Contents lists available at ScienceDirect

Digital Chinese Medicine

journal homepage: http://www.keaipublishing.com/dcmed

Protective effects of ginseng total saponins on reward-directed operant conditioning in hindlimb suspension rats

DONG Lijinchuan[a†], CHEN Ying[a†], HU Qin[b], BAO Yu[c], PAN Ruile[c], ZENG Guirong[d], PENG Bo[a], LIU Xinmin[c, e*], SHI Zhe[f*]

a. Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China

b. The Faculty of Environment and Life, Beijing University of Technology, Beijing 100124, China

c. Institute of Medicinal Plant Development, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing 100193, China

d. Hunan Key Laboratory of Pharmacodynamics and Safety Evaluation of New Drugs & Hunan Provincial Research Center for Safety Evaluation of Drugs, Changsha, Hunan 410331, China

e. Institute of Drug Discovery Technology, Ningbo University, Ningbo, Zhejiang 315000, China

f. Division of Stem Cell Regulation and Application & Key Laboratory for Quality Evaluation of Bulk Herbs of Hunan Province, Hunan University of Chinese Medicine, Changsha, Hunan 410208, China

ARTICLE INFO

Article history
Received 15 September 2023
Accepted 14 November 2023
Available online 25 December 2023

Keywords
Ginseng total saponins
Weightlessness simulation
Learning and memory
Reward-directed instrumental learning
N-Methyl-D-aspartic acid receptors

ABSTRACT

Objective To explore the therapeutic effects of ginseng total saponins (GTSs) on cognitive impairments in astronauts caused by prolonged exposure to microgravity environment.

Methods Fifty specific pathogen-free (SPF) male Wistar rats were randomized into control, hindlimb suspension (HLS), Huperzine A (HLS-Hup A 0.1 mg/kg), low-dose GTSs (HLS-GTSs 100 mg/kg), and high-dose GTSs (HLS-GTSs 200 mg/kg) groups, based on the completion time of reward-directed conditioning tasks. Except for rats in the control group, the others were subjected to HLS and treated with drugs (day 20 – 58), received reflex test under the condition of rewarding, and underwent Nissl body staining and Western blot detection on hippocampal.

Results After modeling, rats in HLS group exhibited a reduction in the number of lever presses and an increase in the completion time of the reward-directed operant conditioning task Ⅰ ($P < 0.05$) when compared with the control group, which were not substantially altered in the HLS-GTSs 100 and 200 mg/kg groups ($P > 0.05$). In the reward-directed operant conditioning task Ⅱ, the HLS group rats demonstrated a marked decrease in the number of lever presses ($P < 0.05$) and nose pokes ($P < 0.01$) when compared with the control group rats; the HLS-GTSs 100 mg/kg showed a significant increase in the number of lever presses and nose pokes ($P < 0.05$), while the HLS-GTSs 200 mg/kg demonstrated a significant reduction in completion time and an elevation in the number of lever presses ($P < 0.05$) when compared with the HLS group rats. In visual signal discrimination task, compared with the control group rats, the HLS group rats showed decrease in the indexes of the visual signal discrimination ($P < 0.01$), while HLS-GTSs 100 and 200 mg/kg groups exhibited manifest increase in it ($P < 0.01$). In reward extinction experiment, the number of lever presses in HLS rats significantly

†The authors contributed equally.

*Corresponding author: SHI Zhe, E-mail: zhezhe1106@163.com. LIU Xinmin, E-mail: liuxinmin@hotmail.com.

Peer review under the responsibility of Hunan University of Chinese Medicine.

DOI: 10.1016/j.dcmed.2024.01.005

Citation: DONG LJC, CHEN Y, HU Q, et al. Protective effects of ginseng total saponins on reward-directed operant conditioning in hindlimb suspension rats. Digital Chinese Medicine, 2023, 6(4): 416-425.

increased when compared with the control group ($P < 0.01$); compared with the HLS group, HLS-GTSs 100 and 200 mg/kg groups demonstrated a marked descrease ($P < 0.05$). The expressions of N-methyl-D-aspartic acid receptor 1 (NR1) and phosophorylated N-methyl-D-aspartic acid receptor 2B (p-NR2B) proteins were markedly decreased in rats in the HLS group ($P < 0.05$ and $P < 0.01$, respectively), while that of NR2B protein maintained the same ($P > 0.05$). GTSs increased the expression levels of p-NR2B ($P < 0.01$).

Conclusion GTSs improved the learning and memory ability of complex operations by regulating the NR1/NR2B phosphorylation pathways in rats.

1 Introduction

The development of aerospace industry has expanded short-term space travel into medium- and long-term journeys. However, this advancement has posed considerable strain on astronaut's physical loads and cognitive functions [1]. During the medium- or long-term orbital flights, astronauts were constantly exposed to a microgravity environment [2], which would cause physiological stress and responses such as damages to cardiovascular function, weightlessness-incluced bone loss, and muscle atrophy [3-5]. In addition, neurological dysfunction was associated with decreased decision-making ability [6]. In the state of weightlessness, the astronaut's blood rushes to the head [7], resulting in reduced osmotic pressure in the cerebrovascular colloid, increased overall intracranial transmural pressure, and a slightly impaired blood-brain barrier [8, 9]. Changes in hemodynamics disrupt microcirculation in the brain, impacting both neural structure and nerve functions [10-12]. A long-term exposure to a microgravity environment resulted in the impairment in learning and memory ability, further influencing peoples' capability of decision-making in the risky and tough space missions that require high precision, hence endangering the safety of space crew [11, 13]. To replicate the weightless environment experienced by astronauts, the hindlimb of the experimental animals were suspended with head-down tilt of 30° [14]. Subsequently, punitive behavioral tests, such as the Morris water maze and shuttle box, two widely recognized approaches, were employed for evaluating the learning and memory impairment [15, 16]. The reward-directed instrumental conditioning tests were deemed a multi-modal approach to evaluate the learning and memory capabilities of animals [17], which was used to measure the operation-based associative learning and memory [18]. The reward-directed instrumental conditioning task was conducted in this study to assess the capacity for operation-based associative learning and memory. This task facilitated target-guided behavior and evaluated the cognitive ability of animals in comprehending the causal relationship between their behaviors and subsequent consequences. It involved conditional stimulus signals (light), unconditional stimulus signals (sugar water), and operational behaviors. By discerning the varied consequences of operant conditioning behaviors triggered by correct or incorrect signals, the task highlighted the animals' conditioned and enhanced operational activities following habituation [19]. Therefore, the assessment of reward-directed instrumental conditioning task reflex in hindlimb suspension (HLS) rats could offer valid evidence for evaluating the astronauts' decision-making ability of astronauts in the aerospace environment, and provide measures for behavioral detection of reward-directed operation-related learning and memory. The HLS rats were observed to exhibit impairment in operating learning and memory, as indicated by a multi-modal and before-and-after-comparison assessment of rewarding-directed operating tasks [20, 21].

Renshen (Ginseng Radix et Rhizoma), a plant from the Araliaceae family, is a representative herb in traditional Chinese medicine with nootropic effects and is known as the "king of herbs" in China, from which the ginseng total saponins (GTSs) are extracted. Previous studies have demonstrated its efficacy in enhancing learning and memory ability in multiple animal models with cognitive impairment [22, 23]. In the tests conducted on HLS rats, it was found that GTSs alleviated the learning and memory impairment caused by HLS using shuttle box in metabolomics studies. In addition, the results of Nissl staining suggested that GTSs treatment increased the number of neurons in HLS rats with improved structural arrangement [20, 24, 25]. According to previous reports, N-methyl-D-aspartate acid (NMDA) receptor-dependent synaptic plasticity played an important role in the acquisition, consolidation, and reappearance of spatial memory in animals. N-methyl-D-aspartate acid receptor 1 (NR1) and phosphorylated N-methyl-D-aspartate acid receptor 2B (p-NR2B) were reported to also affect the acquisition and reproduction of animal memory [26-28]. The phosphorylation state of NR2B regulated the signaling pathway and mechanism of NMDA receptors presented in different cellular domain [29]. The expression levels of NR2B and p-NR2B produced by the onset of nerve inflammation and oxidative stress were markedly reduced, signifying the pathogenesis of cognitive dysfunction [30].

In this study, the multi-mode reward operant conditioning was employed to evaluate the protective effects of GTSs on learning and memory impairment in rats after

being HLS. The neuron morphology of the rat hippocampus and the expression of synaptic plasticity-related proteins, including NR1, NR2B, and p-NR2B, were measured to elucidate the mode of action of GTSs on operational associative learning and memory.

2 Materials and methods

2.1 Animals and environmental conditions

A total of 70 specific pathogen-free (SPF) male Wistar rats (7 weeks old) with weight ranging from 200 to 220 g were purchased from Beijing Charles River Experimental Animal Company [SCXK (Jing) 2016-0006], and housed in a SPF environment in the Institute of Medicinal Plants, Chinese Academy of Medical Sciences [SYXK (Jing) 2017-0020], with 12 h lighting (8:00 am – 8:00 pm) and 12 h darkness (8:00 pm – 8:00 am) in alternatives, along with sterile food and ample water supply. All experiments were conducted in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication No. 86-23, revised in 1996), and received approval from the Animal Care and Use Committee of the Institute of Medicinal Plants, Chinese Academy of Medical Sciences [2020(5)032].

2.2 Main reagents and instruments

The GTSs used in this study were obtained from the Institute of Medicinal Plants, Chinese Academy of Medical Sciences, China, which contained 258.89 mg/g Rb1, 65.77 mg/g Rb2, 12.87 mg/g Rg1, 26.14 mg/g Rg2, 93.72 mg/g Rc, 90.90 mg/g Rd, 105.34 mg/g Re, and 20.87 mg/g Rf. Huperzine A (Hup A) was purchased from Henan Tailong Pharmaceutical Co., Ltd., China. The reward-directed instrumental conditioning task was conducted in four operant chambers (dimensions: 30 cm × 33 cm × 90 cm). These chambers were developed by the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, and the Chinese Astronaut Research and Training Center [20]. The operant chambers were situated in sound-attenuated rooms, each equipped with two recessed food magazines and two retractable levers (4 cm wide, positioned 10 cm from the side walls). Three color light-emitting diode signal lights (red, blue, and yellow) were positioned above the lever. An infrared photocell, situated just inside the recessed magazine, was monitored by computer circuitry to record the duration of time each rat spent with its head in the magazine.

2.3 Animal grouping and modeling

The 70 rats were acclimated for 10 d before the experiment. After training, 63 rats had acquired the reward operant conditioning, 50 of them performed similarly and were randomly divided into five groups (10 rats in each group): (i) control group, with rats received distilled water treatment; (ii) HLS group, with rats bound by tape and iron chain hooks in the hindlimb, 30° between torso and the floor, and received distilled water; (iii) HLS-Hup A 0.1 mg/kg group as the positive drug group, with HLS rats treated with Hup A (0.1 mg/kg); (iv) HLS-GTSs 100 and 200 mg/kg groups, with rats orally administered with 100 and 200 mg/kg GTSs for 38 d, respectively, once a day [24]. All animals were housed in separate cages with free access to water, except during the periods of controlled dietary regulation to maintain 80% – 85% of their free-feeding weight before initiating behavioral tests. This protocol ensures optimal performance in the operant chambers, where food reinforcement was employed.

The establishment of reward-directed conditioning was conducted for 3 d (day 11 – 13), and the reward-directed operant conditioning task I was conducted for 6 d (day 14 – 19). After screening and grouping according to the task completion time, the rats received HLS with oral administration of drugs for 38 d (day 20 – 58). The rats were food restricted between 80% – 85%, and double-bottle feeding before and during behavioral training and testing (day 1 – 20 and day 41 – 58). The reward-directed operant conditioning task Ⅱ was performed for 3 d (day 48 – 50), and then the visual signal discrimination task was performed for 6 d (day 51 – 56). The reward extinction experiment was performed for 2 d (day 57 and 58); following the behavioral tests, the rats were sacrificed for Nissl staining and Western blot. The experimental design setup is shown in Figure 1.

2.4 Behavioral experiments

This method preserves the entire reward detection stage [19]. In the reward-directed conditioning task, rats

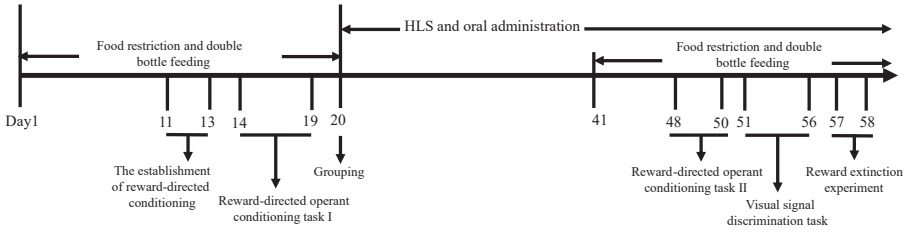

Figure 1 The experiment design procedure

underwent 20 min training tests for 3 d. During the training sessions, no lever was utilized, and the blue signal light was the only stimulus signal. The light was periodically turned on for 10 s, with intervals maintained at 30 ± 1 s. When the blue signal light was on, a drop of 20% sucrose solution was delivered simultaneously. In the reward-directed operant conditioning tasks Ⅰ and Ⅱ, the rats were first subjected to 6 d of training followed by a 3 d test. A fixed ratio schedule was performed in the test. When the animal stepped on the pedal, the blue signal light was turned on for 10 s, and a drop of 20% sucrose solution was delivered as a reward (the maximum number of rewards per session was 50). In the visual signal discrimination task assisted by blue and yellow lights, the lights were activated alternatively, with 120 s each time and a total of 28 min for the entire experimental session. When the blue signal light was on, the animals could receive a reward every time they stepped on the unilateral lever; and the yellow signal light was indicated an incorrect response. The two-color signal lights were on alternately for 120 s, and the test lasted for 28 min. In the reward extinction experiment, the setting was the same as the conditioned reflex of recognizing the reward visual signal. In this session, rats received no reward substance even if they performed correct behavior. The regression of conditioned reflex was recorded.

2.5 Nissl staining

Five rats from each group were randomly selected for Nissl staining, which was used to observe the hippocampal tissues. The rats in each group were anesthetized by 2% isoflurane (RWD, China), perfused with 4% paraformaldehyde to remove their brains, dehydrated with 30% sucrose, sectioned after embedding of their hippocampal tissues with the use of paraffin (6 – 8 μm thick), deparaffinized and immersed in tar violet staining solution for 3 h, followed by differentiation with 95% ethanol. The Nissl bodies were stained purple, then the tissue sections underwent dehydration in absolute ethanol (5 min), were subjected to two rounds of xylene transparency lasting 5 min each, and finally, were sealed with neutral gum. The morphological changes of neurons in CA1 and CA3 regions of the rat hippocampus were photographed with a light microscope (DM3000, Leica), and neuron numbers were counted using Image-Pro Plus analysis software (V 6.0, Media Cybernetics).

2.6 Western blot analysis

Five rats from each group were randomly selected for the experiment. The hippocampal tissues of rats were homogenized in radio immunoprecipitation assay (RIPA) lysis buffer, including 50 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 1% Triton X-100, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), and 100 μg/mL phenylmethanesulfonyl fluoride (PMSF). The supernatants were collected following centrifugation (12 000 rpm, 5 min, 4 °C) and quantified using a bicinchoninic acid (BCA) assay kit (Multi Sciences, China). The protein was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Genstar, China) and transformed into polyvinylidene difluoride (PVDF, Absin, China) membranes. After blocking with 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies at 4 °C overnight. The antibodies used were anti-rat pancadherin, anti-rat NR1, anti-rat p-NR2B, and anti-rat NR2B (1 : 1 000, Abcam, UK). Then, the membranes were washed thrice with Tris buffered saline + Tween (TBST, Cwbio, China), and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The protein bands were detected using the enhanced chemiluminescence (ECL) detection kit (Cwbio, China), and analyzed via the AlphaEase FC software (V 6.0, Alpha Innotech Corp).

2.7 Statistical analysis

All data were expressed as mean ± standard error (SEM). The data were analyzed using SPSS software (V 21.0) with one-way or repeated measures analysis of variance where statistically appropriate. The data from behavioral tests were analyzed using repeated measures analysis of variance (RM ANOVA). When significant effects were detected, post hoc multiple pairwise comparisons were made using the least significant difference (LSD) comparisons test after ANOVA. One-way ANOVA was used to test differences between groups in biochemistry and molecular biology tests. Moreover, $P < 0.05$ (two-tailed) was considered statistically significant.

3 Results

3.1 The establishment of the reward-directed operant conditioning

The number of nose pokes serves as a behavioral indicator, reflecting the rats' spatial memory and exploration interest in the drinking water box. It plays a crucial role in determining whether the animal progresses to the next stage of training. As shown in Table 1, the number of nose pokes and its completion time gradually decreased, while the number of lever presses gradually increased with each successive training day. Furthermore, there was a steady rise in the proportion of rats successfully accomplishing the task within the designated time frame. By day 6, 63 rats had acquired the reward-directed operant conditioning, constituting 90% of the total number.

Table 1 The establishment of the reward-directed operant conditioning

Training day	Number of nose pokes (*n*)	Number of lever presses (*n*)	Completion time (s)	Completion number of rats (*n*)
1	137.69 ± 7.27	22.66 ± 2.55	1 623.51 ± 36.91	22
2	120.22 ± 7.51	30.90 ± 2.66	1 303.62 ± 66.72	34
3	106.91 ± 5.95	39.23 ± 2.33	957.51 ± 67.95	53
4	91.89 ± 6.07	42.97 ± 2.04	729.83 ± 64.07	59
5	82.48 ± 4.43	44.44 ± 1.86	598.66 ± 57.55	62
6	71.74 ± 2.57	45.20 ± 1.74	565.01 ± 54.99	63

3.2 GTSs improving the cognitive performance of HLS-induced spatial learning and memory deficits in rats

3.2.1 Effects of GTSs on the behavior in the reward-directed operant conditioning task Ⅰ of HLS rats In the reward-directed operant conditioning task I, comparisons were made before and after modeling on the first day as shown in Figure 2A, the HLS rats showed a reduction in lever presses, which was restored after GTSs treatment (HLS-GTSs 100 and 200 mg/kg). The rates in HLS-Hup A 0.1 mg/kg group did not reverse the reduction of lever presses. In addition, the HLS rats took significantly longer time to complete the task ($P < 0.05$, Figure 2B). However, rats undergoing GTSs treatment exhibited a reduction in the task completion time, whereas no effect was observed in rats receiving Hup A.

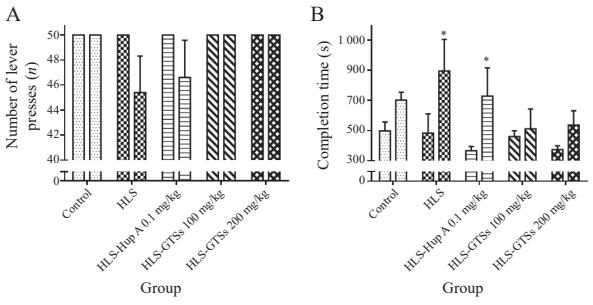

Figure 2 Effects of GTSs on the behavior in the reward-directed operant conditioning task Ⅰ before and after modeling in rats

A, the number of lever presses. B, completion time. Left bars in each graph represent before modeling; right bars in each graph represent after modeling. Data were represented as mean ± SEM (n = 10). $^{*}P < 0.05$, compared indicators before and after modeling.

3.2.2 Effects of GTSs on the behavior in the reward-directed operant conditioning task Ⅱ of HLS rats In the reward-directed operant conditioning task Ⅱ, no difference in the number of lever presses was detected in the control group for three consecutive days ($P > 0.05$). In contrast, a significant reduction was observed in HLS rats on day 2 ($P < 0.05$). HLS-GTSs 100 and 200 mg/kg groups both induced a significant up-regulation in lever presses when compared with the HLS rats ($P < 0.05$, Figure 3A). HLS rats also demonstrated a decrease in the number of nose pokes on day 1 ($P < 0.05$) and day 2 ($P < 0.01$). The rats in HLS-GTSs 100 mg/kg exhibited a significant improvement in the number of nose pokes on day 2 ($P < 0.05$, Figure 3B). Furthermore, a longer completion time was detected in HLS rats on day 3 compared with the control rats ($P < 0.05$); however, when compared with rats in the HLS group, those receiving 200 mg/kg GTSs treatment exhibited a significantly reduced task completion time on day 3 ($P < 0.05$, Figure 3C). Compared with the HLS group, the HLS-Hup A 0.1 mg/kg group showed opposite changes in the levels of the above indicators, but there was no significant difference ($P > 0.05$). The data suggested that GTSs increased the number of lever presses and nose pokes, decreased the task completion time of HLS rats in the reward-directed operant conditioning task II.

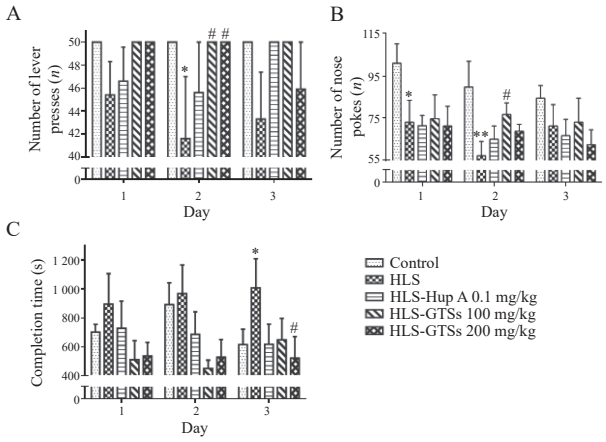

Figure 3 Effects of GTSs on the behavior in the reward-directed operant conditioning task Ⅱ of HLS rats

A, the number of lever presses. B, the number of nose pokes. C, completion time. Data were represented as mean ± SEM (n = 10). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$, compared with the HLS group.

3.2.3 Effects of GTSs on the discrimination index in the visual signal discrimination task of HLS rats As shown in Figure 4, in the two-color lights visual signal discrimination task, the discrimination index was calculated as: (the number of correct lever presses – number of incorrect lever presses)/the total number of lever presses. Notably, it exhibited a positive correlation with the number of training days. A significant decrease was observed in the discrimination index of HLS rats on day 3 when compared with the control group on day 3, 4, and 6 ($P < 0.05$), while a marked increase was shown in HLS-GTSs 100 mg/kg ($P < 0.01$, day 6), HLS-GTSs 200 mg/kg ($P < 0.05$, day 2, 3, and 6), and Hup A 0.1 mg/kg ($P < 0.05$, day 4 and 6) groups.

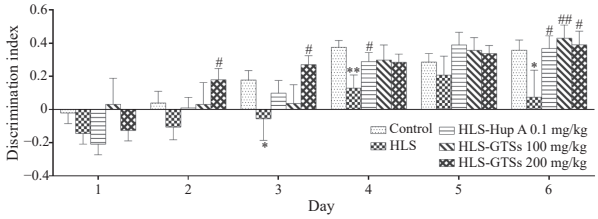

Figure 4 Effects of GTSs on the discrimination index in the visual signal discrimination task of HLS rats

Data were represented as mean ± SEM (n = 10). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the HLS group.

3.2.4 Effects of GTSs on the the behavior in the reward extinction experiment of HLS rats As shown in Figure 5, in the reward extinction experiment, rats received no reward and gradually decreased the numbers of lever presses and nose pokes. On day 2, there was a significant increase in lever presses in HLS rats ($P < 0.01$), and HLS rats undergoing 100 and 200 mg/kg GTSs treatment showed a descrease ($P < 0.05$, Figure 5A). The ratio of the number of lever presses/nose pokes also increased in HLS rats on day 1 and 2 ($P < 0.01$), and reduced in HLS-GTSs 100 mg/kg ($P < 0.05$) and HLS-Hup A 0.1 mg/kg ($P < 0.01$) rats (Figure 5B).

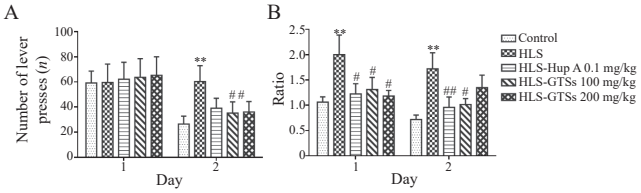

Figure 5 Effects of GTSs on the reward extinction experiment on HLS rats

A, the number of lever presses. B, the ratio of the numbers of lever presses/nose pokes. Data were represented as mean ± SEM (n = 10). $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the HLS group.

3.3 GTSs ameliorating HLS-induced neuronal damage in the hippocampus

The hippocampal tissues of rats in the control group exhibited abundant Nissl bodies, and the neurons were arranged neatly and densely, according to observations (Figure 6A). There was a significant reduction in the number of Nissl bodies and neurons in the hippocampus of HLS rats ($P < 0.05$). In HLS rats, the fibers were partially dissolved, and the neuron arrangement was messy. After the treatment of GTSs, the number of neurons in HLS rats significantly increased ($P < 0.05$, Figure 6B and 6C).

NMDA receptors levels were altered after HLS and drug treatment (Figure 7A). Compared with the control rats, the expression levels of NR1 and p-NR2B in the HLS rats were significantly reduced ($P < 0.01$ and $P < 0.05$, respectively, Figure 7B and 7D), whereas the NR2B expression level was not significantly affected ($P > 0.05$, Figure 7C). HLS rats receiving 100 and 200 mg/kg GTSs treatment induced the increased expression level of p-NR2B when compared with HLS rats ($P < 0.01$). In addition, the proportion of NR2B phosphorylation decreased in the HLS group, which was elevated by GTSs and Hup A, without statistically significant differences observed ($P > 0.05$).

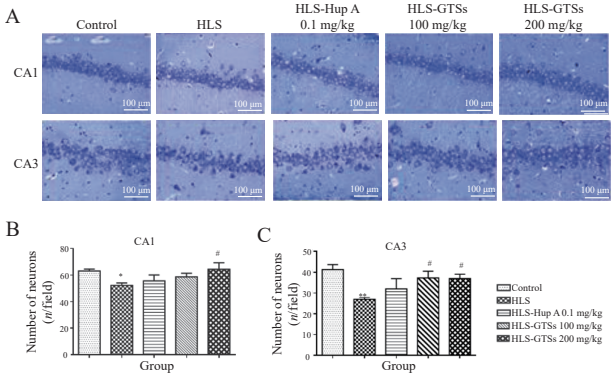

Figure 6 Nissl staining of the CA1 and CA3 areas in the rats' hippocampus

A, the CA1 and CA3 areas of the hippocampus under a 400 × microscope. B, the number of neurons in the hippocampus CA1 area. C, the number of neurons in the hippocampus CA3 area. Data were represented as mean ± SEM (n = 5). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$, compared with the HLS group.

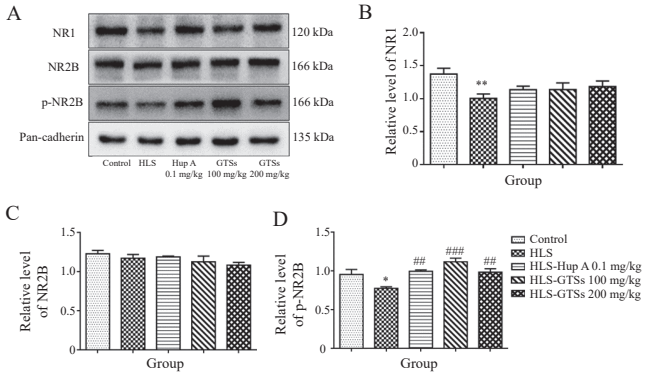

Figure 7 Effects of GTSs on the expression levels of NR1, NR2B, and p-NR2B proteins in the hippocampus of rats

A, the protein bands of Western blot of NR1, NR2B, and p-NR2B. B – D, the quantitative evaluation of the proteins of NR1, NR2B and p-NR2B, respectively. Data were represented as mean ± SEM (n = 5). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the HLS group.

4 Discussion

4.1 GTSs reversing the cognitive impairment caused by HLS

In our study, the multi-mode reward-directed instrumental conditioning task was carried out to monitor the changes in behaviors, neuron morphology, and synaptic plasticity-related expression levels of NR1 and p-NR2B

proteins in HLS rats as well as GTSs treated HLS rats. The data showed that HLS caused impairment of cognitive behaviors and changes in neuron morphology in rats. GTSs treatment could partially ameliorate the impairment of learning and memory function via the up-regulation of NR1 and p-NR2B expression levels.

To replicate a weightless environment, the hindlimbs of rats were suspended specifically, resulting in their hindlimbs being elevated 30° higher than their head while their forelimbs remained on the ground. In the test, the observed physiological changes were partially attributed to changes in hemodynamics following exposure to a weightless environment and the absence of gravitational stimulation in the hindlimbs. It was reported that the physiological changes in the central nervous system in HLS rats were consistent with changes in actual weightlessness stimulation [1]. In addition, the test was characterized by an extended suspension time and exposure to light stress stimulation. Consequently, the obtained results were not significantly affected by stress-induced interference in the animals.

Many behavioral tests, such as the Morris water maze, X-maze shutter, and the step-down test, were developed to evaluate the learning and memory functioning ability of rats [31]. However, during the tests, animals were exposed to noxious stimuli or punitive damages [32]. In the reward-directed instrumental learning tests, the conditioned memory was formed via acquisition, consolidation, reproduction, and reconsolidation, without or with little negative impacts on behaviors and cognitive functions [33]. These findings were associated with both the goal-directed process and the habituation of the stimulus and response mechanism, representing two distinct forms of learning [34-36]. The findings suggested that the number of lever presses decreased and the completion time increased in HLS rats in the reward-directed operant conditioning reflex test, indicating that HLS affected the learning ability of memory reconsolidation. Besides, the number of nose pokes was also counted to measure the interest of the HLS rats in exploration, which showed a significant reduction, suggesting reduced interest of the HLS rats in exploration.

The discrimination index is important for visual signal recognition in reward-directed operant conditioning reflexes. It evaluates animals' ability to discriminate between correct and incorrect signal lights. These results indicated that HLS severely down-regulated the discrimination index. In addition, a reward extinction experiment was performed to monitor the existence of the rat's conditioned reflex which was not reinforced. The lever presses of the HLS rats increased significantly on day 2, indicating that their learning ability was reduced and the learning and memory function impaired. The ratio of lever presses to nose pokes serves as a causal judgment of the operational behavior of rats in the reward extinction experiment. We found the rate of HLS rats significantly increased, indicating that they were not sensitive to causal connection, with reduced learning ability as well as impaired learning and memory observed [17, 18, 37]. After GTSs treatment, the operational decision-making capability was improved in the HLS rats.

4.2 GTSs reversing the neurological damage caused by HLS through the NR1/p-NR2B pathway

HLS also changed the hemodynamic status and cerebrospinal fluid's circulation status in rats' brains, causing morphological changes and apoptosis of nerve cells [20]. In previous studies, having HLS for 7 or 14 d caused apoptosis of hippocampal neurons and reduced neuron numbers in the CA1 area in rats [38, 39]. Based on the above studies, we conducted long-term simulated weightlessness experiments, it was found that after HLS in a simulated weightless environment, the number of neurons and blue-stained Nissl bodies in the hippocampus was significantly reduced, the nerve fibers were partially disintegrated, and the arrangement of the neurons was disorganized. In contrast, the hippocampal neurons of rats in the control group were rich in Nissl bodies and arranged neatly and densely. In HLS rats undergoing GTSs treatment, the number of neurons increased, with regular shapes arranged in a nice order. Hup A, isolated from the Chinese herb Huperzia serrata, can improve learning and memory ability, and has neuroprotective effect by influencing the induction of long-term potentiation (LTP) in CA1 area [40]. Our study and previous studies show that both Hup A and GTSs have modulatory effects on NMDA receptors, but GTSs treatment could produce better learning and memory performance for complex manipulations [21].

Under simulated microgravity conditions, changes in gravity significantly regulate critical genes associated with learning and memory, leading to macroscopic physiological effects [41]. Among them, NMDA receptor-dependent synaptic plasticity played a vital role in the acquisition, consolidation, and reproduction of animal spatial memory. NR1 was an essential subunit of a functional NMDA receptor, and the knockout NR1 affected the acquisition and reproduction of animal memory as well [42]. In addition, the p-NR2B subunit enhanced the current status through the NMDA receptor channel. It regulated the receptor interactions in synapses, affecting LTP mediated by NMDA receptors [43]. Previous studies indicated that reversing the decrease of p-NR2B protein and improving synaptic plasticity could improve cognitive function in AD mice and their offspring exposing to bisphenol A [28, 44]. In addition, continuous microwave exposure research also found that NR2B and p-NR2B might lead to cognitive impairment [45].

Our study preliminarily found that GTS affected the learning and memory in HLS rats by acting on NMDA receptors. HLS significantly inhibited NR2B receptor

phosphorylation without affecting the expression of NR2B protein, and GTSs stimulated NR2B receptor phosphorylation to affect neuronal viability. GTSs can improve the learning and memory impairment of rats in complex operation tasks, making it a promising candidate for addressing learning and memory impairments induced by microgravity during spaceflight. Regardless, further in-depth verification appears imperative to expand and enrich this research. Hence, multiple approaches have been contemplated for future investigation into the roles of the NR1/p-NR2B pathway in GTSs treatment with regards to improving cognitive function in HLS animals.

5 Conclusion

In summary, this study conducted multi-mode reward-directed instrumental conditioning tasks. The findings indicated that HLS could induce learning and memory impairment in rats, which were ameliorated by treatment with GTSs. This improvement could be attributed to the improvement of the neuron morphology in the hippocampus and up-regulated expression of synaptic plasticity-related NR1 and p-NR2B proteins.

Fundings

Space Medical Experiment Project of Chinese Manned Space Program (HYZHXM05003), and Scientific and Technological Innovation Project of the China Academy of Chinese Medical Sciences (CI2021A04905).

Competing interests

The authors declare no conflict of interest.

References

[1] NDAY CM, FRANTZIDIS C, JACKSON G, et al. Neurophysiological changes in simulated microgravity: an animal model. Neurology India, 2019, 67(Supplement): S221–S226.

[2] ZHANG YL, WANG Q, CHEN HL, et al. Involvement of cholinergic dysfunction and oxidative damage in the effects of simulated weightlessness on learning and memory in rats. BioMed Research International, 2018, 2018: 2547532.

[3] SHIBATA S, WAKEHAM DJ, THOMAS JD, et al. Cardiac effects of long-duration space flight. Journal of the American College of Cardiology, 2023, 82(8): 674–684.

[4] TRAPPE TA, TESCH P, ALKNER B, et al. Microgravity-induced skeletal muscle atrophy in women and men: implications for long-duration spaceflights to the Moon and Mars. Journal of Applied Physiology, 2023, 135(5): 1115–1119.

[5] ZHU M, LIU ZY, GAO MZ, et al. The effect of Bu Zhong Yi Qi decoction on simulated weightlessness-induced muscle atrophy and its mechanisms. Molecular Medicine Reports, 2017, 16(4): 5165–5174.

[6] TAHIMIC CGT, PAUL AM, SCHREURS AS, et al. Influence of social isolation during prolonged simulated weightlessness by hindlimb unloading. Frontiers in Physiology, 2019, 10: 1147.

[7] ZAHID AM, MARTIN B, COLLINS S, et al. Quantification of arterial, venous, and cerebrospinal fluid flow dynamics by magnetic resonance imaging under simulated micro-gravity conditions: a prospective cohort study. Fluids and Barriers of the CNS, 2021, 18(1): 8.

[8] IWASAKI KI, OGAWA Y, KURAZUMI T, et al. Long-duration spaceflight alters estimated intracranial pressure and cerebral blood velocity. The Journal of Physiology, 2021, 599(4): 1067–1081.

[9] BELLONE JA, GIFFORD PS, NISHIYAMA NC, et al. Long-term effects of simulated microgravity and/or chronic exposure to low-dose gamma radiation on behavior and blood-brain barrier integrity. NPJ Microgravity, 2016, 2: 16019.

[10] KERMORGANT M, NASR N, CZOSNYKA M, et al. Impacts of microgravity analogs to spaceflight on cerebral autoregulation. Frontiers in Physiology, 2020, 11: 778.

[11] VAN OMBERGEN A, DEMERTZI A, TOMILOVSKAYA E, et al. The effect of spaceflight and microgravity on the human brain. Journal of Neurology, 2017, 264(1): 18–22.

[12] GOTO M, SHIBATA Y, ISHIYAMA S, et al. Brain microstructure and brain function changes in space headache by head-down-tilted bed rest. Aerospace Medicine and Human Performance, 2023, 94(9): 678–685.

[13] ROBERTS DR, ASEMANI D, NIETERT PJ, et al. Prolonged microgravity affects human brain structure and function. American Journal of Neuroradiology, 2019, 40(11): 1878–1885.

[14] LIU HY, LIANG M, DENG YL, et al. Simulated microgravity alters P-glycoprotein efflux function and expression via the Wnt/β-catenin signaling pathway in rat intestine and brain. International Journal of Molecular Sciences, 2023, 24(6): 5438.

[15] WANG Q, ZHANG YL, LI YH, et al. The memory enhancement effect of Kai Xin San on cognitive deficit induced by simulated weightlessness in rats. Journal of Ethnopharmacology, 2016, 187: 9–16.

[16] FENG L, YUE XF, CHEN YX, et al. LC/MS-based metabolomics strategy to assess the amelioration effects of ginseng total saponins on memory deficiency induced by simulated microgravity. Journal of Pharmaceutical and Biomedical Analysis, 2016, 125: 329–338.

[17] XU P, WANG KZ, LU C, et al. Effects of the chronic restraint stress induced depression on reward-related learning in rats. Behavioural Brain Research, 2017, 321: 185–192.

[18] SHI Z, CHEN LL, LI SD, et al. Chronic scopolamine-injection-induced cognitive deficit on reward-directed instrumental learning in rat is associated with CREB signaling activity in the cerebral cortex and dorsal hippocampus. Psychopharmacology, 2013, 230(2): 245–260.

[19] WANG KZ, XU P, LU C, et al. Effects of ginsenoside Rg1 on learning and memory in a reward-directed instrumental conditioning task in chronic restraint stressed rats. Phytotherapy Research, 2017, 31(1): 81–89.

[20] LV JW, JIANG N, WANG HX, et al. Simulated weightlessness induces cognitive changes in rats illustrated by performance in operant conditioning tasks. Life Sciences in Space Research, 2021, 29: 63–71.

[21] JIANG N, LV JW, ZHANG YW, et al. Protective effects of

ginsenosides Rg1 and Rb1 against cognitive impairment induced by simulated microgravity in rats. Frontiers in Pharmacology, 2023, 14: 1167398.

[22] ZHENG CX, LI XP, CHEN LP. Effects of ginseng total saponin combined with icariin on learning and memory and apoptosis of hippocampal nerve cells in vascular dementia rats. China Pharmacist, 2014, 17(9): 1444–1447

[23] GONG YG, LIU Y, ZHOU L, et al. A UHPLC-TOF/MS method based metabonomic study of total ginsenosides effects on Alzheimer disease mouse model. Journal of Pharmaceutical and Biomedical Analysis, 2015, 115: 174–182.

[24] FENG L, LIU XM, CAO FR, et al. Anti-stress effects of ginseng total saponins on hindlimb-unloaded rats assessed by a metabolomics study. Journal of Ethnopharmacology, 2016, 188: 39–47.

[25] WANG TM , ZHANG YL , WANG YL, et al. Effects of tail-suspension on memory and apoptosis related protein expression in rat hippocampus. Space Medicine & Medical Engineering, 2016, 29(4): 235–239.

[26] GALAJ E, BARRERA ED, LYNCH OL, et al. Muscarinic and NMDA receptors in the substantia nigra play a role in reward-related learning. The International Journal of Neuropsychopharmacology, 2023, 26(1): 80–90.

[27] SENGAR AS, LI HB, ZHANG WB, et al. Control of long-term synaptic potentiation and learning by alternative splicing of the NMDA receptor subunit $GluN_1$. Cell Reports, 2019, 29(13): 4285–4294. e5.

[28] MAHAMAN YAR, FENG J, HUANG F, et al. Moringa oleifera alleviates Aβ burden and improves synaptic plasticity and cognitive impairments in APP/PS1 mice. Nutrients, 2022, 14(20): 4284.

[29] GOEBEL-GOODY SM, DAVIES KD, ALVESTAD LINGER RM, et al. Phospho-regulation of synaptic and extrasynaptic N-methyl-D-aspartate receptors in adult hippocampal slices. Neuroscience, 2009, 158(4): 1446–1459.

[30] CAO MY, FANG JK, WANG XQ, et al. Activation of AMP-activated protein kinase (AMPK) aggravated postoperative cognitive dysfunction and pathogenesis in aged rats. Brain Research, 2018, 1684: 21–29.

[31] ZHANG RY, WANG JM, HUANG L, et al. The pros and cons of motor, memory, and emotion-related behavioral tests in the mouse traumatic brain injury model. Neurological Research, 2022, 44(1): 65–89.

[32] WELLS DJ. Animal welfare and the 3Rs in European biomedical research. Annals of the New York Academy of Sciences, 2011, 1245: 14–16.

[33] TRUCKENBROD LM, BETZHOLD SM, ALEXARAE W, et al. Circuit and cell-specific contributions to decision making involving risk of explicit punishment in male and female rats. The Journal of Neuroscience, 2023, 43(26): 4837–4855.

[34] BALLEINE BW, DICKINSON A. Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. Neuropharmacology, 1998, 37(4/5): 407–419.

[35] COURTIN J, BITTERMAN Y, MÜLLER S, et al. A neuronal mechanism for motivational control of behavior. Science, 2022, 375(6576): eabg7277.

[36] KRUGLANSKI AW, SZUMOWSKA E. Habitual behavior is goal-driven. Perspectives on Psychological Science, 2020, 15(5): 1256–1271.

[37] SHI Z, CHEN SG, CHEN LL, et al. Evaluation of reward-relevant learning and memory behavior with operant conditioning task in rats. Acta Laboratorium Animalis Scientia Sinica, 2012, 20(4): 9–15.

[38] LI JH, XUE CY, YANG HY, et al. Simulated weightlessness induces hippocampal insulin resistance and cognitive impairment. Life Sciences, 2023, 333: 122112.

[39] LI Y, CAO L, LI J, et al. Influence of microgravity-induced intervertebral disc degeneration of rats on expression levels of p53/p16 and proinflammatory factors. Experimental and Therapeutic Medicine, 2019, 17(2): 1367–1373.

[40] YE L, QI JS, QIAO JT. Long-term potentiation in hippocampus of rats is enhanced by endogenous acetylcholine in a way that is independent of N-methyl-D-aspartate receptors. Neuroscience Letters, 2001, 300(3): 145–148.

[41] MCNEARNEY TA, WESTLUND KN. Pluripotential GluN1 (NMDA NR1): functional significance in cellular nuclei in pain/nociception. International Journal of Molecular Sciences, 2023, 24(17): 13196.

[42] ZHANG XL, XIE YB, XU WQ, et al. Effects of 5-Aza on p-Y1472 NR2B related to learning and memory in the mouse hippocampus. Biomedicine & Pharmacotherapy, 2019, 109: 701–707.

[43] LI X, HAO LY, ZENG FC, et al. Effect of decabrominated diphenyl ether exposure on spatial learning and memory, the expression and phosphorylation of hippocampal glutamate receptor subunits in adult Sprague-Dawley rats. The Journal of Toxicological Sciences, 2018, 43(11): 645–657.

[44] WU D, WU FJ, LIN R, et al. Impairment of learning and memory induced by perinatal exposure to BPA is associated with ERα-mediated alterations of synaptic plasticity and PKC/ERK/CREB signaling pathway in offspring rats. Brain Research Bulletin, 2020, 161: 43–54.

[45] WANG H, TAN SZ, XU XP, et al. Long term impairment of cognitive functions and alterations of NMDAR subunits after continuous microwave exposure. Physiology & Behavior, 2017, 181: 1–9.

人参总皂苷对后肢悬吊大鼠奖励性操作式条件反射能力的保护作用

董李晋川[a†], 陈颖[a†], 胡秦[b], 鲍余[c], 潘瑞乐[c], 曾桂荣[d], 彭博[a], 刘新民[c, e*], 石哲[f*]

a. 中国中医科学院中药研究所, 北京 100700, 中国
b. 北京工业大学环境与生命学院, 北京 100124, 中国
c. 中国医学科学院北京协和医学院药用植物研究所, 北京 100193, 中国
d. 新药药效与安全性评价湖南省重点实验室 & 湖南省药物安全性评价研究中心, 湖南 长沙 410331, 中国
e. 宁波大学新药技术研究院, 浙江 宁波 315000, 中国
f. 湖南中医药大学药学院湘产大宗药材品质评价湖南省重点实验室 & 干细胞中药调控与应用实验室, 湖南 长沙 410208, 中国

【摘要】目的 本研究旨在探讨人参总皂苷（GTSs）对持续暴露于微重力环境导致的宇航员认知障碍的治疗作用。**方法** 按完成奖赏条件反射适应任务的时间，将 50 只无特定病原体（SPF）雄性 Wistar 大鼠随机分为对照组、后肢悬吊（HLS）组、石杉碱甲（HLS-Hup A 0.1 mg/kg）组、GTSs 低剂量（HLS-GTSs 100 mg/kg）组和 GTSs 高剂量（HLS-GTSs 200 mg/kg）组。除对照组外，其余各组大鼠后肢悬吊并给予药物治疗（第 20－58 天），进行奖赏反射测试，并对大鼠的海马进行尼氏体染色和蛋白质印迹检测。**结果** 造模后，与对照组比较，HLS 组大鼠按压杠杆次数减少，完成奖赏条件反射任务Ⅰ的时间延长（$P < 0.05$），而 HLS-GTSs 100 和 200 mg/kg 组大鼠上述指标无明显变化（$P > 0.05$）。奖赏操作条件反射Ⅱ实验中，与对照组相比，HLS 组大鼠按压杠杆次数明显减少（$P < 0.05$），触摸鼻腔次数明显减少（$P < 0.01$）；与 HLS 组大鼠比较，HLS-GTSs 100 mg/kg 组大鼠杠杆按压次数和鼻触次数均明显增加（$P < 0.05$），HLS-GTSs 200 mg/kg 组完成时间显著缩短，杠杆按压次数显著增加（$P < 0.05$）。视觉信号辨别实验中，与对照组大鼠相比，HLS 组大鼠视觉信号辨别测试指标降低（$P < 0.01$），HLS-GTSs 100 和 200 mg/kg 组大鼠视觉信号辨别能力明显升高（$P < 0.01$）。奖赏消退实验中，与对照组相比，HLS 组大鼠的杠杆按压次数显著增加（$P < 0.01$）；与 HLS 组相比，HLS-GTSs 100 和 HLS-GTSs 200 mg/kg 组大鼠的杠杆按压次数显著降低（$P < 0.05$）。HLS 组大鼠 N-甲基-D-天冬氨酸受体 1（NR1）和磷酸化 N-甲基-D-天冬氨酸受体 2B（p-NR2B）蛋白表达明显降低（分别为 $P < 0.01$ 和 $P < 0.05$），而 NR2B 蛋白表达无明显变化（$P > 0.05$）。GTSs 可上调 p-NR2B 的表达水平（$P < 0.01$）。**结论** GTSs 通过调节 NR1/NR2B 磷酸化通路改善大鼠对复杂操作的学习记忆能力。

【关键词】人参总皂苷；模拟失重；学习记忆；奖励奖赏条件反射；N-甲基-D-天冬氨酸受体