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Different Durations of Simulated Weightlessness Induced Depressive-like Behaviors in Rats*

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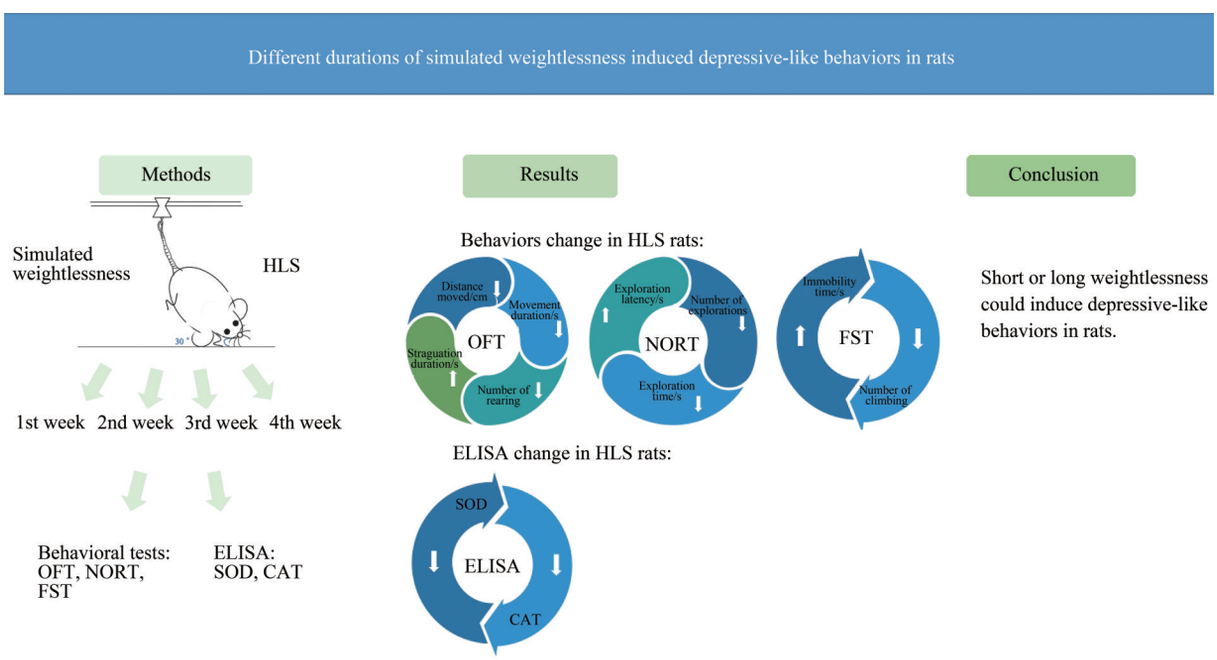
Graphical abstract



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Abstract **Objective** This study was designed to observe different durations of simulated weightlessness induced depressive-like behaviors and the change of superoxide dismutase (SOD) and catalase (CAT) in hippocampi of rats, so as to explore its effects and possible mechanism. **Methods** Weightlessness was simulated by hindlimb suspension by tails (HLS) in rats. The rats were divided into the control and the HLS groups for each respective week (s), *i.e.*, 1, 2, 3, and 4. The depressive-like behaviors in rats were investigated by using the open field test (OFT), novel object recognition test (NORT), forced swim test (FST), followed by hippocampal protein level estimation of SOD and CAT by ELISA. **Results** The OFT results showed increased stagnation time among HLS rats in each respective group compared to their corresponding control groups (P<0.05, P<0.01). In the NORT, the latency of exploration increased, while the number and the time of exploration decreased in the HLS groups in each week, compared to their corresponding control rats (P<0.05, P<0.01). Similarly, FST displayed enhanced immobility with the reduced climbing rate in HLS rats in each respective week, compared to the normal rats (P<0.05, P<0.01). Simulated weightlessness significantly reduced the SOD and CAT levels in the hippocampi in HLS rats compared to the normal rats (P<0.05, P<0.01). **Conclusion** Results suggest that short or long weightlessness could induce depressive-like behaviors in rats.

Key words simulated weightlessness, depressive like behaviors, open field test, forced swim test, superoxide dismutase, catalase
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During space flight, astronauts face extreme conditions, *i.e.*, radiations, zero-gravity, weightlessness, isolation, and circadian asynchrony, which have negative impact on their physiological and psychological state[1]. Numerous studies have reported the adverse effect of microgravity on the cardiovascular system, musculoskeletal system, and neuropsychiatric system in human beings or animals[2-8]. The advancement in space sciences will result in longer duration of stay on-orbit residence tasks in the future[1-2]. Weightlessness is one of the most important and constant factors during space flight which could induce depressive like symptoms in astronauts and lead to their dependency on drug, or even resulted in suicide[4, 9-10]. However, the extent of depressive-like symptoms may vary with flight duration, *i.e.*, short or long stay in space flight[11-13]. Animal models of simulated weightlessness have provided an economical way to understand its role in different physiological and psychological outcomes. Among them, the hindlimb suspension (HLS) model was commonly used[1,5]. This model has been showed to mimic the weightlessness faced during astronaut's flight in space. Oxidative damage was another key factor which could influence organs such as brain and negatively impact cognitive or depressive-like symptoms[14-16]. Rat exposed to HLS model have shown cognitive decline with elevated lipid peroxidation and impaired antioxidant enzymes, *i.e.*, catalase (CAT) or superoxide dismutase (SOD) in the brain samples[17-18]. But only few report focused on depressive-like behaviors induced by different HLS durations. So, in this study the HLS model was used to understand the effect of different time durations of simulated weightlessness to induce depressive-like behaviors in rats and its correlation with the antioxidant system. This study may provide referential evidence to screen effective measures to overcome the damage to astronauts during their space flight.

1 Materials and methods

1.1 Animals

Fifty-six SPF male Sprague-Dawley (SD) rats (weighing 200−220 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co, Ltd (Qualified No. SCXK 2016-0001, Beijing, China). They were housed under standard experimental conditions, *i.e.*, 20−22ºC temperature, 55% humidity, 12/12 h light-dark cycle. Rats were access to feed and water ad libitum. The ethical committee approved the protocols for the Care and Use of Laboratory Animals of IMPLAD, CAMS & PUMC, China (No. 2016515). All experiments were conducted according to the "Principles of Laboratory Animal Care" (N. I. H. publication No. 86−23, 1996) and P. R. China legislation for the use and care of laboratory animals.

1.2 Experimental design

Rats were acclimatized for 3 d in the experimental room before model development. HLS tail model was induced by hanging the rat in a computer-aided controlling and imagine analyses system for hindlimb by tail suspension (Chinese patent No. ZL201310228949.2, designed by Southwest Medical University, China Astronaut Research and Training Center, and Chinese Academy of Medical Sciences and Peking Union Medical

College).

Rats were randomly divided into 8 groups, with 7 rats per group: HLS-1w, HLS-2w, HLS-3w, HLS-4w, and their respective controls: C-1w, C-2w, C-3w, C-4w. 1−4w refers to the time of mold marking using ZL201310228949.2 system, which are 1−4 weeks. All rats were housed in the same environment with a natural light-dark cycle. After each time point, rats were evaluated for depression-like symptoms using a battery of behavioral tests such as the open field test (OFT), the novel object recognition test (NORT), and the forced swim test (FST). After the behavioral assessment, rats were anesthetized, and the hippocampi were dissected out for biochemical analysis.

1.3 The hindlimb suspension （HLS） rat model

The HLS was performed by tail suspension as described before[19-22]. Briefly, a black plexiglass cage with 26 cm×26 cm×30 cm dimension was used with a crossbar. The medical adhesive tapes were tied around the rat tails, and the rats were fixed with a small hook in a stainless chain mounted at the top of the cage. The length of the chain was adjusted to prevent the hind limbs' contact with any supportive surfaces in the cage floor while the forelimbs kept contact with the cage floor. Rats were maintained at a 30° head-down tilt position and allowed to rotate freely at 360°. The body mass of the rats was weighed every three days and compared among groups.

1.4 Behavioral tests and biochemical assay

1.4.1 The open field test（OFT）

The OFT was used to assess spontaneous locomotion, exploration, and anxiety-like activity among rodents[23-24]. The open field instrument comprises of four cylindrical containers, with the dimension of 80 cm in diameter and 50 cm in height. Each cylinder is equipped with a computer (software) aided camera to record locomotory activities such as movement time, stagnation time, speed of rats, number of rearing as mentioned in previous studies[25-26]. Each cylindrical of the open field arena is further divided into three portions, *i.e.,* the center area (40 cm diameter), the peripheral area (10 cm wide), and the exploration area between the central and peripheral area. The locomotory pattern of the rats was recorded for 10 min after placing the rat in the center of the area. After every experiment, the rats were placed back in the cage, and the open field arena was cleaned with 70% alcohol solution and dried. The total movement distance, average speed, duration of movement, number of rearing were recorded as the activity of every rat.

1.4.2 The novel object recognition test（NORT）

The NORT was introduced to evaluate the depressive like behavior in rats[27-28]. After the OFT, the NORT was performed as described before[29]. Briefly, a blue cylindrical object (4 cm×4 cm in high and diameter) was placed in the middle of the box. The explorative behavior of rats within 10 min was recorded after placing them in the corner of the arena. Parameters such as latency, number, and time of exploration were recorded by a software-aided camera placed above the arena. The exploratory behavior of rats was defined as the animal's mouth or nose less than 1 cm away from the object or directly touching the object. However, the behavior of animals' standing on object did not consider as explorations.

1.4.3 The forced swim test（FST）

The FST was a popular method to observe depressive like behavior in rodents[30-32]. The test included two days' detection and used to evaluate the relation of simulated spaceflight on the depression-like behavior in rats[33]. On day 1, rats were placed in a plexiglas cylinder (46 cm in height, 18 cm in diameter) which was filled with water up to 30 cm deep. Rats were allowed to explore the environment for 15 min, then rats were removed, dried and returned to the home cage. On day 2, the procedure was repeated for 5 min and the total mobility (rats moving their forepaws up along the edge) or immobility (rats keeping their heads above the water face without moving) time was recorded.

1.4.4 Biochemical assay

After behavioral tests, each rat was anesthetized, and the brain was dissected out to remove the hippocampus. Tissue samples were homogenized with a glass homogenizer in a tube containing RIPA lysis buffer (Solarbio Science & Technology, China) with protease and phosphatase inhibitors (Thermo Scientific, USA). The homogenates were centrifuged at 3 500 r/min at 4ºC for 10 min. The supernatant was transferred to a new tube, and protein concentration was estimated by bicinchoninic acid (BCA) kit (Solarbio Sciences and Technology, China). The prepared homogenate was used to quantify the level of SOD and CAT in each respective sample. The level

of SOD and CAT were analyzed using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, China). Activities of SOD and CAT were expressed as units/mg.

1.5 Statistical analysis

All data were analyzed using SPSS 17.0 software (IBM Corp.). Values are expressed as the mean± standard error of the mean (SEM). GraphPad Prism 5 (San Diego, CA, USA) was used to make figures. One-way ANOVA used to determine the statistically significant differences between groups with Tukey-Kramer and standard's *t*-test. The *P*-value below 0.05 was considered as significant difference.

2 Results

2.1 Effects of HLS different weeks on the body mass of rats

All the rats in the HLS groups, *i.e.*, 1, 2, 3, and 4 w, showed a significant decrease in mass as compared to rats in the respective control groups (P<0.05, Figure 1).

2.2 Effect of HLS different weeks on the locomotory activity in the open field test in rats

The OFT was used to observe the locomotory activities of rodents. All durations (1, 2, 3, 4 w) of HLS negatively impacted the locomotory activities of rats compared to their respective controls (Figure 2). The HLS rats in their respective time point showed significant reduction in distance traveled (Figure 2a), movement duration (Figure 2b), and rearing (Figure 2d), and increased in stagnation duration (Figure 2c) as compared to their respective controls (P<0.05). But the movement speed had no difference between HLS rats and their controls (data not show). The locomotory activities of the rats in different region of the open filed *i.e.* central, peripheral and exploratory areas were also monitored. The rats showed significantly reduced distance traveled (Figure 2a), movement duration (Figure 2b), with significantly increased stagnation duration (Figure 2c) as compared to respective controls in the central and exploration area. On the contrary rats preferred activities in the peripheral area compared to the central area (Figure 2).

2.3 Effects of HLS different weeks in the novel object recognition test in rats

In the NORT test, HLS rats in each respective group showed significantly increased latency of exploration compared to the control rats in each group (P<0.05, Figure 3a). On the contrary, the number and time of exploration significantly decreased in the HLS group at each time point as compared to their respective control group (P<0.05, Figure 3b, c). Results suggested more noticeable changes appear after 2 weeks of HLS, which impairs further in 3 and 4 weeks.

2.4 Effects of HLS different weeks in the forced swim test in rats

In the FST, the immobile nature of rats suggests depressive like symptoms among rats. On the whole, the rats in the HLS groups showed significant increased immobile time with a significantly decreased number of climbing as compared to their respective control groups, except the rats in the HLS-2w

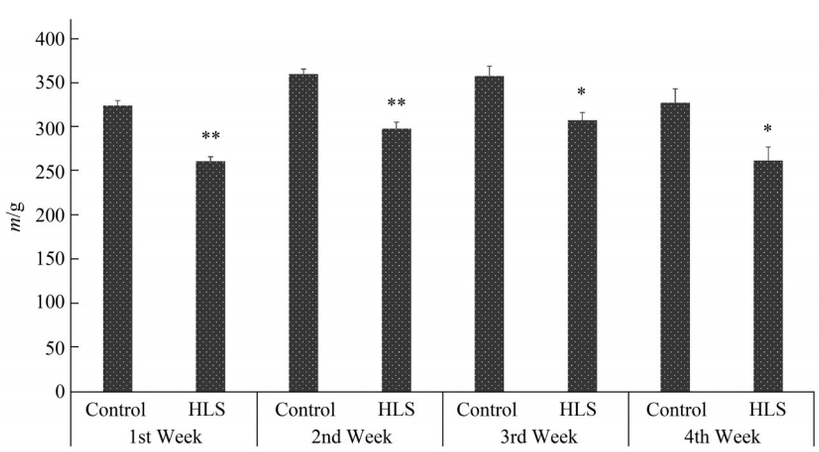

Fig. 1 Effects of HLS on rats' body mass

Each bar represents value as means±S. E. M. Statistical significance of the data was represented as *P<0.05, **P<0.01 compared with the corresponding control group.

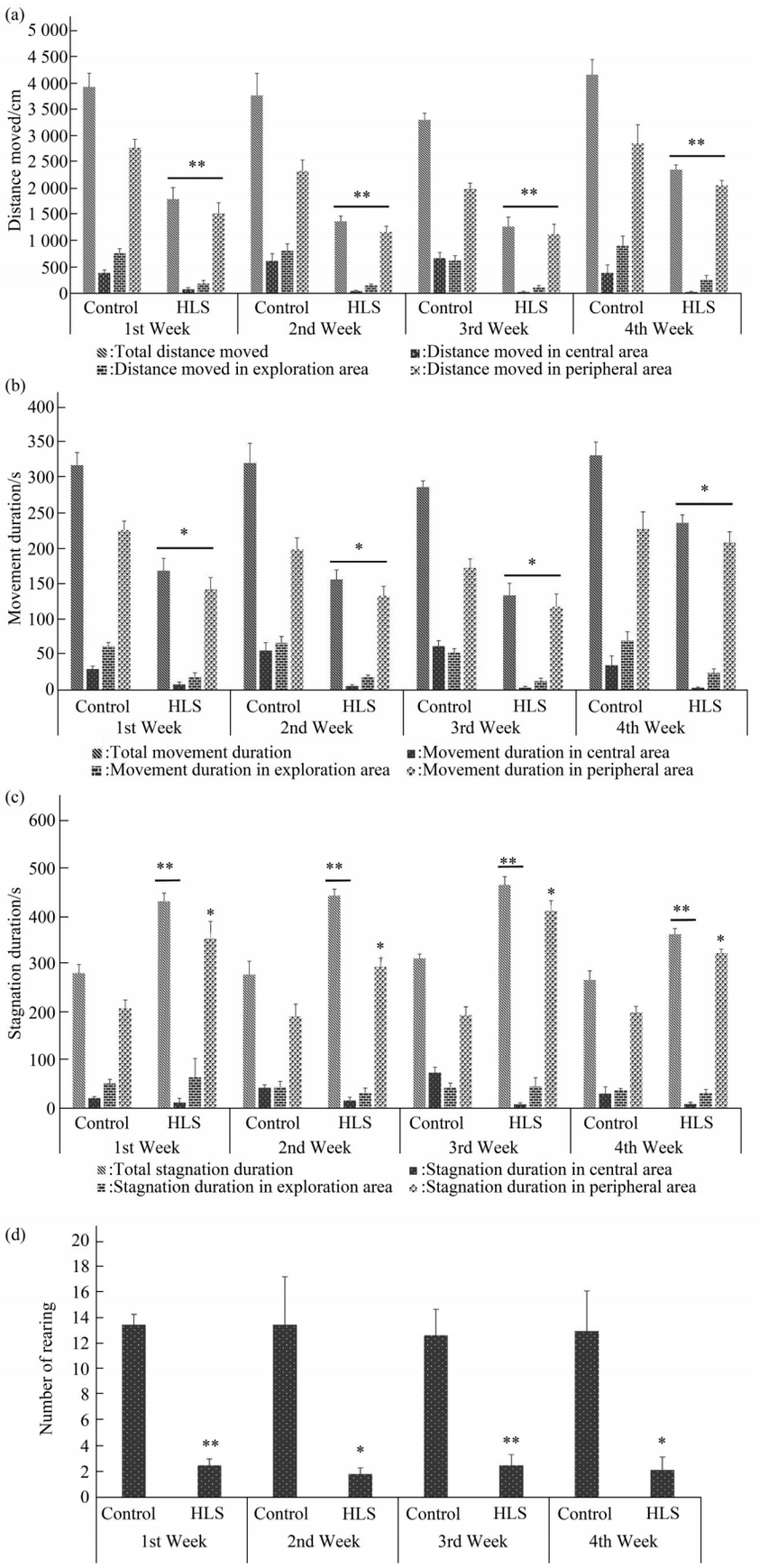

Fig. 2　Effect of HLS different weeks on the locomotor activity of rats in the respective in the open field test

(a) Movement distance, (b) movement duration, (c) stagnation duration, (d) number of rearing. Each bar represents values as mean±S.E.M. Statistical significance of the data are represented as **P*<0.05 and ***P*<0.01 compared to their respective control group.

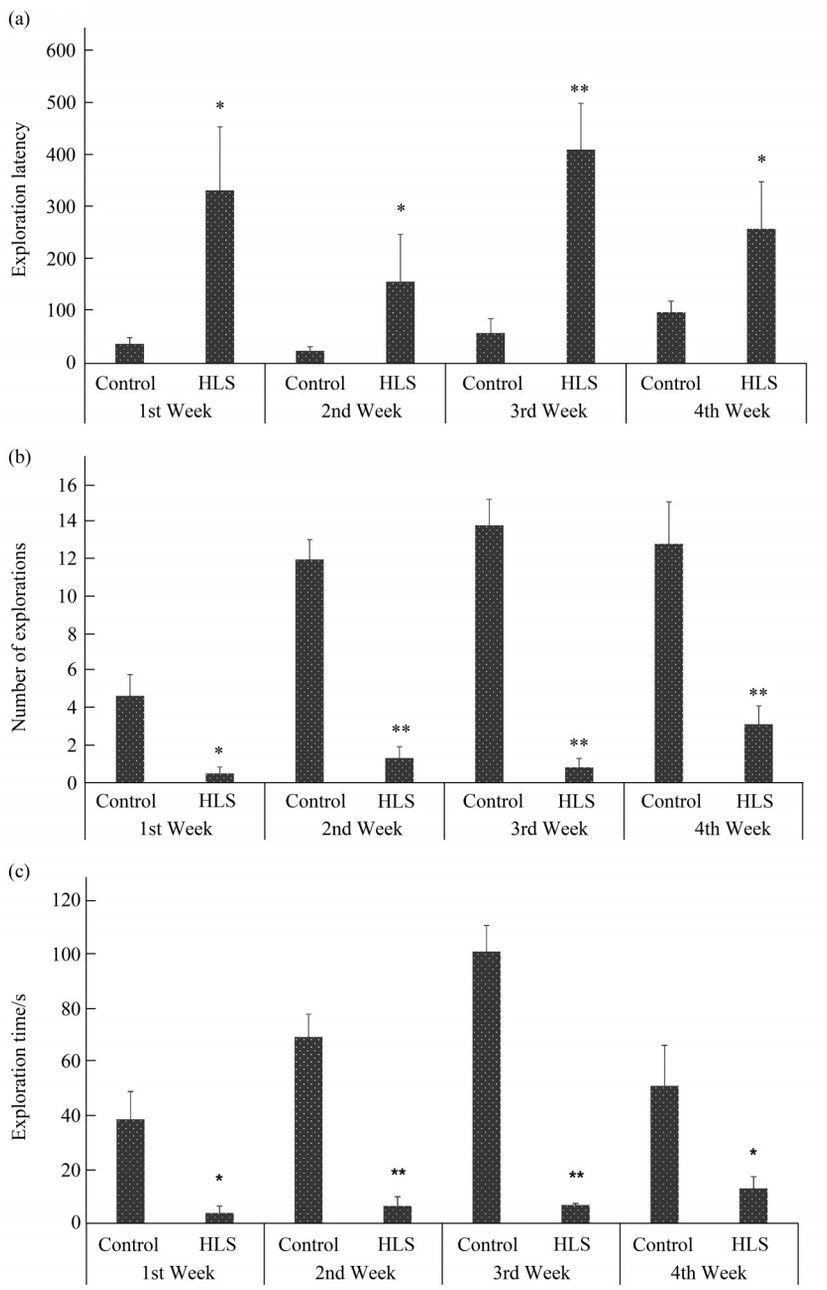

Fig. 3　Effect of HLS different weeks on the exploration in the novel object recognition test in rats

(a) Latency of exploration, (b) number of explorations, (c) time of exploration. Each bar represents values as mean±S.E.M. Statistical significance of the data are represented as **P*<0.05 and ***P*<0.01 compared to their respective control group.

group tried to struggle to escape and the immobile time decreased (*P*<0.05, Figure 4a, b).

2.5　Effect of HLS different weeks on the SOD and CAT activities in the hippocampi in rats

The oxidative stress and antioxidant system play a crucial role in depicting any disease or psychological condition. In this study, effect of HLS on antioxidant enzymes, *i. e.*, SOD and CAT were evaluated in the rat's brain hippocampus. The HLS treatment at each time point significantly downregulated the SOD and CAT activities in the rat's hippocampus (*P*<0.05, Figure 5a, b).

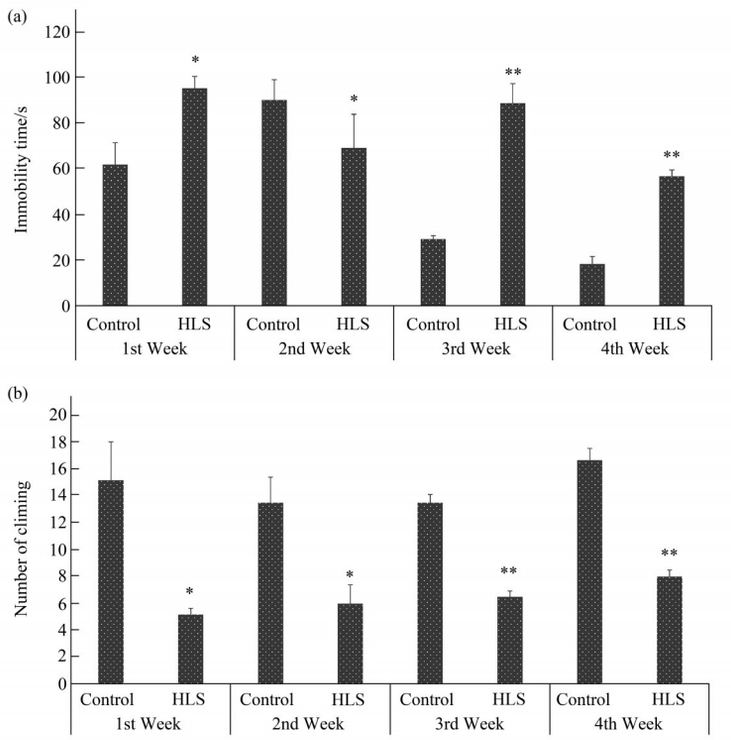

Fig. 4　Effect of HLS different weeks on the depressive like behavior in the forced swim test in rats

(a) Immobility time, (b) number of climbing. Each bar represents values as mean±S.E.M. Statistical significance of the data are represented as **P*<0.05 and ***P*<0.01 compared to their respective control group.

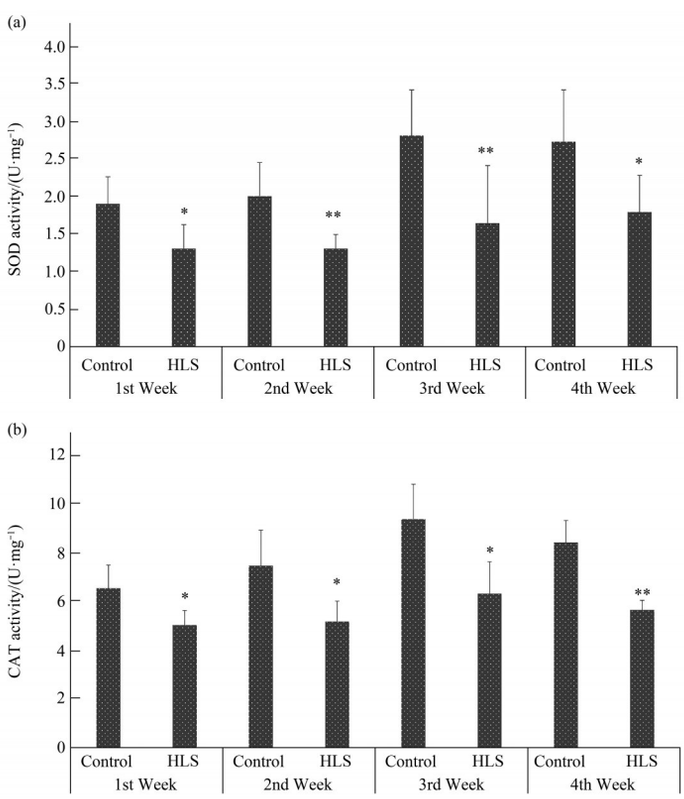

Fig. 5　Effects of HLS different weeks on the SOD and CAT activities in the hippocampi in rats

(a) SOD activity, (b) CAT activity. Each bar represents values as mean±S.E.M. Statistical significance of the data are represented as **P*<0.05 and ***P*<0.01 compared to their respective control group.

3 Discussion

The present study aimed to study the depressive like behaviors induced by simulated weightlessness in rats. To understand the effect of time duration of HLS towards depressive like behavior, rats were exposed to HLS for 1, 2, 3, and 4 weeks and matched with their respective control groups. It was reported that profound change occurred in the human body in response to microgravity[13]. Microgravity induces changes in the cardiovascular system, nervous system, skeletal and muscular system[6, 34-35], but it also impacts on psychology of the astronauts[13, 36]. The report suggested that long-term space missions significantly affected the psychology system negatively in astronauts during 438 d stay in space[13]. Among different models of microgravity, hindlimb unloading model was used in various studies, which suggested this model induces anxiety and depressive like behaviors in mice. Different from other models of depression which mostly induced by the supply of food and water (no water and fasting), change in living environment (moist bedding), change in temperature (high temperature stimulation, low temperature stimulation), swim (cold water swim, ice water swim, forced swim) and other factors, HLS is trying to simulate the real aerospace environment. In space, astronauts often encounter an “extreme environment” such as weightlessness, radiation, changed circadian rhythm which are completely different from earth. Exposed to such “extreme environment”, astronauts often suffer psychological and physical damage which may be aggravated with the extension of duration. So this study aimed to assess whether the simulated weightlessness could induce depression-like behaviors in rats. As mentioned in previous studies, depression was manifested in passive coping strategies, such as enhancing avoidance and immobility and reducing curiosity and exploratory ability. Our study found the simulated weightlessness could induce depressive like behaviors in rats, such as less locomotor activity, less exploration, and more immobility in rats, consistent with the previous reports[14, 37-38].

At an emotional level, depression can affect food intake and lead to body mass changes[39]. However, there is no consensus on the relationship between body mass and depression. The desire for high-fat foods in most people with depression is increased[40-41]. Some studies focused on correlates obesity and depression, while some other studies focused on the correlation between underweight and depression[42-43]. In addition to that, a study highlighted using the U-curved association that depression was more pronounced among underweight or overweight individuals than the subject having normal body mass[44]. This study showed that the body mass of the rats in the HLS groups decreased significantly than the control groups at each respective time point, suggesting that HLS-induced body loss can contribute to the depressive like behavior in rats.

The OFT is a signature behavioral test to explore the locomotory activities of rodents. In the OFT, a circular or rectangular arena with bright lighting bordering by the high walls to prevent escape was used. Normally, the arena was divided into two areas, *i.e.*, the central and peripheral areas. The movement in the central area reflects the conflict between fear and exploration in mice. However, the activity in the peripheral area reflects that rats seem to stay close to the wall get unnoticed, while the vertical movement reflects the anxiety-like behavior, especially the high-leaning behavior[23, 25]. In this study, an exploration area was introduced between the central and periphery areas, which was used to explore the hesitation behavior in rats. The locomotor activities in the HLS group rats at each time point were significantly reduced, as evidenced by reduced distance traveled and movement time with increased stagnant time. The rats in HLS groups showed the least tendency towards exploration and central area. The unfamiliar open space and bright lighting combinations will force animals to stay near the walls or freeze[24]. Even in the peripheral area, rats preferred to stay still, as evident by significantly increased stagnant time as well as the reduced central area’s stagnation time. It indicates that rats preferred stagnation over movement in the peripheral area compared to the central area. In the case of vertical movement, rats in the HLS group showed less rearing with less exploration rate. These results also hinted at the depressive like behavior in HLS treated rats for 1−4 weeks’ duration.

The NORT monitors the rodents’ spontaneous exploration behavior in the unique environments. When the rodents are faced with the novel object in a familiar open field, they produce conflicts between

curiosity and fear towards the novel object, which define their approach-avoidance contradictory behavior[45]. The rat's mouth and nose touching novel objects is a sign of curiosity or exploratory behavior reduced in depressed animals. The results showed that the HLS rats showed depressive behavior in NORT, as evidenced by reduced latency toward novel objects with reduced number and time to explore novelty. The depressive like behavior was present at all the time points (1−4 weeks) of HLS exposure. The results are consistent with the previous study[22].

The FST is a trademark experiment to observe depressive like behavior or screen antidepressant effects of drugs in depressive animals. In the FST, the rodents encountered an inescapable environment filling with water and were forced to swim for 15 min. After 24 h of exposure, the depressive nature of rodents was evaluated by noticing increase passive action (*e. g.*, immobility) and decrease positive activities (*e. g.*, climbing and swimming) [31]. The immobility among rats was considered behavioral despair (depression). On the contrary, climbing and swimming are regarded as active responses[46]. Despite the different arguments on the reliability of FST, such as hypothermia, the independent environment, the noise from other animals, it is still one of the most commonly used tests to evaluate depression[47-48]. In our results, The HLS rats showed significantly increased immobility time with a reduced number of climbing at each respective time point of HLS exposure. The results are consistent with studies that defined FST as a useful test, suggesting that simulated weightlessness may induce passive actions in rats and showed depressive like behaviors in rats[22].

Although previous studies have shown that tail suspension adversely affects hindlimb muscle volume and strength in rats[29], In theory, this injury should be assessed in conjunction with the characteristics of motor deficits in rats, as well as hindlimb exertion force testing, cage-wheel running, and *in vitro* muscle electrophysiology. However, our article is to detect the psychological tendency of rats, which is a curiosity-driven exploration of unknown areas and novelties, and a desire to survive rather than physical strength. Although the muscle mass and strength of the rats have not been tested for systematic evaluation, combined with our experimental findings, the rats did not have motor deficits when they explored after the tail suspension, that is, there was no hyperextension of the knee and ankle joints and forward movement of the limbs. At the same time, the movement speed acting as an important index to evaluate the damage of locomotor activity ability in rats had no difference between HLS rats and their controls in the OFT. Therefore, we believe that even if the tail suspension might play some part in the activities of rodents, this influence is not crucial for us to judge its psychological tropism.

Depression is closely linked to increased oxidative stress with an impaired antioxidant system in the brain[49]. Impaired oxidative burst was also evident in people during space flight[50]. Different studies also highlighted the presence of oxidative stress with the reduced antioxidant system, *i. e.*, CAT and SOD and other in the HLS rat model, which is responsible for muscle atrophy, bone damage, brain functions[14, 51-52]. In this study, the antioxidant enzymes were downregulated in the HLS rats at each time point which indicates the exposure to microgravity will result in oxidative damage with negative impact on the body.

4 Conclusion

In conclusion, our findings demonstrate that HLS 1−4 weeks could induce depressive like behaviors in rats, cause body mass loss, reduce less exploration and struggle in the NORT and FST, respectively. Most interesting, the movement is much less in the central area than the other two areas, with less rearing and normal movement speed in the OFT in the HLS rats, which suggest a lack of interest to explore new area (depression) and no impairment on movement ability in the HLS rats. Synchronously, the significantly downregulate of the antioxidant enzymes in the HLS rats suggests oxidative stress's role in depressive like behavior. These results may give the reader better knowledge of depressive like behavior induced by simulated weightlessness and help to provide method for screening effective countermeasures preventing risk during space flight.

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模拟失重不同时长对大鼠抑郁样行为的影响*

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摘要　目的　本研究旨在观察不同持续时间的模拟失重对大鼠抑郁样行为和海马超氧化物歧化酶（SOD）和过氧化氢酶（CAT）的影响，以探究其影响及可能的作用机制。**方法**　采用后肢悬挂（HLS）尾吊法模拟大鼠失重状态。将大鼠分为对照组和不同模拟失重时间尾吊组（尾吊时长分别为1、2、3、4周）。采用旷场实验（OFT）、新物体识别实验（NORT）、强迫游泳实验（FST）观察大鼠抑郁样行为，采用酶联免疫吸附试验（ELISA）法测定海马SOD和CAT活性。**结果**　OFT结果显示，与对照组相比，HLS不同时间大鼠的僵滞时间增加（$P<0.05$，$P<0.01$）。在NORT中，与对照组相比，HLS不同时间大鼠对新物体的探索潜伏期增加，探索次数和时间减少（$P<0.05$，$P<0.01$）。在FST中，与对照组相比，HLS不同时间大鼠在FST中的不动时间增加，攀爬次数减少（$P<0.05$，$P<0.01$）。与对照组相比，HLS不同时间组大鼠海马组织中SOD和CAT水平均下降（$P<0.05$，$P<0.01$）。**结论**　短时间或长时间的失重都会导致大鼠产生类抑郁样行为。

关键词　模拟失重，抑郁样行为，旷场实验，强迫游泳实验，超氧化物歧化酶，过氧化氢酶
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