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*Original Article*

# Ciliary neurotrophic factor protects retinal structure against hypergravity stimulation with short time and high +Gz value in rat

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**Abstract:** Objective: To investigate the effects of hypergravity on retinal structure in rat. Methods: Retinal structure and protein expression were examined by hematoxylin and eosin stain, immunohistochemistry and Western blot in the Sprague-Dawley® rats induced by hypergravity with short arm centrifuge (+10 Gz/3 min). Results: Retinal structure had no substantial change after hypergravity stimulation, but the expression levels of glial fibrillary acidic protein and ciliary neurotrophic factor were significantly increased by hypergravity stimulation. Conclusion: Hypergravity induced the expression levels of ciliary neurotrophic factor, which protect retinal structure against hypergravity stimulation with short time and high +Gz value in rat.

**Keywords:** Hypergravity, retina, glial fibrillary acidic protein, ciliary neurotrophic factor

## Introduction

Hypergravity is referred to the condition under which the force of gravity exceeds 1 G on the surface of the Earth. In the aerospace field, both astronauts in the manned space crafts and pilots in the modern fighters during missions are under hypergravity, which can reach to +8-+15 G [1]. Hypergravity may induce the transformation, translocation and blood supply changes of organs, which may suffer functional and structural change and even impairment [2-4].

Previous reports have stated that higher +G acceleration can lead to arrhythmia and mild changes in systolic and diastolic function, and edema, denaturation and apoptosis of cardiomyocytes on ultrastructural levels [5-10]. The effects of hypergravity on brain tissue include impairment of learning and memory, G-induced loss of consciousness when cerebral blood flow reduces to critical value, and neuronal edema and apoptosis in the brain [1, 11, 12].

The eye is the dual receptor for both vision and acceleration stress. It is important and necessary to understand the change of visual function under hypergravity. A survey recruiting 594 Chinese Air Force pilots stated that the percentages of pilots suffered visual blurring, grayout and blackout were 18.5%, 38.9% and 18.7% respectively. The Air Force pilots from other countries were also reported to similar visual change under hypergravity [11, 13, 14]. Tsai et al. examined visual function and ocular structure of 14 healthy volunteers under +9 Gz hypergravity and reported transient vision loss, increased corneal thickness and anterior chamber depth, and continuously decreased contrast sensitivity to low and intermediate frequency [15]. Matsunami et al. examined the visual evoked potential of rat eyes under +3 Gz hypergravity and found substantially lower amplitude and longer latency, suggesting hypergravity affects visual function [16]. The reports of hypergravity-induced impairment of retinal ultrastructure in animal models were limited. Zhang et al. investigated retinas from rabbits exposed to +5 Gz/1 min hypergravity and found complete retinal structure and normal subretinal layers under optical microscope. Electron microscopy also indicated complete retinal


**Figure 1.** The Short-arm Centrifuge for Hypergravity Model. Vertical overload of +10 Gz can be achieved by the animal centrifuge with a diameter of 2 m and programmed by computers. The rats were placed with prone position and head toward the centrifuge axis in the box fixed on the distal end of the centrifuge arm. During the whole centrifugation, the longitudinal axis of the rat body is always parallel to the centrifuge arm.

outer segment and normal inner nuclear layer (INL) and ganglion cell layer (GCL), suggesting little retinal impairment of hypergravity with short duration and low G value [17]. Barnstable et al. examined the frozen section of retinas from rats under +2 G hypergravity for 14 days and found thinner retinal outer layer and fewer rod cells. Retinal impairment of hypergravity with long duration and low G value might result from increased intraocular pressure and decreased choroidal blood supply [18].

Better understanding of hypergravity's impact on eye structure will help protect the visual function of pilots under hypergravity environment and ensure the successful completion of flying missions. In this presented study, we reported the effects of hypergravity with short duration and high G value on retinal structure in rat for the first time.

## Materials and methods

### *Animal*

Sprague-Dawley rats were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. Male rats with body weight of around 200 g were bred under ordinary conditions for one week and then used for experiments. During the experiment, the feed and water were consumed normally. The indoor lighting was 12-on, 12-off schedule with the ambient temperature of about 20°C, keeping proper humidity and ventilation. Animals were randomly grouped into the control (CON) group bred under normal 1 G condition and the hypergravity (HG) group exposed to +10 Gz with the peak duration of 3 min.

### *Short-arm centrifuge for hypergravity model*

The animal centrifuge with a diameter of 2 m was provided by Astronaut Center of China. Vertical overload of +1-+26 Gz can be controlled and programmed by computers. The rats were placed with prone position and head toward the centrifuge axis in the box fixed on the distal end of the centrifuge arm. During the whole centrifugation, the longitudinal axis of the rat body is always parallel to the centrifuge arm (**Figure 1**). The onset rate was 1.5 G/s, and the duration of +10 Gz peak was 3 min, which was selected according to previous publications [1, 19]. Our preliminary experiments also suggested rats could tolerate this condition.

### *Paraffin section and hematoxylin and eosin staining*

Rats were euthanized with chloral hydrate (300 mg/kg intraperitoneal injection) and perfused

1 hour, 6 hours, 1 day, 3 days and 7 days after hypergravity stimulation. After opening the pleural cavity and exposing the heart, a small incision was made to the posterior end of the left ventricle and a 12-gauge blunt-tipped perfusion needle was passed through the cut ventricle into the ascending aorta. An incision as made to the right atrium and phosphate-buffered saline solution (50-70 ml) was quickly and evenly pumped into the aorta. After the running fluid became clear, paraformaldehyde (4%, 100 ml) was slowly infused until general stiffness and tail rigidness. Eyeballs were enucleated from the cut eyelid and infused with paraformaldehyde (4%, 5 ml) from the anterior segment. After incubated for 1 hour at room temperature, cornea, lens and vitreous body were gently removed under dissecting microscope. The posterior segment of eyeballs was dehydrated by ethanol sequentially from 70% to 100%. After embedded in paraffin, vertical sections with the thickness of 4 μm were made around optic nerve head. After conventional dewaxing, the sections were stained with hematoxylin and eosin.

*Examination of retinal structure*

Retinal thickness and the number of ganglion cells were assessed under optical microscope to examine the effects of hypergravity on retinal structure. The sections were scanned by Pannoramic Digit Slide Scanners (3DHISTECH, Hungary). Retinal thickness was measured on images with 20 times magnification at 5 different locations within 1 mm away from the optic nerve head. The number of retinal ganglion cells (RGC) was counted on images with 40 times magnification at 5 random areas within 2/3 diameter of the retina.

*Immunohistochemistry*

After deparaffinization and rehydration, antigen was unmasked. To quench endogenous peroxidase activity in samples, the sections were placed in 3% hydrogen peroxide for 10 min. After blocking, the samples were incubated with primary antibodies at 4°C overnight. Rabbit polyclonalciliary neurotrophic factor (CNTF) antibody (1:300) and mouse monoclonal glial fibrillary acidic protein (GFAP) antibody (1:7500) were purchased from Wuhan Boster Biological Technology, Ltd., and goat serum was used as negative control. After washing, the samples were incubated with corresponding secondary antibodies conjugated with biotin at 4°C for 45 min. Diaminobenzidine was applied and the samples were monitored closely. After counterstained with hematoxylin, the sections were dehydrated again with gradient ethanol, cleared with xylene, mounted with DPX, and examined under optical microscope.

*Western blot*

Rats from both CON and EXP groups were euthanized with chloral hydrate (300 mg/kg ip injection) and eyeballs were enucleated. The sclera was cut and opened 2 mm away from corneal limbus. The retina was then gently taken out and used for protein extraction. The concentration of protein was assessed with the bicinchoninic acid assay. After denaturation, the samples were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (80 V for concentration and 120 V for separation). The protein was then transferred (30 V) from the gel to the nitrocellulose membrane at 4°C overnight. After blocking with 5% skim milk at room temperature for 60 min, the samples were incubated with rabbit polyclonal CNTF antibody (1:300, R&D Systems, USA) or mouse monoclonal GFAP antibody (1:10000, Abcam, USA) at 4°C overnight. After washing, the samples were incubated with corresponding secondary antibodies at 37°C for 60 min. The membrane was then incubated with developing solution at room temperature for 1 min and exposed to X-ray film. The optical density of target band was analyzed with gel image processing system.

*Statistical analysis*

The data was analyzed with Statistical Package for the Social Sciences 17.0. The results were presented as mean ± standard deviation. The comparison between groups was analyzed with independent-sample t test and one-way analysis of variance. The criterion for significance was set at a probability of $\leq 0.05$.

## Results

*Hypergravity at +10 Gz for 3 min has few effects on retinal structure*

The paraffin sections of rat retinas indicated ordinary arrangement and clear hierarchy of

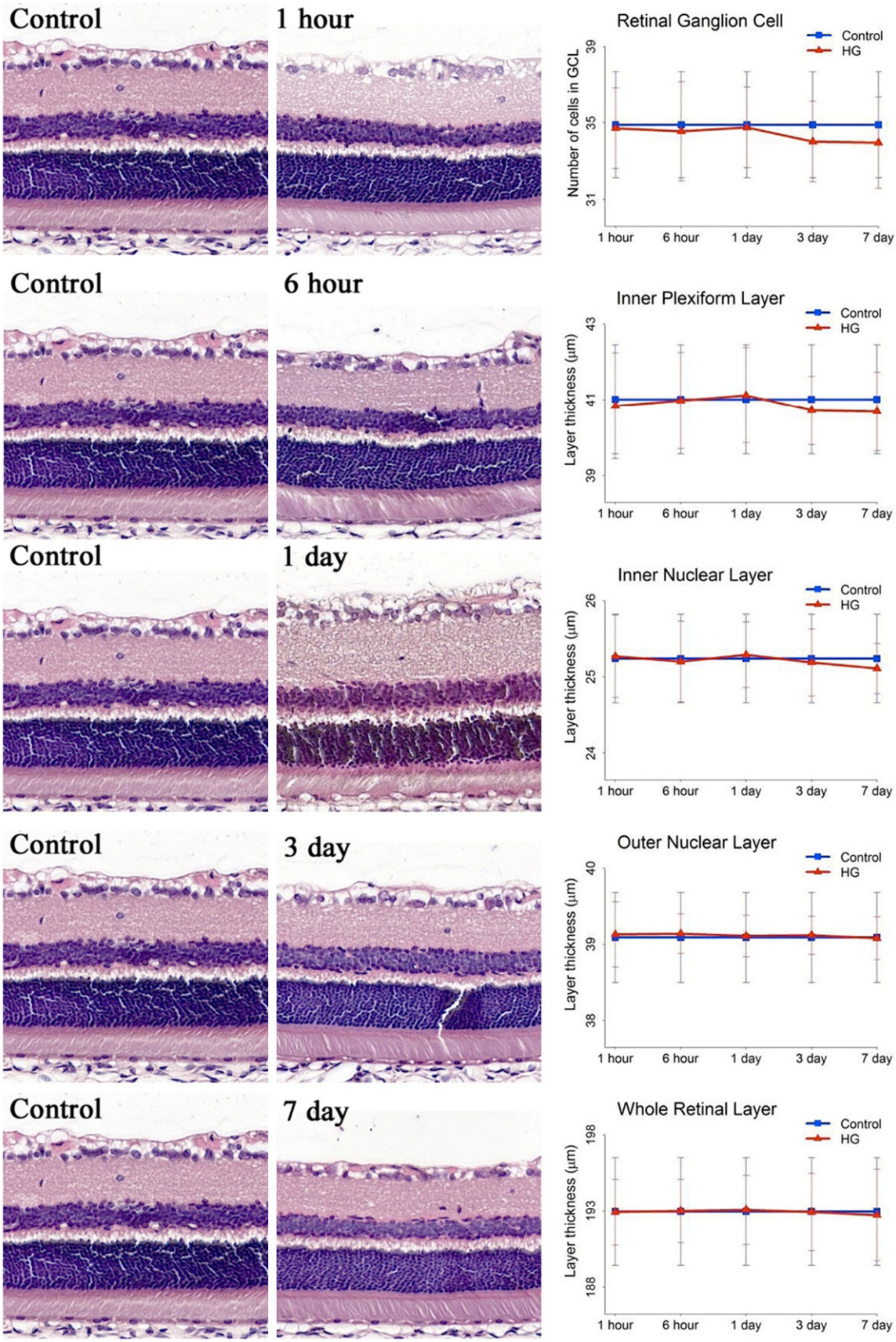
Control
1 hour
Retinal Ganglion Cell
Control
HG
Number of cells in GCL
39
35
31
1 hour
6 hour
1 day
3 day
7 day
Control
6 hour
Inner Plexiform Layer
Control
HG
Layer thickness (μm)
43
41
39
1 hour
6 hour
1 day
3 day
7 day
Control
1 day
Inner Nuclear Layer
Control
HG
Layer thickness (μm)
26
25
24
1 hour
6 hour
1 day
3 day
7 day
Control
3 day
Outer Nuclear Layer
Control
HG
Layer thickness (μm)
40
39
38
1 hour
6 hour
1 day
3 day
7 day
Control
7 day
Whole Retinal Layer
Control
HG
Layer thickness (μm)
198
193
188
1 hour
6 hour
1 day
3 day
7 day

**Figure 2.** Retinas under hypergravity stress have regular and clear layers and no significant morphological change. The thickness of inner retina including inner nuclear layer and inner plexiform layer trends to increase at 24 hours after hypergravity and decrease at day 7 without statistical significance. The thickness of outer nuclear layer is consistent, and the decrease of the number of retinal ganglion cells is not significant.

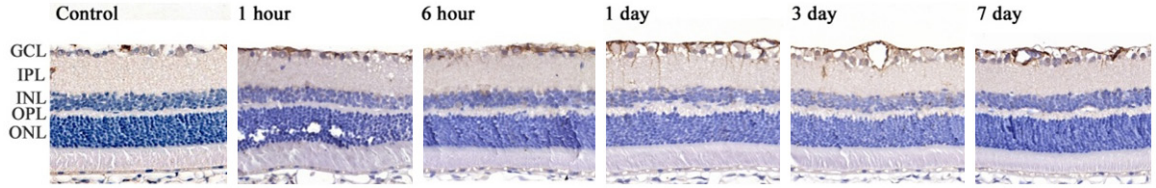


**Figure 3.** Immunohistochemistry of GFAP in rat retinas. The weak expression of GFAP in retinas from CON rats was distributed in the endfoot of astrocytes and Müller cells distributed in both the NFL and GCL. HG retinas collected 1 hour after hypergravity had stronger staining in the NFL and GCL, and had filamentary staining perpendicular to the direction of inner limiting membrane in the IPL. The filamentary staining penetrated into the INL and turned deeper color, more number and thicker distribution with time, which reached the peak at day 1 and began to decrease from day 3.

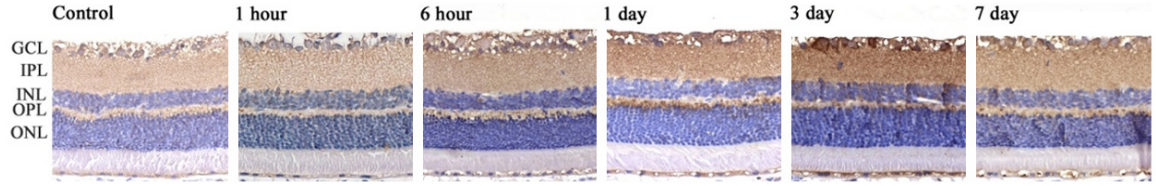


**Figure 4.** Immunohistochemistry of CNTF in rat retinas. CNTF was undetectable in the CON group and detectable in the IPL and INL in the HG group 1 hour after hypergravity. The staining became stronger as time, reached the peak at day 3 and began to decrease from day 7.

retinal layers, suggesting no substantial change of retinal structure. The thickness of the inner plexiform layer (IPL) trended to increase ($P > 0.05$) 24 hours after exposure to hypergravity compared with CON group, showing mild edema. The thickness of the IPL and inner nuclear layer (INL) trended to decrease ($P > 0.05$) at day 3 and 7 compared with CON group. The outer nuclear layer (ONL) and whole retina had no significant change on the thickness (**Figure 2A**). The number of retinal ganglion cells (33.97±2.39) trended to decrease 7 days after exposure to hypergravity compared with CON group (34.90±2.77), but there is no significant difference ($P > 0.05$) (**Figure 2B**).

*Hypergravity induces glial fibrillary acidic protein and ciliary neurotrophic factor*

GFAP was detected in the nerve fiber layer (NFL) and ganglion cell layer (GCL) of rat retinas in the CON group. This weak staining was believed to be the end foot of astrocytes and Müller cells distributed in both the NFL and GCL. Compared with CON group, the retinas collected 1 hour after hypergravity stimulation had stronger staining in the NFL and GCL, and had filamentary staining perpendicular to the direction of inner limiting membrane in the IPL. The filamentary staining penetrated into the INL and turned deeper color, more number and thicker distribution with time, which reached the peak at 1 day after hypergravity stimulation and began to decrease from day 3 (**Figure 3**). CNTF was undetectable in the CON group and detectable in the IPL and INL in the HG group 1 hour after hypergravity stimulation. The staining became stronger as time, reached the peak at 3 days after hypergravity stimulation, and began to decrease from day 7 (**Figure 4**). The consistent results were also observed in Western blot. The protein levels of GFAP increased in HG 1 hour group, reached the peak in HG day 1 group, and began to decrease in HG day 3 group. Compared with the CON group, the protein levels of GFAP were significantly induced in all HG groups (**Figure 5**). The protein levels of CNTF increased in HG 6 hour group, reached

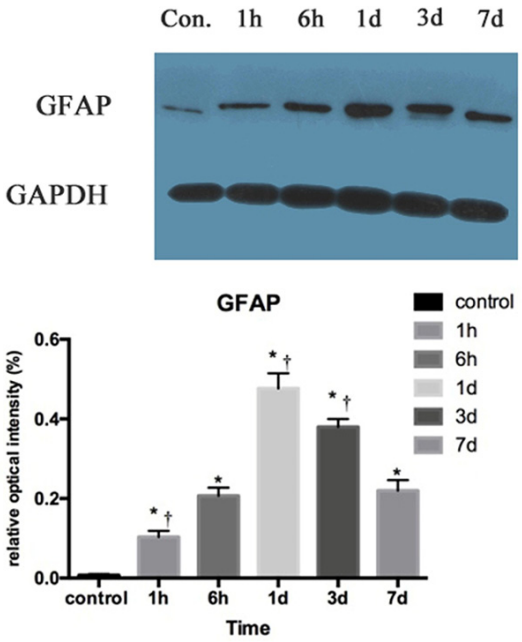


Figure 5. Western blot of GFAP in rat retinas. The protein levels of GFAP increased 1 hour after hypergravity, reached the peak at day 1, and began to decrease at day 3. Compared with the CON group, the protein levels of GFAP were significantly induced in all HG groups. N = 4, *P < 0.05 compared with CON group, †P < 0.05 compared between HG groups.

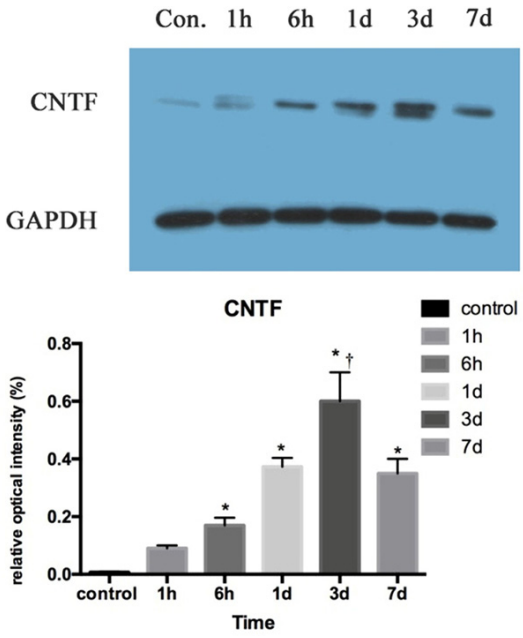


Figure 6. Western blot of CNTF in rat retinas. The protein levels of CNTF increased 6 hours after hypergravity, reached the peak at day 3, and began to decrease at day 7. Compared with the CON sample, the protein levels of CNTF were significantly induced in HG samples collected 6 hours after hypergravity. N = 4, *P < 0.05 compared with CON group, †P < 0.05 compared between HG groups.

the peak in HG day 3 group, and began to decrease in HG day 7 group. Compared with the CON sample, the protein levels of GFAP were significantly induced in all HG samples collected 6 hours after hypergravity stimulation (**Figure 6**).

## Discussion

Gravitational overload, also known as craniocaudal overload, leads to blood transfer from the head to the feet, causing ischemia in organs located over the heart [20, 21]. Craniocaudal overload in rats at +9 Gz for 12 min was reported to make the pressure in meningeal arteries drop to 0 [22]. Iwasaki et al. found decreased cerebral blood flow in 15 healthy patients under hypergravity at +1.5 Gz for 6 min stimulated by the short-arm centrifuge [23]. Although the conclusions may vary due to the difference of hypergravity stimulation, Gz value, duration time and assessment methods in both animal experiments and real-time monitoring of the human body, there is consistent reports of reduced blood flow in organs located over the heart, especially the brain. Larger Gz value causes more severe ischemia [24, 25]. The blood supply to the eyeballs comes from the carotid artery as the same as the brain tissue, therefore theoretically eyeballs become ischemic when the brain tissue is ischemic under hypergravity condition. Laughlin et al. measured CBF in miniature swine under +Gz stress with the radio-labeled microsphere technique. Exposure to +5 Gz hypergravity had no significant effect on CBF, but blood flow to the retina was significantly decreased and ceased during exposure to +7 Gz stress, confirming the ischemic status of eyeballs in hypergravity condition [26]. The hypergravity condition (+10 Gz, 3 min) we reported here caused a transient ischemia status with a higher Gz value. After that, immunohistochemistry indicated complete and normal structures of retinal layers in rats. The IPL thickness increased 1 day after exposure to hypergravity, showing mild edema. While the ONL had no significant change on the thickness, the inner retinal layers' thickness showed a decreasing trend 7 days after hypergravity stimulation. The number of RGC trended to

decrease at day 7 compared with CON group, but there is no significant difference. There results suggested that transient hypergravity with high Gz value had no substantial effects on retinal structure in rates.

Previous studies indicated that retinal glial cells were activated at the early stage in response to retinal damage such as ischemia, mechanical injury, retinal detachment, and high intraocular pressure. Müller cells are the primary glial cells in the neuroretina. Under normal circumstances, Müller cells do not express or express a small amount of GFAP. When the retina is damaged, Müller cells are activated, showing hypertrophy and upregulating GFAP, nestin and vimentin. The high expression of GFAP is the most sensitive index of Müller cells in response to retinal disorders and reactive hyperplasia, known as the marker for retinal stress [27-30]. Our results indicated that while there is no change on retinal structure, immunohistochemistry and Western blot data showed increased expression of GFAP 1 hour after hypergravity stimulation with the peak at 24 hours and decreasing at day 3. The induced expression of GFAP in Müller cells was previously reported in glaucomatous rats with the rise of the intraocular pressure beginning at 2 hours and before RGC degeneration [31-33]. These results suggest that rat retinal Müller cells are very sensitive to hypergravity (+10 Gz/3 min) and activated at the early stage to upregulate GFAP expression. Early activation of glial cell proliferation confines the scope of damage and protects the integrity of tissues by multiple pathways including releasing neurotrophic factor and promoting the absorption of glutamate. This is called conservative gliosis and beneficial to the damaged or irritated nerve tissues [34]. However, excessive activation of glial cells eventually form the scar tissues to replace the damaged nerve tissues, leading to structural and functional changes to nerve tissues when the damage is severe and lasting. Therefore we believe that the early activation of rat retinal Müller cells in response to transient ischemia caused by hypergravity (+10 Gz/3 min) is beneficial to retinal ganglion cells.

CNTF is one of the family members of interleukin-6 distributed in non-neuronal cells of the central and peripheral nervous systems. Previous studies indicated that endogenous or exogenous CNTF as a neuroprotective factor protect retinal ganglion cells, photoreceptors and retinal pigment epithelium against ischemia, high intraocular pressure, photodamage and denaturation through different signaling pathways in vertebrates [35-38]. In 1999, Won-Kyu et al. found that CNTF supplied by Müller cells has a protective function for damaged neurons following transient ischemia [39]. Other investigators also reported the neuroprotective effects of CNTF in Müller cells [40, 41]. Our results show that CNTF levels begin to increase 6 hours after hypergravity (+10 Gz/3 min) stimulation, reach the peak at day 3 and then decrease. Immunohistochemistry data indicates that CNTF is present only in inner retina, which is colocolized with GFAP. Therefore we believe that the early activation of retinal Müller cells protect retinal ganglion cells against hypergravity with short duration and high Gz value by releasing neuroprotective CNTF.

## Conclusion

This research investigated retinal changes under hypergravity stress with high Gz Value using the short-arm centrifuge to mimicking craniocaudal overload. While there is no substantial change of retinal structure after exposure to hypergravity (+10 Gz/3 min), the expression levels of the Müller cell marker GFAP and the neuroprotective factor CNTF were significantly increased compared with CON group. High strength hypergravity (+10 Gz) may lead to retinal ischemia, but short duration (3 min) is not enough to cause substantial changes of retinal structure. Moreover, the CNTF released by early activated retinal Müller cells are beneficial to the retina. In future studies, we will investigate the potential damage of retinal structure and function by hypergravity with high Gz value and long duration, and the mechanisms how CNTF protect retina against hypergravity. More researches on the retinal structure and function under hypergravity stress will provide more theoretical support for the visual health of air astronauts.

## Disclosure of conflict of interest

None.

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