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Short Communication

Neuroprotective Effect of Excitatory Amino Acid Receptor Antagonist on Retina of Rat

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ABSTRACT

The objective of this study was to investigate the protective effect of the excitatory amino acid receptor antagonist, α-melanocyte stimulating hormone, on the retina. Twenty-four Sprague Dawley (SD) rats were randomly divided into normal control group, glutamate induced injury group and α-melanocyte stimulating hormone pretreatment group each with 8 rats, 16 eyes in each group. The content of neurotransmitter amino acids and the survival rate, apoptosis rate and mitochondrial membrane potential of the retinal nerve cells were compared between the three groups. According to our results the contents of free glutamate, γ-aminobutyric acid, taurine, glycine and aspartate were all higher in the induced injury group than in the normal control group, which were significantly lower in the intervention group than in the induced injury group, though higher than the normal control group, showing statistically significant difference between the three groups (P<0.05). Absorbance value of retinal nerve cells was significantly higher in the normal control group than in the induced injury group and the intervention group, which was significantly higher in the intervention group than in the induced injury group, showing statistically significant difference between the three groups (P<0.05). Retinal ganglion cell apoptosis rate was significantly higher in the induced injury group than in the normal control group, which was significantly lower in the intervention group than in the induced injury group, though higher than the normal control group, showing statistically significant difference between the three groups (P<0.05). The mitochondrial membrane potential of retinal nerve cell was obviously lower in the induced injury group than in the normal control group, which was obviously higher in the intervention group than in the induced injury group, though still lower than the normal control group, showing statistically significant difference between the three groups (P<0.05). It is concluded that excitatory amino acid receptor antagonist, α-melanocyte stimulating hormone, can effectively antagonize glutamate-induced apoptosis of retinal nerve cells, whose mechanism may be related to the enhancement of mitochondrial transmembrane potential.

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CF and WF collected the samples. JY and QC analysed the data. YW conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
Glutamate, α-melanocyte stimulating hormone, Retina, Apoptosis.

Glaucoma, diabetic retinopathy, age-related cataracts, and age-related macular degeneration are currently the four blinding eye diseases with the highest incidence in the world. The literature points out that all the above-mentioned blinding eye diseases may be accompanied by rising excitatory amino acid levels in the retina (Wang *et al*., 2016; Harada *et al*., 2013). Clinical studies have confirmed that diabetic retinopathy can increase the release of glutamate in patients; some cataract patients may have increased secondary intraocular pressure after surgery, which may ultimately cause an increase in glutamate expression. A large number of studies at home and abroad have confirmed that high concentrations of glutamate can cause excitotoxicity of the retina (Wang *et al*., 2017; Li *et al*., 2017), seriously affecting patients' quality of life. In recent years, some scholars have proposed that α-melanocyte stimulating hormone can effectively antagonize the apoptosis of hippocampal neurons in the brain caused by glutamate-induced excitotoxicity. Moreover, several studies have pointed out that α-melanocyte stimulating hormone also protects other parts of the nervous system (Zhang *et al*., 2016; Bertolesi *et al*., 2015). Retina is an important extension of the central

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nervous system. Whether α-melanocyte stimulating hormone has an effect on glutamate-induced retinal excitotoxicity is worth investigation. To this end, this study took SD rats as the experimental objects and induced rat retinal nerve cell apoptosis using high concentrations of glutamate to investigate the effect of α-melanocyte stimulating hormone on rat retinal nerve cell apoptosis.

Materials and methods

In this study, 24 SD rats (Experimental Animal Center of Sun Yat-Sen University) 7-8 days old, half male and half female healthy without eye diseases were used. They were randomly divided into three groups: normal control group, glutamate-induced injury group, and α-melanocyte stimulating hormone pretreatment group, each containing 8 rats and 16 eyes. Under topical anesthesia, the rats in the normal control group and induced injury group were intravitreally injected with 3μL autoclaved pure water. The pretreatment group rats were intravitreally injected with 3μL α-melanocyte stimulating hormone (Cclbiochem, USA) with a concentration of 3.3mg/mL, and levofloxacin eye drops were used (Ningxia Kangya Pharmaceutical Co., Ltd., National Medicine Permission Number H20103313) to prevent infection. The rats were then fed in an incubator for 24 h. Under topical anesthesia, the rats in the induced injury group and pretreatment group were intravitreally injected with 3 μL sodium glutamate solution at a concentration of 3.3 mg/mL, while the normal control group was intravitreally injected with 3 μL pure water. Levofloxacin eye drops were used for infection prevention, followed by placement in an incubator for 48 h before testing.

For determination of the content of free amino acids (FAA) in rat's retina 0.2g samples were homogenized in a 1.5ml centrifuge tube, in 1ml of 10% sulfosalicylic acid by ultrasonication . Blood samples (0.6ml) were treated with 10% sulfosalicylic acid solution (0.4ml). Supernatants obstipated with centrifugation at 14000r/min for 20min were analyzed for FAA by automatic amino acid analyzer (Hitachi L-8900, Japan).

For determination of rat retinal nerve cell density, survival rate, and apoptosis rate MIAS2000 image analysis system was used to analyze the density of retinal nerve cells. The tetrazolium salt (MTT) colorimetric method was used to determine survival rate of the rat retinal nerve cells indicated by absorbance value. AnnexinV/PI kit (Shenzhen Jingmei Electronic Technology Co., Limited) was used to detect cell apoptosis, for which AnnexinV/FITC (3 μL) was used to resuspend the cells with diluted binding buffer (200 μL) and incubated at room temperature for 10min in the dark. PI (2μL) was added, mixture incubated for 5min at room temperature in the dark and then subjected to flow cytometry (Beckman Coulter, USA), after addition of 300μL binding buffer.

For determination of mitochondrial membrane potential of rat retinal nerve cells, 500 μL of phenol red-free Dulbecco minimal essential medium (DMEM) was added to cells to configure JC-1 dye solution with a concentration of 0.2μL/mL (JC-1 fluorescent probe purchased From Molecular Probe, USA). The mixture was incubated for 30 min at 37°C in the dark and centrifuged for 5 min at 300 x g. PBS (500μL) to added to pellet to measure the cell mitochondrial membrane potential using a flow cytometer (Beckman Coulter, USA).

The data in this study were all processed by SPSS20.0 statistical analysis software (IBM, USA); measurement data is expressed by "mean ± standard deviation", and comparisons between multiple groups are performed by one-way analysis of variance or repeated measures analysis of variance. Pairwise comparisons are performed by LSD-t test; count data are expressed by percentage (%), and comparisons between multiple groups are performed by χ^2 analysis; $P<0.05$ indicates statistically significant difference.

Results and discussion

Table I shows the FAA in rat retina and blood serum which are significantly higher in retina of the induced injury group than in the normal control group ($P<0.05$). The free glutamate content is significantly lower in the intervention group than in the induced injury group, though higher than the normal control group, showing statistically significant difference between the three groups ($P<0.05$). There are no significant differences in serum FAA levels between the normal control group, induced injury group and intervention group ($P>0.05$).

Table II shows the density, apoptosis rate and mitochondrial membrane potential of retinal cells. The density of retinal nerve cells, absorbance values and mitochondrial membrane potential in the normal control group are significantly higher than those in induced injury group and intervention group, showing statistically significant difference ($P<0.05$). The intervention group has significantly higher density absorbance and mitochondrial membrane potential of retinal nerve cells than the induced injury group, showing statistically significant difference ($P<0.05$).

On the other hand, the apoptosis rate of retinal nerve cells was significantly higher in the induced injury group than in the normal control group (48.71±6.89 vs 5.36±0.92), showing statistically significant difference ($P<0.05$). The apoptosis rate of retinal nerve cells is 12.69±1.97% in the intervention group, which is significantly lower than that in the induced injury group, but still higher than the normal control group. The difference between the three groups is statistically significant ($P<0.05$).

Table I.- Effect of injury on free amino acid content in retina (mg/kg) and blood serum (mg/L) of rats (Mean ±SD).

Group	Control group (n=16)	Induced injury group (n=16)	Intervention group (n=16)	F value	P value
Amino acids of retina (mg/kg)					
Glutamate	248.72±23.15	438.29±34.66	314.36±28.17	15.231	0.001
γ-Aminobutyric acid	18.14±2.36	28.66±3.09	23.50±2.81	14.663	0.001
Taurine	1008.65±187.64	1219.44±180.39	1125.39±179.62	10.124	0.001
Glycine	130.81±19.32	201.33±19.40	168.27±17.20	15.896	0.001
Aspartate	134.70±18.33	197.65±23.17	162.34±20.05	15.636	0.001
Amino acids of blood serum (mg/L)					
Glutamate	24.91±3.26	24.68±3.31	25.64±3.29	0.631	0.259
γ-Aminobutyric acid	0.04±0.01	0.05±0.01	0.04±0.01	0.559	0.357
Taurine	24.68±3.18	25.11±3.10	25.02±3.09	0.628	0.751
Glycine	18.49±4.02	18.61±3.94	18.40±3.87	0.307	0.592
Aspartate	4.67±0.63	4.71±0.58	4.70±0.62	0.832	0.229

Table II.- Effect of injury on retinal nerve cell (RNC) density (cell/3 fields), absorbance value, apoptosis rate (70%) and mitochondrial membrane potential of retinal nerve cells of rats (Mean ± SD).

Group	Control group (n=16)	Induced injury group (n=16)	Intervention group (n=16)	F value	P value
Ganglion cell density	16.80±2.32	10.65±1.97	14.33±2.11	10.259	0.002
Absorbance value of RNC	0.82±0.13	0.49±0.08	0.70±0.10	11.163	0.002
Apoptosis rate (70%)	5.36±0.92	48.71±6.89	12.69±1.97	27.122	0.001
Mitochondrial membrane potential	0.835±0.132	0.483±0.067	0.687±0.101	15.625	0.001

In addition to being important nutrients and metabolites, amino acids *in vivo* also play an important role in the nervous system. Amino acids can be classified into excitatory amino acids and inhibitory amino acids, including glutamate, γ-aminobutyric acid, glycine, and aspartate, all of which belong to classic neurotransmitters. Where, glutamate and aspartic acid are excitatory amino acids, γ-aminobutyric acid and glycine are inhibitory amino acids (Zhang *et al*., 2016; Luo and Du, 2017). A large number of studies have pointed out that glutamate is an important excitatory neurotransmitter in a variety of optical signal transduction pathways, which plays an important role in the conduction of bipolar cells, photoreceptor cells and ganglion cells; aspartate as an important excitatory neurotransmitter in the central nervous system is closely related to physiological and pathological processes such as neuronal damage. When the body is in a healthy state, γ-aminobutyric acid in the retina can block the excitotoxicity of glutamate by stimulating γ-aminobutyric acid receptors to maintain the balance between excitatory neurotransmitters and inhibitory neurotransmitters of the retina (Kimura *et al*., 2015; Li and Jia, 2014). In this study, glutamate-induced injury group rats had significantly higher contents of free glutamate, γ-aminobutyric acid, taurine, glycine and aspartate in the retina, showing statistically significant difference (P<0.05), indicating that the neurotransmitter amino acids in rats increased significantly after retinal injury.

The excitotoxicity of glutamate has relation to various neurological diseases such as glaucoma, diabetic retinopathy and Alzheimer's disease. The excitotoxicity of glutamate in retinal tissues can cause excessive activation of glutamate N-methyl-D-aspartate (NMDA) receptor subtypes, which then triggers calcium ion overload and ultimately leads to retinal nerve cell apoptosis (Zhou *et al*., 2016; Asami *et al*., 2015). α-melanocyte stimulating hormone is the 13 amino acid residues released by pre-pro-opiomelanocortin under the action of prohormone-converting enzyme, which has biological activity after chemical modification of the terminal (Zhang *et al*., 2020). Studies have shown that α-melanocyte stimulating hormone and its receptors are expressed in both the eye and central nervous system, whose primary functions include thermoregulation, anti-inflammatory, and immune regulation (Cheng *et al*., 2014). A large number of studies have pointed out that α-melanocyte stimulating hormone has protective effect against ischemic damage in the organs like brain, kidney, gastrointestinal tract, as well as eye. Moreover, studies have confirmed that intraperitoneal injection of α-melanocyte stimulating hormone can rescue

brain hippocampal neuron apoptosis caused by excessive activation of glutamate receptors (Liu *et al*., 2013; Zhang and Si, 2020). To this end, this study used α-melanocyte stimulating hormone to prevent glutamate-induced retinal excitotoxicity. The experimental results showed that compared with the glutamate-induced injury group, α-melanocyte stimulating hormone intervention group had significantly increased cell density and cell survival rate in the tested rats, while its apoptosis rate decreased significantly, suggesting that α-melanocyte stimulating hormone has a protective effect against glutamate-induced retinal damage.

Mitochondria plays a significant role in the regulation of apoptosis. Changes in mitochondrial membrane permeability are related to multiple pro-apoptotic cascade transduction signals. Where, Δψm damage of mitochondrial transmembrane potential is an event that occurs first in the apoptosis cascade reaction, which is before the cell nucleus shows apoptotic features. When the mitochondrial transmembrane potential collapses, cell apoptosis is irreversible. To further analyze the mechanism by which α-melanocyte stimulating hormone antagonizes glutamate-induced apoptosis, flow cytometry was used in this study to measure the mitochondrial membrane potential of the three groups of tested rats. It was found that rat mitochondrial membrane potential was significantly higher in the intervention group than in the induced injury group, showing statistically significant difference (P<0.05). It suggests that the inhibitory effect of α-melanocyte stimulating hormone on glutamate-induced apoptosis is achieved by up-regulation of mitochondria membrane potential, which in turn blocks the downstream apoptosis cascade reaction, ultimately inhibiting the release of apoptosis-related factors.

Conclusion

The excitatory amino acid receptor antagonist, α-melanocyte stimulating hormone, can effectively antagonize glutamate-induced apoptosis of retinal nerve cells and increase the density and survival rate of retinal nerve cells, the mechanism of which may be related to the increase of mitochondrial transmembrane potential.

Statement of conflict of interest

The authors have declared no conflict of interests.

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