·Basic Research·

Effect of long-term weightlessness on retina and optic nerve in tail-suspension rats

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Abstract

• **AIM: To evaluate the effect of long-term weightlessness on retina and optic nerve in tail-suspension (TS) rats.**

• **METHODS: A stimulated weightlessness model was established by suspending rats´ tail. After 12wk, the ultrastructure and the number of optic nerve axons were observed by transmission electron microscope. The number of survival retinal ganglion cells (RGCs) was calculated by fluorescent gold retrograde labeling. Retina cells apoptosis was detected by TUNEL staining. The function of optic nerve and retina was evaluated by the visual evoked potential (VEP) and oscillatory potentials (Ops).**

• **RESULTS: The optic nerve axons were swollen and sparsely aligned, and the lamellar separation and myelin disintegration occurred after 12wk in TS rats. The density of optic nerve axons was 32.23±3.92 (*vs* 37.43±4.13, *P*=0.0145), the RGCs density was 1645±46 cells/mm² (*vs* 1867±54 cells/mm² *P*=0.0000), the incidence rate of retinal cells apoptosis was 5.38%±0.53% (*vs* 4.75%±0.54%, *P*=0.0238), the amplitude of VEP-P100 was 15.43±2.14 μV (*vs* 17.67±2.17 μV, *P*=0.0424), the latency of VEP-P100 was 69.05±5.34ms (*vs* 62.43±4.87ms *P*=0.0143) and the sum amplitude of Ops was 81.05±8.34 μV (*vs* 91.67±10.21 μV, *P*=0.0280) in TS group and the control group, respectively.**

• **CONCLUSION: Long-term weightlessness can induce the ultrastructural changes and functional depress of the optic nerve, as well as retinal cell damages in TS rats.**

• **KEYWORDS:** weightlessness; retina; optic nerve; tail suspension
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INTRODUCTION

With the depth of space exploration, the health and safety of space explorers becomes more and more under the spotlight and attracts much attention. The effects on astronauts' eyes during space flight were still not fully known. It has been well-known that the significant ocular disorders could happen on astronauts during space flight, including decreased visual acuity, hyperopic shifts, papilledema, globe flattening, choroidal folds, retina damage and so on [1-5]. The collective effects of space radiation and weightlessness during space flight are believed to be the main reasons [6-8]. However, whether and how long-term weightlessness alone could contribute to the damage of optic nerve still remains largely unknown.

Animal studies have confirmed that weightlessness had remarkable effects on dorsal root ganglion neurons, and the metabolism and excitation of neurons in the spinal cord [9-12]. These studies provided convincing evidences that weightlessness might cause central nervous system (CNS) damage. Since optic nerve was composed of retinal ganglion cells (RGCs) axons without Schwann cells, belongings to a part of the CNS, it was reasonable that weightlessness, especially for long-term, might cause optic nerve damage. Therefore, we hypothesized that long-term weightlessness may be associated with the injury of optic nerve and further affect visual function. This study aimed to evaluate the effect of long-term weightlessness on optic nerve and retina by using tail-suspension (TS) rats as weightlessness models.

MATERIALS AND METHODS

Animals and Tail-suspension Model Adult Sprague

Dawley rats, without limitation of sex, weighing 200-300 g, aging 6-8wk, were obtained from the Experimental Animal Center of Beijing Medical University. Each rat was caged separately at 22℃-24℃ and controlled in light/dark cycles (12h/12h). One week later, 18 rats were randomly assigned to two groups evenly: control group (without tail suspension) and TS (with tail suspensions) group. TS model was made according to the method described by Morey-Holton and Globus[13]. The rats were suspended by the tail at an angle of about 30° from the head down to avoid contact between the hind limbs and the ground. The rats could walk freely on their forelimbs for access to food and water.

Experimental Design All rats received visual evoked potential (VEP) and oscillatory potentials (Ops) tests at 12wk. The ultrastructure and density of optic nerve axons was observed by transmission electron microscope (TEM) in 3 rats of each group at 12wk. Three rats in each group received retrograde fluorescent gold labeling with intracranial injection at 11wk, and then was analyzed the density of survival RGCs by counting RGCs labeled with fluorescent gold in retina flatmount under fluorescence microscope at 12wk in each group. The incidence of RGCs apoptosis was analyzed by TUNEL assay in other 3 rats of each group at 12wk.

Ultrastructure and number of optic nerve axons Rats were anesthetized, eyeballs and optic nerve were removed. Optic nerve (at a distance from retrobulbar 3-5 mm) was taken and fixed in a cold solution consisting of glutaraldehyde and paraformaldehyde, and postfixed with osmium tetroxide in the same buffer at 4℃ for 2h. Then the samples were dehydrated in a graded ethanol series, and treated with propylene oxide, and finally embedded in Spurr's resin. Ultrathin cross sections of optic nerve (approximately 50 nm in thickness) were made by an ultramicrotome (Leica UC7, Germany) with a diamond knife. The stained cross sections were examined with a transmission electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV. Three sections of each sample were randomly selected. The number of optic nerve axons was manually counted in 5 fields randomly selected on each section and averaged.

Retrograde fluorescent gold labeling It was performed according to the literature [14]. Briefly, rats were deeply anesthetized (10% hydrate of chlorine aldehyde), then placed in a small stereotactic instrument. The skull was opened. The Bregma point was identified and the injection points overlying the lateral geniculate body was marked. The holes were drilled at demarcated points with a 25 Gauge needle. 2 μL of 2% fluorescent gold (Biotium, 80014, USA) was injected into the superior colliculus and lateral geniculate body of each hemisphere through the bony surface of the brain by using a Hamilton syringe. And then the incision of skin was sutured.

Retina flatmount and retinal ganglion cellscounting Rats were anesthetized, eyeballs were enucleated and postfixed for 1h in 4% paraformaldehyde. Cornea and the lens were removed and the eyecups were incubated in phosphate buffer saline (PBS). The whole retina was then carefully dissected, flat mounted on slides, and cover slipped. Fluorescent RGCs were observed with fluorescent microscope (Japan OlympusBX-53, Fluorescence attachment). Each retina was divided into four quadrants (superior, inferior, nasal and temporal), and images of retina within 2.0 mm from the center of the optic disc in each quadrant were obtained. The size of counted area in each quadrant was 0.153 mm^2 (450× 340 μm^2). Fluorescent RGCs on the images were measured using Image Pro plus 6.0. Finally, the number of RGCs was obtained by dividing the area and expressed as number per square millimeter.

TUNEL assay and the analysis of retinal ganglion cells apoptosis TUNEL assay was used for RGCs apoptosis detection. It was performed according to the CFTM488A TUNEL Assay Apoptosis Detection Kit (American Biotium Corporation, 30063) protocol. Negative controls were incubated with TUNEL reaction buffer without TdT Enzyme. Photographs were taken by using a fluorescent microscope (OlympusBX-53, Fluorescence attachment, Japan). Nine sections were randomly selected in each eye, and one field was randomly selected on each section. TUNEL-positive cell counts were calculated manually as percentages of total cell number.

Visual evoked potential and oscillatory potentials waves cording After dark-adaptation, rats were anesthetized with intraperitoneal 10% chloral hydrate. VEP signals were recorded in the scalp covering the visual cortex using a stainless steel recording electrode (Gift from The 4th Military Medical University) placed subcutaneously 1 cm anterior to the midpoint of a line connecting the two back ear edges. A stainless steel reference electrode was placed in the mouth, and a stainless steel grounding electrode was placed in the tail. The flash stimulus parameter was 3.5 $cd \cdot s \cdot m^{-2}$, 1.3 Hz. The amplitude and latency of the VEP-P100 component were recorded automatically (GUOTE MEDICAL, V8.1, China). After VEP cording, rats were dilated pupils using 1% tropicamide eye drops. The corneal surfaces were anesthetized using 0.4% hydrochloric acid oxybuprocaine eye drops. The reference electrode was placed on the foreheads of the rats, the ground electrode was placed on their ears, and the ring-recording electrode was placed on the surface of the corneas of the rats. The pass band of full-field stroboscopic white light stimulus was 75-300 Hz. The flash stimulus parameter was 3.0 $cd \cdot s \cdot m^{-2}$. The sum of Ops amplitudes was recorded automatically.

Statistical Analysis The experimental data are expressed as the mean±standard deviation and was analyzed by Student's

t-test. Each *P*-value was calculated by two-tail Student's *t*-test with $P<0.05$ considered significant.

RESULTS

Long-term Weightlessness Caused Optic Nerve Injury and Reduced the Density of Optic Nerve Axons in Tail-suspension Rats To observe the effect of long-term weightlessness on optic nerve axons in TS model rats, the ultrastructure of cross section of the optic nerve was observed by TEM at 12wk after TS. Normal optic nerve axons are closely aligned and have compact myelin, normal microfilaments, microtubules and mitochondria (Figure 1A). While in TS group, optic nerve axons were swollen and sparsely aligned, and were observed to have abnormal proliferation of neural connective tissue between axons, lamellar separation and disintegration of myelin (Figure 1B). The number of optic nerve axons was 32.23±3.92 in TS group, and 37.43±4.13 in control group respectively (P=0.0145). Optic nerve axons density was decreased approximately 15% in the TS group compared with that of the control group (Figure 1C).

Long-term Weightlessness Decreased the Number of Survival Retinal Ganglion Cells in Tail-suspension Rats To explore the effect of long-term weightlessness on RGCs number. We countered the number of survival RGCs in retina flatmount at 12wk after TS. RGCs were presented as green- fluorescent spots. The number of RGCs was 1645±46 cells/mm^2 in TS rats group, and 1867±54 cells/mm^2 in control group (Figure 2A, 2B, P=0.0000). RGCs density was decreased 13% in the TS group compared with that of the control group (Figure 2C).

Long-term Weightlessness Increased the Incidence Rate of Retinal Cells Apoptosis in Tail-suspension Rats In order to examine the effect of long-term weightlessness on retinal cells apoptosis in TS model rats. Next, we detected retinal cells apoptosis using the TUNEL assay at 12wk after TS. The TUNEL results showed there are very few numbers of retinal apoptosis cells presented as bigger green-fluorescent spots in normal rats (Figure 3A), while the number of retinal apoptosis cells, especially in RGCs layer, was significantly increased in TS rats group (Figure 3B). The incidence rate of retinal cells apoptosis was 4.75%±0.54% in control group, and was 5.38%±0.53% in TS group (P=0.0238). The incidence rate of retinal cells apoptosis was increased 13% in the TS group compared with that of the control group (Figure 3C).

Long-term Weightlessness Depressed Visual Evoked Potential and Oscillatory Potentials Waves in Tail-suspension Model Rats In order to value the function of optic nerve and retina after long-term weightlessness in TS model rats. We detected VEP and OPs waves of rats by visual electrophysiology instrument at 12wk after TS. The results showed the amplitude of VEP-P100 was decreased approximately 13% in the TS group (15.43±2.14 μV)

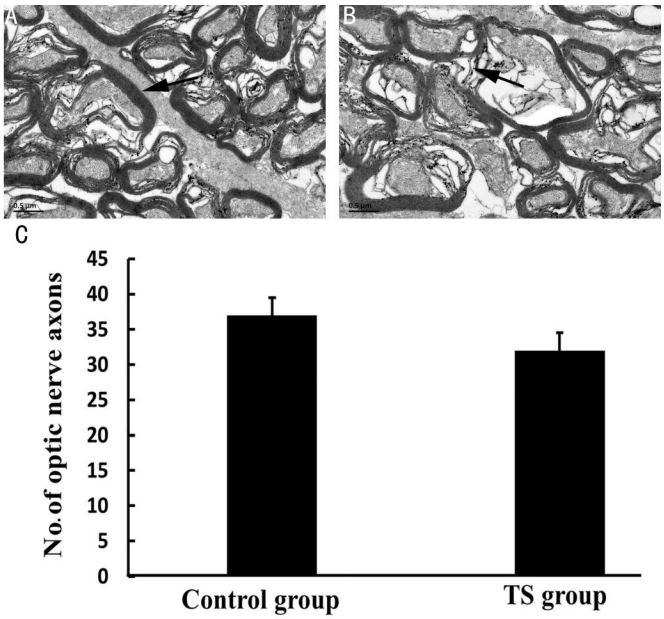

Figure 1 The ultrastructure of optic nerve axons by TEM and comparison of optic nerve axon density in rats between control group and TS group A: Optic nerve axons were closely aligned, and had compact myelin in normal rats (arrow); B: Optic nerve axons were swelling and sparsely aligned, even occurred in the lamellar separation and disintegration of myelin (arrow); C: The number of optic nerveaxons in TS group was 32.23±3.92 in TS group, *vs* 37.43±4.13 in control group in the same magnification field of view (P=0.0145). Scale bar=0.5 μm.

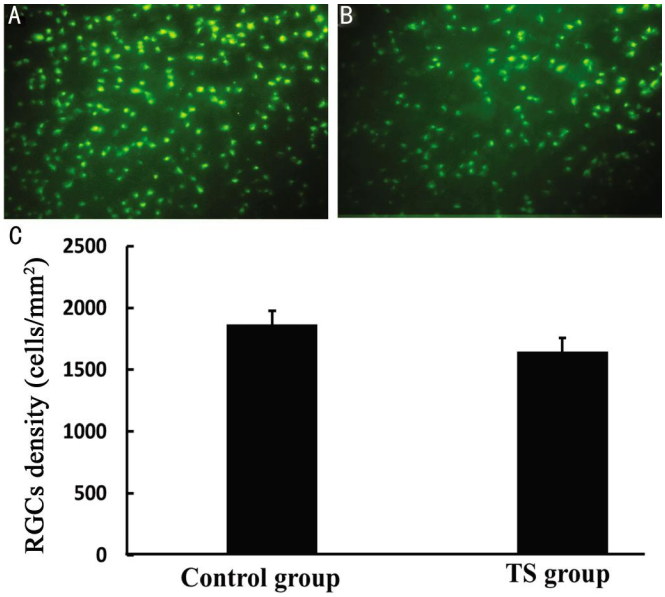

Figure 2 RGCs numbers in rats with fluorescent gold retrograde-labeling between control group and TS group A: Images of representative retinal flat mounts in control group. A lot of RGCs were present as big and bright green-fluorescent spots in retinal preparations (×400 magnification). B: Images of representative retinal flat mounts in TS group. The number of big and bright green-fluorescent spots of RGCs in retinal preparations was decreased (×400 magnification). C: The RGCs density was 1645±46 cells/mm^2 in TS group, *vs* 1867±54 cells/mm^2 in control group (P=0.0000).

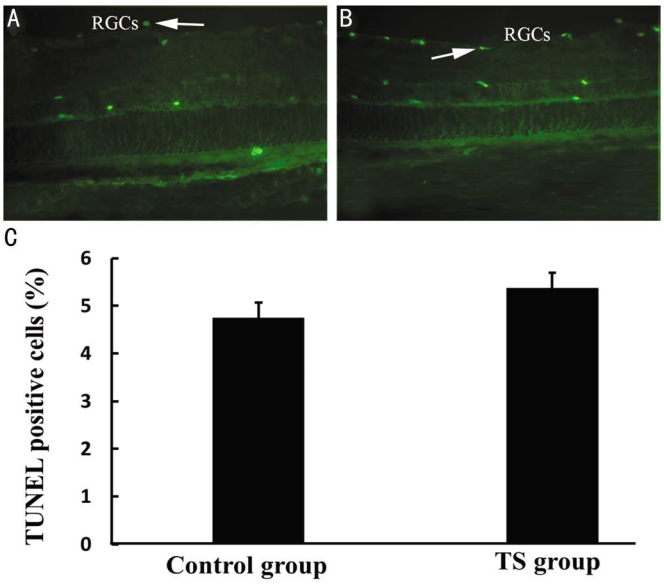

Figure 3 Retinal cells apoptosis in rats by TUNEL assay between control group and TS group A: Images of representative retinal cells apoptosis in control group. Apoptotic retinal cells were present as big and bright green fluorescent spots (arrow, ×400 magnification). B: Images of representative retinal cells apoptosis in TS group. The number of apoptotic retinal cells presented as green fluorescent spots was increased, especially in RGCs layer (arrow, ×400 magnification). C: The incidence rate of retinal cells apoptosis was 4.75% ±0.54% in control group, *vs* 5.38%±0.53% in TS group (P=0.0238).

compared with that of the control group (17.67±2.17 μV). There was statistical significance of amplitude of VEP-P100 between two groups (P=0.0424) (Figure 4A). The latency of VEP-P100 was increased approximately 11% in the TS group (69.05 ±5.34ms) compared with that of the control group (62.43±4.87ms). There was statistical significance of latency of VEP-P100 between two groups (P=0.0143) (Figure 4B). The sum amplitude of Ops was decreased approximately 13% in the TS group (81.05 ±8.34 μV) compared with that of the control group (91.67±10.21 μV). There was statistical significance of the sum amplitude of Ops between two groups (P=0.0280) (Figure 4C).

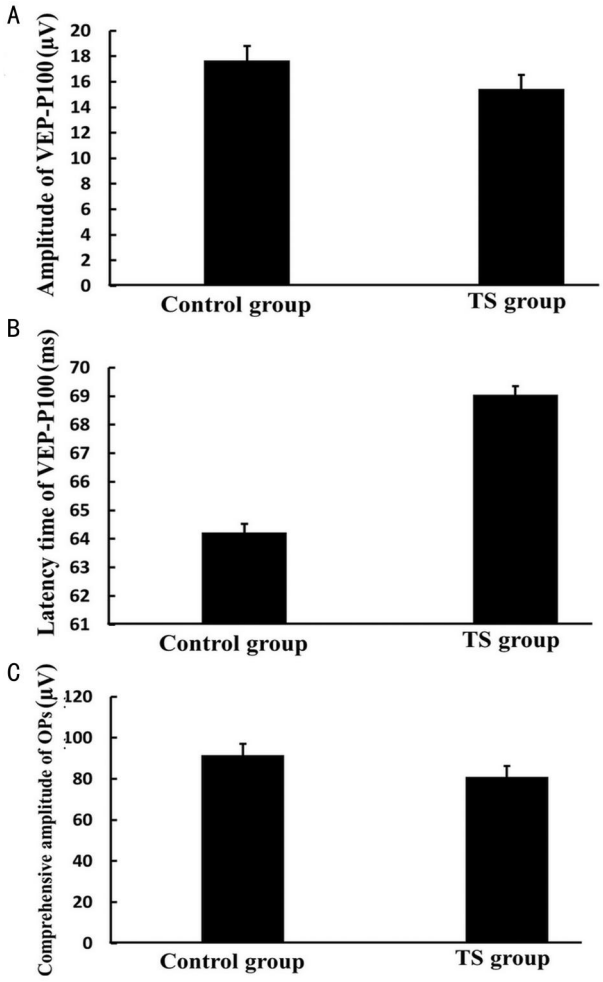

Figure 4 The amplitude and latency of VEP –P100 and the sum amplitude of Ops in control group and TS group A: The amplitude of VEP-P100 was 17.67±2.17 μV in control group, *vs* 15.43 ±2.14 μV in TS group (P=0.0424); B: The latency of VEP-P100 was 62.43±4.87ms in control group, *vs* 69.05±5.34ms in TS group (P=0.0143); C: The sum amplitude of Ops was 91.67± 10.21 in control group, *vs* 81.05±8.34 in TS group (P=0.0280).

DISCUSSION

In this study, we demonstrated that the optic nerve ultrastructure was damaged, including optic nerve axons swelling, sparsely alignment, lamellar separation and even disintegration of myelin. The density of optic nerve axons and the numbers of survival RGCs was significantly decreased. The incidence of RGCs apoptosis was increased. VEP and Ops waves were exacerbated due to weightlessness in TS rats.

Weightlessness can cause the disorders of multiple organs or organizations, such as bone, muscle, cardiopulmonary, immune system and some ocular disorders as well [15-18]. Mader *et al* [2] reported that 5 out of 7 astronauts in space for 6mo happened optic papilla edema, suggesting that optic nerve could be damaged during space flight. However, regardless space radiation factor, whether and how the weightlessness factor alone could induce this damage still remains unclear. Our study demonstrated that weightlessness in TS rats can cause both optic nerve injury and RGCs apoptosis. To our knowledge, it was the first report on documenting it.

Although the mechanism about optic nerve injury by weightlessness factor is not completely understood, we speculate that it may be related to the following factors: 1) increased intracranial pressure. Loss of gravitationally induced cranial outflow of blood in the vertebral veins and collaterals, may lead to intracranial venous hypertension and subsequently cause papillary edema. Long-term papillary edema could produce expansion of optic nerve sheath, which further compress the optic nerve; 2) intraocular pressure (IOP). Long-term high IOP in a state of weightlessness could compress the optic disc and result in optic nerve injury; 3)

imbalanced translaminar pressure difference (TLPD). The difference in IOP and cerebrospinal fluid (CSF) across the lamina cribrosa is known as the TLPD. It was reported astronauts returning from prolonged space flight on the International Space Station with papilledema [2]. But papilledema has not been observed in shorter duration space flight. A study has demonstrated that CSF no longer pools in the caudal spinal column as it does in the upright position on earth. Instead, CSF diffuses throughout the subarachnoid space resulting in a moderate but persistently elevated cranial CSF pressure, including the region just posterior to the lamina cribrosa known as the optic nerve subarachnoid space. This small but chronically elevated CSF could lead to papilledema when CSF pressure is greater than the IOP[19].

Apart from optic nerve injury, our study further demonstrated that the numbers of survival RGCs was significantly decreased and might subsequently contributed to a decline of visual function which evaluated by VEP. A recent report revealed that spaceflight conditions induce oxidative damage that results in significant apoptosis of retina cells in rats, especially inner nuclear layer and ganglion cell layer [8]. But the conclusion of above study cannot be excluded the result of joint action both weightlessness and space radiation. Our study indicated that long-term weightlessness alone could enough increase the apoptosis RGCs, as well as that of retinal cells.

Retinal ischemia might play an important role in the process of RGCs and retinal cells' apoptosis during weightlessness. A decreased Ops in electroretinogram (ERG) was found in this study is as an evidence for this speculation. Ops is the sub-component of ERG, it can objectively and sensitively reflect inner retinal blood circulation. Previous studies have confirmed that the weightlessness could cause ocular hemodynamics chang [2,4] and vascular endothelial cell apoptosis in rats of spaceflight conditions[8]. Redistribution of blood in the head and face due to weightlessness can cause ocular venous congestion. Short-term redistribution of blood produces autonomous adaptation, but which for long time will produce a series pathophysiology retinal changes. Our study provided a proof that the function of the retina was damaged might through changing inner retinal blood circulation due to long-term weightlessness in TS rats.

Notably, other important factors could not be excluded in the process of apoptosis of RGCs and retinal cells, such as oxidative stress. Several studies have suggested that weightlessness resulted in increased oxidative stress in the CNS [20-21], which might have profound implications in the pathogenesis of retinal cells death [22-23]. Moreover, it was reported that Bcl-2 signaling pathways may represent an event upstream of the retina cells apoptosis step in rats of spaceflight conditions [8]. However, more investigations should be taken to clarify this issue.

In summary, our study confirmed that long-term weightlessness alone could enough cause morphological and functional optic nerve damage, and induce RGCs and retinal apoptosis. But a simulated weightlessness animal model will never reflect fully the picture of actual spaceflight in human. This limitation was well known and needed to further overcome to extrapolate reasonably from a simulated weightlessness animal model to human. Nevertheless, these simulated animal studies do provide ideas to further evaluate the optic nerve and retina changes in human weightlessness.

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