreal injection of NMDA, the concentration of 40mmol/L, The results of the preliminary study showed that MDHB could improve the visual behavior of the model rats, alleviate NMDA-induced apoptosis, maintain the relative density of RGCs and improve the photoreaction intensity of RGCs, that is, MDHB had neuroprotective effects and nutrition effect.

The results have shown that the neuroprotective effect of MDHB on RGCs. In this study, the behavioral changes of the rats were measured by black and white box. The behavior test was performed after five consecutive days of administration. The results showed that the blank group. The time of the rats in the black box was 192.80 ± 21.17 s. The model group had a significant difference ($P<0.05$) in the black box time of 141.15±34.17s. The time of the drug group was prolonged in the black box, the time of the positive drug group was 184.75±33.32s, the time duration of MDHB25mg/kg group was 183.70±237.95s, the MDHB 50mg/kg was 188.98±33.36s, the MDHB100mg/kg group ($P <0.05$). The experimental results showed that the visual behavior of the rats was significantly improved after administration. The results showed that the visual behavior of the rats was significantly higher than that of the control group ($P <0.05$).

In this study, immunofluorescence was used to detect the survival rate of rat ganglion cells. Brn-3a was a subfamily of POU family transcription factor [28], The family member of POU includes Brn-3a, Brn-3b and Brn-3c in rat RGCs. The expression of Brn-3a can be a marker of the survival RGCs in rat retina in vitro, besides Brn-3a does not change the pattern of expression after retinal damage, so Brn-3a can be used as a marker for survival of RGCs. Brn-3a can be used as a marker of surviving RGCs. Reliable markers and the number of RGCs that can be quantified with Brn-3a [29]. The number of positive cells in the blank control group was 861.69±123.79, and the number of viable cells in the model group was 217.97± 46.47. The number of ganglion cells in the model group was significantly lower than that in the model group ($P<0.05$). Positive group, MDHB 25mg/kg group, MDHB50mg/kg group and MDHB100mg / kg group, the positive drug group was 429.77±44.24, the MDHB 50mg/kg group was 482.57±155.96, and the MDHB100mg/kg group was 432.49±25.53 Compared with the model group was statistically significant ($P<0.05$). MDHB can also improve the sensitivity of ganglion cells to photoreaction. ERG is an important tool to detect visual electrophysiology. This experiment is used to detect the change of the negative reaction wave of the ganglion, that is, the change of PhNR wave, PhNR wave in the electroretinogram The amplitude of the first trough after the b-wave is reflected by the change in the light-sensitive intensity of the nodal cells. Under the condition of photopic 10.0, the amplitude of each group was improved compared

with the model group, the intensity of light reaction increased after treatment. The amplitude of the blank control group was 18.16±3.29 and the model group was 8.23±4.55. The results showed that the nodal cells in the model group were significantly lower than those in the control group ($P<0.05$). ($P<0.05$). The results showed that MDHB50mg/kg was significantly higher than that of model group ($P<0.05$).

Although most of the drugs cannot pass through the blood-eye barrier, such as neurotrophic factors, neurotrophic factors in the nervous system development, to maintain the nervous system, but also in the treatment of neuropathy[30], Function has an important role in the early neurotrophic agent is a nerve growth factor to ensure and maintain the survival of cholinergic neurons such as BDNF, NGF, CNTF, these drugs cannot pass through the blood-brain barrier must be through the extracorporeal circulation system connected to the ventricle Intubation to achieve, and can cause significant headache, pain and other symptoms, thus hindering the in-depth study of such drugs, while the eye drops of the bioavailability of low[31]. It is important to look for drugs that can pass through the blood-eye barrier and have a protective target on the retina and play a protective role [32]. The molecular weight of MDHB used in this experiment is 168.15, the small molecular weight make it easy to pass through the blood eye barrier, after 2 minutes of intraperitoneal injection can be detected by HPLC in the retina. If an agent could come into the market, it should be comply with the criterion as follow: the presence of specific receptors on the retina and the optic nerve; the activation of the target receptor has the effect of protecting the cells in the retina and the optic nerve; the drug should be distributed to the retina in a safe pharmacological range dose; the optic nerve protection effects should be validated clinically [33]. In combination with the experimental data, MDHB as a small molecular weight drug basically meets the above requirements, in addition to the distribution of adenosine receptors in the eye, there are currently listed drugs blocking adenosine A3 receptor treatment of glaucoma, previous studies have shown that MDHB may be Through the adenosine receptor play a role, pending further experimental study [34]. In addition, in vitro experiments, MDHB found that anti-oxidative stress and other damage to play the role of protection of RGC-5. MDHB therefore has an effect on NDMA-induced apoptosis of retinal ganglion cells.

The formula of MDHB shows that it contains two adjacent hydroxide groups, which suggests that MDHB may have antioxidant and anti-free radicals effects. 3,4-DHBA can inhibit Aβ-induced calcium overload, inhibit apoptosis, and MDHB structural formula contains ester bond, which enhances the ability of lipophilic, make it traverse the blood-brain blood barrier easily. Research have shown that the Aβ content increased in glaucoma patients [35], early experiments confirmed that MDHB can resist Aβ-induced neuronal apoptosis, the mechanism is by up-regulation of Bcl-2 expression, down-regulation of Bax expression, inhibition of Cleaved caspase-3 apoptosis pathways, this experiment also confirmed that the protective mechanism of MDHB on RGCs can be attribute to inhibit the expression of the apoptotic pathway in retina in vivo[36]. Western blot analysis showed that MDHB could enhance the expression of anti-apoptotic protein Bcl-2 and decrease the level of Bax. When the vitreous injection of NMDA, the concentration in the retina, activation of NMDA receptors lead to a large number of calcium influx, and then increase free radicals and activation of nitric oxide synthase, activation of apoptosis pathway leading to RGCs apoptosis[37]. The expression of Bax/Bcl-2 protein was statistically significant. Bcl-2 was associated with apoptosis-related gene family [38]. The family was divided into two groups: one was the gene that promoted apoptosis, the representative genes is Bax, Bad, the other is anti-apoptotic gene, the representative gene is Bcl-2, Mcl-2[39]. The expression of Bax was up-regulated in the progression of apoptosis, and the expression of Bcl-2 protein was decreased according to the expression of two proteins. The ratio of MDHB 50mg/kg and MHDB 100mg/kg group was significantly higher than that of model group ($P<0.05$). The results indicating that MDHB has the ability to inhibit the expression of apoptotic protein Bax and enhance the expression of anti-apoptotic Bcl-2. Caspase-3 plays an important role in the Caspase family, it is an executor of apoptosis. If Caspase-3 is activated, it can also stimulate the downstream pathway, causing apoptosis cascade response [40]. Celaved Caspase-3 is a spliceosome that performs an upstream apoptotic signal [41]. The increase protein expression suggests that the

increase in cell apoptosis is based on this mechanism. In this study, the classical protein of apoptotic pathway was used for the detection of apoptosis [42]. The Cleaved Caspase-3/Caspase3 ratio was used to calculate the condition of apoptosis. The ratio of MDHB50mg/kg was statistically different from that of model group ($P<0.05$). The expression of Bax/Bcl-2, Celaved Caspase3 / Caspase-3 in each group showed that MDHB can resistant the injury induced by NMDA by inhibiting apoptosis process.The results show that MHDB can activate phosphorylated Akt, Akt plays an important role in signal transduction, while it take participates in multiple signaling pathways. Phosphorylated Akt may play a protective role in activating downstream GSK-3β [43,44], which needs to be further confirmed. In this study, phosphorylation of ERK and BDNF was also observed. Phosphorylated ERK plays an important role in cell migration and survival [45]. MDHB has protective effect on rat retinal ganglion cells, it is concluded that MDHB can protect retinal ganglion cells, the optimal dosage is 50mg/kg group.

5. Statistical Analysis

In this study, the data of each group were expressed by Mean ± SD, and SPSS16.0 was used for statistical analysis. Single factor analysis of variance was used to compare the two groups. After variance homogeneity test, the variance is homogeneous with S-N-K, and the variance is different. Tamhane's is used to compare the two groups. To check the difference between the significance of each group, $P<0.05$ that there are significant differences. $P<0.01$ was considered very significant difference.

6. Conclusion

MDHB can protect the retina from injury against NMDA toxicity, the mechanism may contribute to the enhance expression of anti-apoptosis proteins such as Bcl-2 and inhibit the expression of apoptosis protein Bax, Caspase-3, increase the expression of Akt protein, finally MDHB promote the survival of RGCs. It may open up a novel agent with small molecular weight, which have the neurotrophic effect therapeutic approach for the retina disease.

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Compliance with Ethical Standards

Conflict of interest No conflicting relationship exists for any author.

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