

Original Article

Transplantation of bone marrow mesenchymal stem cells reduces GluR1 expression in the hippocampus and amygdala and affects secretion of stress-related hormones in rats with spinal cord injuries: a potential anti-chronic stress mechanism

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Abstract: There have been many studies about bone marrow mesenchymal stem cell (BMSCs) transplantation therapy for spinal cord injuries (SCI), achieving positive results both in laboratory and clinical applications. However, its exact repair mechanisms are still under investigation. The authors of this present study hypothesized that chronic stress plays an important role in SCI and investigated a potential anti-chronic stress mechanism of transplantation of BMSCs into rat SCI. A total of 48 adult male SD rats were randomly and equally allocated into three groups (control, model, and treatment). Lower thoracic partial SCI was induced in both the model and treatment group, while the control group received only laminectomy. The control and treatment group were injected with 100 μ l of Hank's buffered saline solution containing 1 million rat BMSCs into the L4-5 intervertebral space. The model group received the same amount of Hank's buffered saline solution via injection. Hind limb motor function was evaluated by Basso, Beattie, and Bresnahan (BBB) scale while enzyme-linked immunosorbent assay (ELISA) detection was performed for these stress-related hormones: plasma adrenocorticotrophic hormone (ACTH), norepinephrine (NE), epinephrine (E), and serum corticosterone (CORT). Immunohistochemistry staining was performed for Glutamate (Glu) receptor 1 (GluR1) in the hippocampus and amygdala of rat brains. Following BMSCs transplantation, motor function of hind limbs of rats in the treatment group achieved sustained restoration. Additionally, 14 days and 28 days after transplantation, levels of plasma ACTH, NE, E, and serum CORT in the treatment group were significantly decreased compared with that in the model group ($P < 0.05$). Positive rates of GluR1 in the hippocampus and amygdala of the treatment group were also dramatically reduced compared with that in the model group ($P < 0.05$). Results showed that BMSCs transplantation is beneficial for motor function recovery of hind limbs in rats with SCI. Furthermore, it may exert a preventive role on chronic stress by downregulating GluR1 expression in the central nervous alpha-amino hydroxymethyl-oxazole propionic acid (AMPA) channel and inhibiting secretion of stress-related hormones, implying a potential anti-chronic mechanism.

Keywords: Bone marrow mesenchymal stem cells, spinal cord injury, stress, stress-related hormones, GluR1

Introduction

Spinal cord injuries (SCI) are characterized by severe trauma to the central nervous system. Manifestations include long-term sensory disturbances below the injured level of the spine, dyskinesia, and sphincter dysfunction. Methods for treating SCI include drug therapies, operative treatments, and physical adjuvant therapies. Outcomes, however, have not been ideal [1]. Bone marrow mesenchymal stem cells (BMSCs) are capable of transdifferentiating to neu-

rons, *in vitro* [2], providing a promising cellular transplantation therapy for SCI.

There have been numerous studies concerning BMSC transplantation therapy for SCI, reporting positive results in both laboratory and clinical applications [3-11]. However, the exact mechanisms have not been fully determined. Many studies have suggested that when transplanting BMSCs at the local site affected by SCI, the damaged spinal cord is directly repaired [12-14]. However, this ignores the fact that BMSCs

potentially play a regulatory role for the whole body. Patients with SCI have been shown to have a relatively high stress state, due to stimulation such as long-term pain [15]. The limbic system, especially the hippocampus and amygdala, is not only the regulatory center of the body's stress reaction but also an area sensitive to stress injuries. A relatively high stress state may affect the limbic system through negative feedback regulation, causing down regulation of relevant stress hormone receptors.

The present study was designed and conducted to verify whether injecting BMSCs into rats with SCI improves their stress state and affects expression of excitatory amino acid receptors in the hippocampus and amygdala. This study aimed to explore mechanisms by which BMSCs provide treatment effects on SCI, hoping to provide new insight into prevention and treatment of SCI.

Materials and methods

Animals

This study used 6-week-old male Sprague Dawley (SD) rats (weight, 98-102 g; n=6) and adult male SD rats (weight, 260-300 g; n=48). They were acquired from the Experimental Animal Center of Soochow University (Suzhou, China; License No. SYXK Su 2002-0037). The animal room was maintained at $25\pm 2^{\circ}\text{C}$, with a relative humidity of $50\pm 5\%$. Rats were fed under conditions of alternative darkness and light (light: 6:00-18:00; darkness: 18:00-6:00). The rats were given food and water freely. They acclimatized to the laboratory environment for 7 days prior to the study, thus, forming circadian rhythms in a specified state. Ethical approval was obtained from the Ethics Committee of Soochow University. Protocol was in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985).

Rat BMSCs isolation and culture

Bone marrow was collected from the femora and middle femoral medullary cavities of the tibia of 6-week-old male SD rats for whole bone marrow culturing. Specific methods used were previously described by Jendelová et al. [16] and other previous work [17]. Tibias and femurs were dissected free and proximal and distal ends were removed to expose the marrow cav-

ity to be aspirated with 10 mL of BMSCs growth medium: (LG-DMEM+10% FBS) (Gibco, Grand Island, MA, USA) with a 21 G needle. The aspirate was twice centrifuged at 1,000 rpm for 5 minutes. Cell pellet containing hematopoietic cells and BMSCs was then suspended in BMSCs culture medium. The suspension obtained was cultured in 25 cm² plastic flasks and incubated at 37°C with 5% humidified carbon dioxide (CO₂) for 24 hours. Flasks were cleansed with phosphate buffered saline (PBS) to leave an adherent layer of cells containing rBMSCs. The interval of replacing fresh culture medium was 3 to 4 days. When these cultures were close to confluence, cells were then harvested, digested with 0.25% trypsin/1 mM EDTA (Gibco, Grand Island, MA, USA), and collected for future experiments under sterile conditions.

Creation of SCI model

Initially, 48 adult SD rats were allocated into three groups (each n=16): control, model, and treatment group. Following intraperitoneal anesthesia with pentobarbital (50 mg/kg) in the prone position, surgical sites centered on the T10 spinous process were disinfected and draped in a sterile manner. Rats in model and treatment groups were given a partial low thoracic spinal cord injury (SCI) by modified Allen's method [18] (weight drop method) at T10. A 10 g impact rod was centered above T10 and dropped through a hollow transparent tube from a height of 5.0 cm to induce a consistent partial SCI. Immediate tail wagging or tremors of both lower extremities of the rats confirmed that the model was successfully established. Rats in the control group received only laminectomies, without spinal cord drop. Each incision was then sutured layer by layer in order. Following recovery of the rats, motor function of bilateral hind limbs were evaluated according to the Basso, Beattie, and Bresnahan (BBB) scale [19]. Intramuscular injections of cefoperazone 100 mg/kg were given to prevent urinary tract infections once per day for 3 days. Urinary bladders were emptied, manually, 3 times per day until reflex bladder emptying was established.

BMSCs transplantation

At 7 days after the rat model of SCI was established, rats in each group were anesthetized (pentobarbital, 50 mg/kg) again. The dura at



Figure 1. BMSCs transplantation using a microinjector via a tip-bent 31 G needle.

L4-L5 intervertebral space was exposed with partial removal of the L5 spinous process and L4-L5 ligamentum flavum through a posterior approach. Rats in the control and treatment groups received a slow subdural injection of a cell suspension in Hank's buffered saline solution (Gibco, Grand Island, MA, USA) containing 1.0×10^6 BMSCs with a total volume of 100 μ l using a microinjector (Hamilton, Switzerland) via a tip-bent 31G needle (Hamilton) (**Figure 1**). Rats in the model group received a subdural injection of 100 μ l Hank's buffered saline solution only. Wounds were then closed layer by layer in order. At 1, 3, 7, and 14 days following BMSCs transplantation, hind limb motor function of all rats was evaluated in accordance with the BBB scale. Postoperative management was the same as previously described.

Enzyme-linked immunosorbent assay (ELISA) detection

Serum and plasma hormone levels of rats were detected by ELISA. Briefly, 14 and 28 days after BMSC transplantation, 8 rats were taken from each group and numbered in order. After each rat was anesthetized with pentobarbital (50 mg/kg), the left ventricle was exposed for collection of 10 mL fresh blood in blood-collection tubes A and B, respectively (5 mL in each tube). Blood-collection tube A was centrifuged at 2000 r/min for 15 minutes. Supernatant (serum) was obtained and placed in another tube. The obtained sample was saved at 4°C. Heparin sodium (1:100, Sigma-Aldrich, St. Louis, MO, USA) was added into tube B as an

anticoagulant. Tube B was then centrifuged at 2000 r/min for 15 minutes at 4°C. Supernatant (plasma) was obtained and placed in another tube and stored in refrigerator at 4°C. Levels of stress-related hormones (ACTH, CORT, NE, and E) in cell-free supernatants of the co-culture system were detected using ELISA detection Kits (Cusabio, Wuhan, China), according to manufacturer instructions. The plates were read at 450 nm and obtained data were processed for calculation of concentrations.

Immunohistochemical staining

Following cardiac perfusion with formaldehyde solution, brains of the rats were removed and placed into molds. With reference to the 3D brain anatomy atlas of rats [20], 0.5-cm brain tissues centered on the cerebral hippocampal area were taken for preparation of conventional paraffin-embedded samples. For each group, eight coronary sections from the same site were removed prior to examination using GluR1. Paraffin-embedded tissue samples were cut into 5 μ m sections. All sections were dewaxed in xylene and rehydrated in alcohol gradient. After 15 minutes of antigen retrieval, these sections were immersed in 0.3% hydrogen peroxide solution to block endogenous peroxidase activity. After sections were washed with PBS three times, they were incubated at 4°C overnight with primary antibody against GluR1 (1:100). These sections were then incubated at 37°C for 20 minutes with secondary antibody (1:250, Abcam, Cambridge, MA, USA). Diaminobenzidine reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was employed to visualize the stain, according to manufacturer instructions.

Image processing

All images were analyzed using image analysis software Image-Pro Plus 5.0 (Media Cybernetics, Inc., Rockville, MD, USA). Under the same conditions, 200 μ m \times 100 μ m sections of hippocampal area CA1 along the pyramidal layer and 200 μ m \times 200 μ m sections of hippocampal area CA3, the dentate gyrus (DG), and basal lateral amygdala (BLA) were selected for counting of stained cells. The mean of measured numbers for the right side and left side was taken as the number of positive cells in the image.

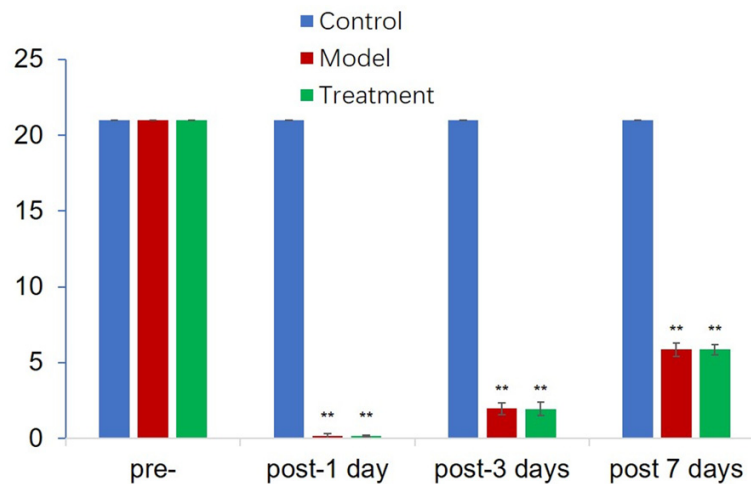


Figure 2. BBB scale score pre- and post-spinal cord injury. **P<0.01, compared with control group.

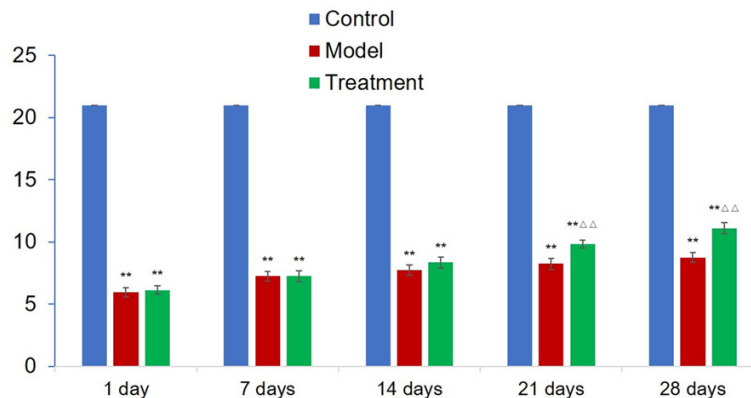


Figure 3. BBB scale scores after BMSCs transplantation. **P<0.01, compared with control group; ΔΔP<0.01, compared with model group.

Statistical analysis

SPSS 18.0 software package was used to process all data (SPSS, Chicago, IL, USA) through repeated measures analysis and one-way analysis of variance. Comparisons between different groups were made by the least significant difference method. Data are presented as mean \pm standard deviation. P<0.05 was considered to indicate statistical significance.

Results

Hind Limb motor function of animals

Movement of the bilateral hind limbs of rats was not observed in the three groups immediately after establishing the model. Therefore,

BBB scores were 0. Following recovery from anesthesia, rats in the control group fully recovered hind limb movement, gradually, and their BBB score was 21. Rats in the model and treatment groups recovered their bilateral hind limb motor function slowly, by day 1 after SCI surgery, and their auto-urination function returned to normal 3 days after SCI surgery. BBB scores of bilateral lower limbs in the model group and treatment group were obviously lower than those in the control group at days 1, 3, and 7 (P<0.01), but no statistically significant differences were detected between these two groups (P>0.05) (Figure 2). All animal urinary function recovered within 3 days after surgery.

After receiving an intrathecal injection of 100 μ l Hank's buffer cell suspension containing 1.0×10^6 BMSCs, there was no change in hind limb motor function for rats in the control group and BBB scores remained unchanged compared to before anesthesia. BBB scores of bilateral lower limbs in the model group and

treatment group were lower than those in the control group at all time points (P<0.01). BBB scores of bilateral hind limbs in the model group were slightly increased over time and BBB scores in the treatment group were significantly higher than those in the model group after BMSCs transplantation at 21 and 28 days (P<0.01) (Figure 3).

ELISA detection

At 14 days after BMSCs transplantation, plasma ACTH levels in the model and treatment groups were remarkably higher than those in the control group (P<0.05). Plasma NE levels in the model and treatment groups were much higher than the control group (P<0.05), while plasma NE levels in the treatment group were

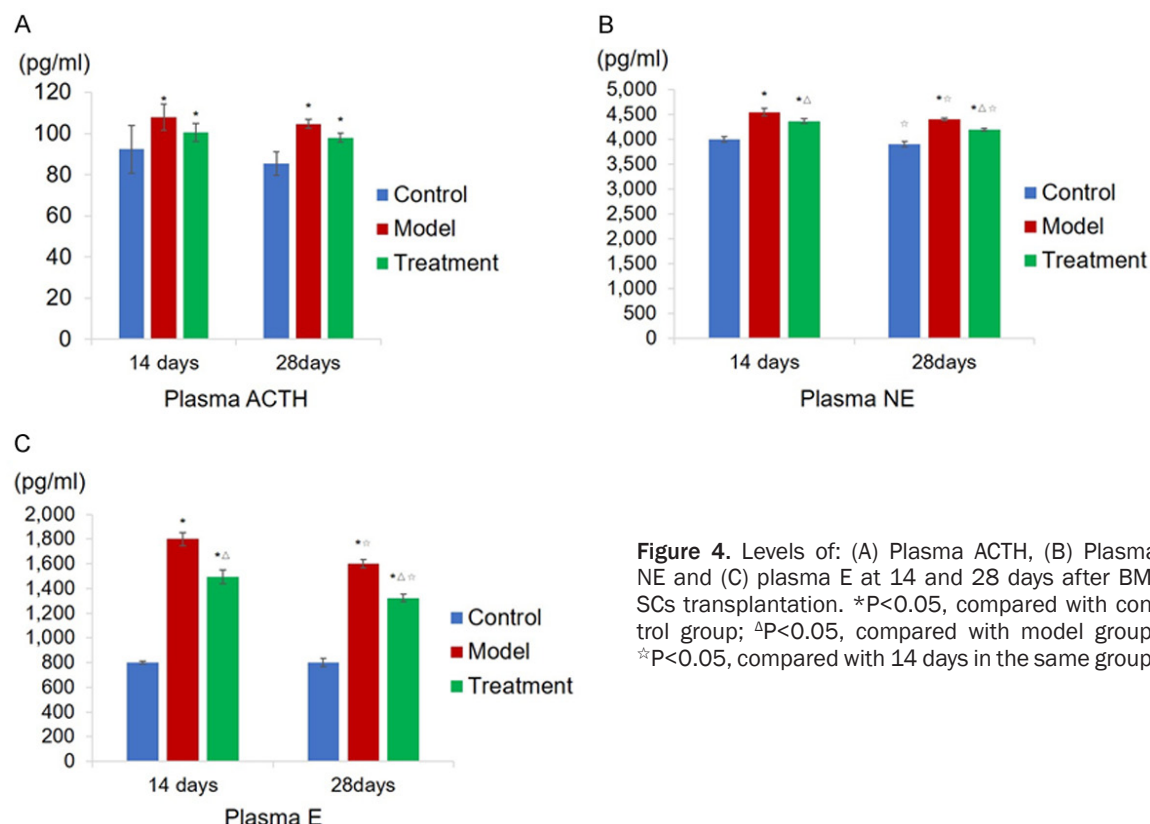


Figure 4. Levels of: (A) Plasma ACTH, (B) Plasma NE and (C) plasma E at 14 and 28 days after BMSCs transplantation. * $P < 0.05$, compared with control group; $^{\Delta}P < 0.05$, compared with model group; $^{\star}P < 0.05$, compared with 14 days in the same group.

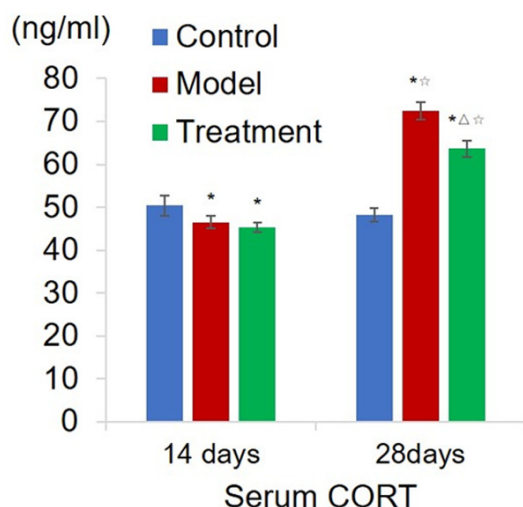


Figure 5. Levels of serum CORT at 14 and 28 days after BMSCs transplantation. * $P < 0.05$, compared with control group; $^{\Delta}P < 0.05$, compared with model group; $^{\star}P < 0.05$, compared with 14 days in the same group.

significantly lower than the model group ($P < 0.05$). Plasma E levels in the model and treatment groups were obviously higher than the control group ($P < 0.05$), while plasma E levels in the treatment group were greater than the

model group ($P < 0.05$). At 28 days after BMSCs transplantation, plasma ACTH levels in the model and treatment groups were lower than those at 14 days, although the differences showed no statistical significance ($P > 0.05$). Plasma NE levels in the control, model, and treatment groups at 28 days were significantly decreased compared with those at 14 days ($P < 0.05$). Plasma NE levels in the treatment group on day 28 were lower than that in the model group at the exact same time ($P < 0.05$). Plasma E levels in the model and treatment groups at 28 days were significantly decreased compared to those at 14 days ($P < 0.05$), while plasma E levels in the treatment group on day 28 were lower than the model group at the same time point ($P < 0.05$) (Figure 4).

At 14 days after BMSCs transplantation, serum CORT levels in the model and treatment groups were significantly lower than the control group ($P < 0.05$). At 28 days after BMSCs transplantation, serum CORT levels in the model and treatment groups were obviously increased compared with those at 14 days ($P < 0.05$), but increase in the treatment group was smaller than that in the model group ($P < 0.05$) (Figure 5).

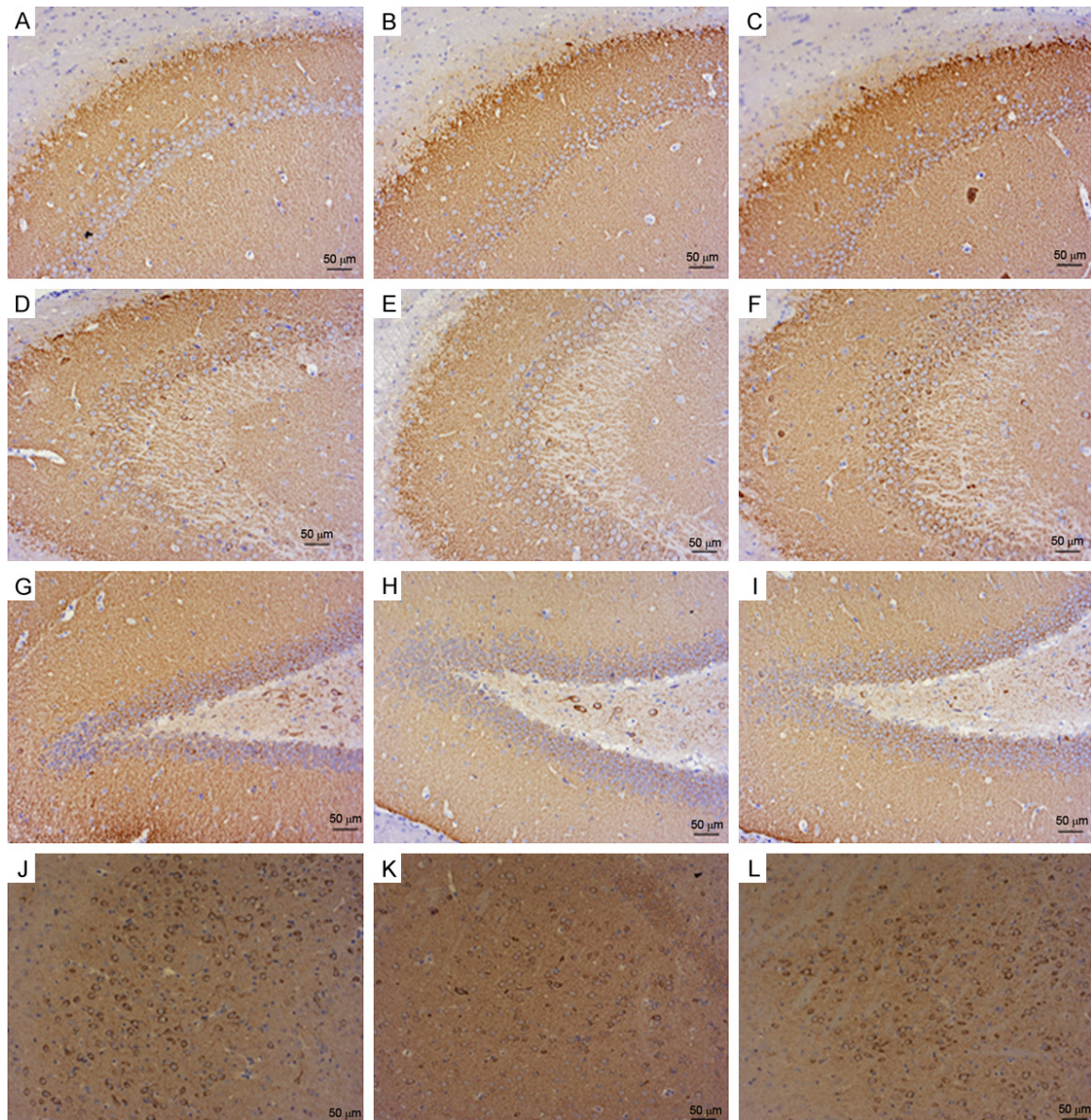


Figure 6. Immunohistochemical staining results of GluR1 in different hippocampal areas. Hippocampal CA1 in the (A) control group, (B) model group, and (C) therapeutic group. Hippocampal CA3 in the (D) control group, (E) model group, and (F) therapeutic group. Dentate gyrus in the (G) control group, (H) model group, and (I) therapeutic group. Basal lateral amygdala in the (J) control group, (K) model group, and (L) therapeutic group. Bar = 50 μ m.

Immunohistochemical staining

Degree of staining in the hippocampus was observed to be higher than other cerebral areas under a microscope. Cell staining for GluR1 was visible in the hippocampal CA1 and CA3 regions, DG, and BLA. Cells in CA1 appeared rounded and cell membranes and processes were stained yellow brown. In the CA3 region, somas were large and oval- or round-shaped with a high degree of staining and yellow-brown positive expression was visible in cell mem-

branes as well as in the cytoplasm and processes near them. The coloring of BLA was slightly darker than that of the rat cerebral cortex and cell morphologies in this area were similar to those in CA1. Cells in the DG were small and dense, while staining was mainly observed in cell membranes (**Figure 6**).

In the brain sections with GluR1 immunohistochemical staining, the positive cell number in the CA1 area at 14 days after BMSC transplantation in the treatment group was lower than

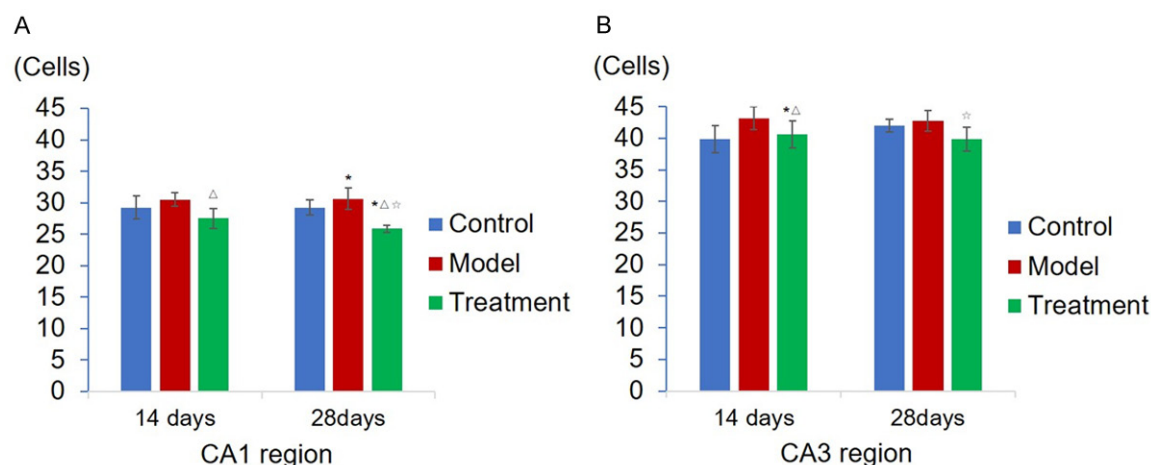


Figure 7. Immunohistochemical staining of GluR1 in Hippocampal: (A) CA1 region and (B) CA3 region at 14 and 28 days after BMSCs transplantation. * $P < 0.05$, compared with control group; [△] $P < 0.05$, compared with model group; [☆] $P < 0.05$, compared with 14 days in the same group.

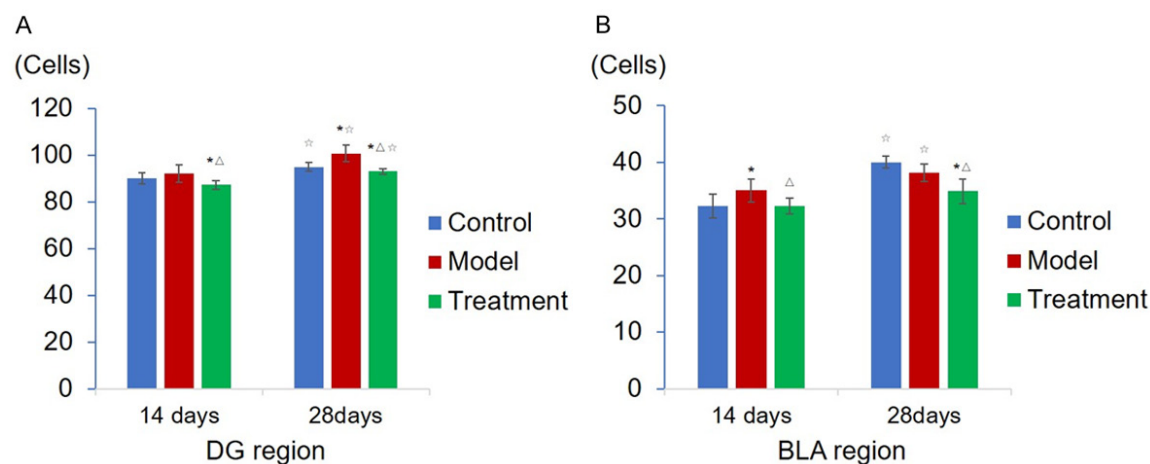


Figure 8. Immunohistochemical staining of GluR1 in: (A) Hippocampal DG region and (B) BLA region at 14 and 28 days after BMSCs transplantation. * $P < 0.05$, compared with control group; [△] $P < 0.05$, compared with model group; [☆] $P < 0.05$, compared with 14 days in the same group.

that in the model group ($P < 0.05$), while the number of positive cells at 28 days after BMSC transplantation in the treatment group was decreased compared to 14 days ($P < 0.05$). The positive cell number in the CA3 area at 14 days after BMSC transplantation in the treatment group was higher than that in the control group, but lower than that in the model group (both $P < 0.05$). The positive cell numbers in the CA3 region at 28 days after BMSC transplantation in the model and treatment groups were decreased compared with those at 14 days in the same groups, although only the difference in positive cell numbers in the treatment group between these two time points was statistically significant ($P < 0.05$) (Figure 7).

Positive cell number in the DG at 14 days after transplantation in the treatment group was decreased compared with that in control and model groups ($P < 0.05$), while those at 28 days after transplantation in the three groups were significantly different from those at 14 days ($P < 0.05$). Furthermore, the GluR1 positive cell number in the treatment group was lower than that in the model group on day 28 ($P < 0.05$). The positive cell number in the BLA at 14 days after transplantation in the model group was slightly higher than the control and treatment groups ($P < 0.05$), while those at 28 days after transplantation were higher than those at 14 days after transplantation for each group, with increases in control and model groups statisti-

cally significant (all $P < 0.05$). Positive cell number in the treatment group was much lower than the model group on day 28 ($P < 0.05$) (Figure 8).

Discussion

Stress is a series of non-specific responses, including sympathetic nervous excitement, an increase in HPA axis secretion, and various functional and metabolic changes, that occur when the body is stimulated by various undesirable factors [21]. Appropriate stress can help the body improve environmental adaptability, while excessive, particularly chronic, stress may lead to physiological or psychological disorders to varying degrees. Stress may even cause physical disease. Most patients with severe SCI will have sensations with movement and/or anal sphincter dysfunction, along with long-term chronic limb pain and progressively severe bedsores, pulmonary infections, and urinary infections. Prolonged existence of these stimulatory factors can cause the body to remain at a relatively high stress level [22]. Studies have demonstrated that when the body is at a high stress level, oxidative stress can trigger sequelae to SCI [23, 24]. Therefore, relieving the stress state after SCI can help reduce sequential injuries of the spinal cord and relieve psychological imbalances in the body, serving an indispensable role in the treatment of SCI.

When the body is under stress, a series of physiological changes occur, including changes in autonomic nervous activity and increased neuroendocrine response [25]. Changes in autonomic nervous activity mainly manifest as increased sympathetic nervous activity, notably that of the sympathetic-adrenal medulla axis. NE and E secretion is also increased [26]. An increase in neuroendocrine activity mainly comprises activation of the HPA axis and a subsequent increase in GC secretion [27]. HPA axis activation and increased GC secretion are important characteristics of systemic stress. Main components of the HPA axis are the hypothalamus, pituitary gland, and adrenal cortex which form a feedback system regulating each other. When the body is stimulated by internal and external stressors, the higher central nervous system transmits this information to the paraventricular nucleus (PVN) of the hypothalamus and PVN cells then synthesize and release polypeptide hormones such as corticotrophin-releasing hormone (CRH) to act on the anterior

pituitary to release ACTH [27]. Along with blood circulation, ACTH stimulates the adrenal cortex to secrete GCs, mainly CORT in rodents, while a continuous increase in GC secretion may have a negative feedback effect on the pituitary gland and hypothalamus. When the body is under chronic stress, HPA axis imbalance occurs, characterized by ACTH and CORT disorders [28]. Therefore, many researchers have regarded them as indices for evaluating the state of chronic stress [29, 30].

The hippocampus, located inside the temporal lobe, is an important structure of the brain. It plays important roles in learning, memory, regulation of autonomic nervous activity, and neuroendocrine function. In addition, the hippocampus is an important zone for regulation of stress. Studies have shown that the limbic system above the hypothalamus, particularly the hippocampus and amygdala, may be involved in negative feedback regulation mechanism of the HPA axis. Therefore, it is quite important in chronic stress regulation [31, 32]. The hippocampus is not only a regulator of the HPA axis but also to stress-induced injuries [33]. The amygdala is the zone where the body executes stress behavior and neuroendocrine response. If the amygdala is injured, the body may be unable to react to stress. Specific mechanisms by which chronic stress results in hippocampus injury remains unknown. However, hyperglucagonemia has been proposed as a potential mechanism [34, 35]. A study by Filipović et al. [36] reported that when the HPA axis was excited, the hippocampus was able to inhibit this excitement and reduce secretion of hormones such as GCs. In contrast, excessive elevation of hormones, such as GCs, could damage the dorsal structure of the hippocampus, reducing inhibition of the HPA axis, leading to an increase in GCs in the plasma. However, certain scholars have hypothesized that excitatory amino acids and abnormal expression of their receptors might be an important cause of hippocampal injuries. Magarinos et al. [37] found that most of the neurons in the hippocampus were glutamatergic neurons and almost all neurons in the CA3 region were glutamatergic neurons. It has been found that when a body is under stress, the Glu content of the brain is clearly higher than normal levels. A large amount of Glu acting on Glu receptors may generate a Glu excitotoxic [38] effect that has damaging effects on the structure and function of the hippocampus.

Glu, at physiological concentrations, maintains the basic physiological activities of the central nervous system. If concentrations and exposure times of Glu exceed the physiological scope, overactivation of the Glu receptor can cause injury to neurons, the so-called excitotoxic effect of Glu [15]. Glu receptors can be divided into metabolic and ionic Glu receptors. In the latter type, the alpha-amino hydroxymethyl-oxazole propionic acid (AMPA) receptor is important for mediation of fast excitatory neurotransmission. Emphasis is placed on it during the Glu excitotoxic effect. The AMPA receptor is a heterologous-tetramer ion channel consisting of Glu receptor protein 1-4 (GluR1-4) [39]. Studies have indicated that riluzole, an AMPA receptor antagonist, may have treatment effects on patients with SCI [40, 41].

Theoretically, there could be a synergic mechanism among SCI recovery, physiological stress state, and AMPA receptor regulation. When the body is in a state of stress, an increase in autonomic nervous and neuroendocrine activity may occur. An increase in autonomic nervous activity is characterized by excitement of the adrenal medulla system along with increased NE and E secretion. In addition, an increase in neuroendocrine activity is mainly characterized by HPA axis hyperfunction along with increases in ACTH and CORT hormone secretion. These two hormones are major indices reflecting a chronic stress state. During chronic stress, HPA function becomes disordered, the hippocampus and amygdala become vulnerable when inhibiting HPA axis excitement, and the content of excitatory amino acid Glu in the brain increases, becoming involved in the Glu excitotoxic injury mechanism in the hippocampus and amygdala.

In the present study, at 14 days after BMSC transplantation, numbers of GluR1 positive cells in the model group were increased compared to those in the control group in each hippocampal region. In the model group and treatment group, plasma ACTH content was higher than that of the normal control group while serum CORT content was lower, suggesting that the rats were in a stress reaction state. Plasma NE and E levels of the model and treatment groups were significantly elevated compared with those of the control group, indicating that sympathetic nerves of the body were in a highly excitatory state. GluR positive expression in rat brains indicated that these rats were in a state of undue stress following SCI, result-

ing in an increase in Glu secretion and number of AMPA receptors. Therefore, strong stress occurred in the rats, stimulating sympathetic nervous system excitement. These results along with secretion levels of NE and E in the model and treatment groups mutually support each other.

Comparing data 28 days after BMSCs transplantation with those 14 days after BMSC transplantation, in the same group, significant differences were found in 13 sets of data. Number of GluR1-positive cells in the CA1 region decreased slightly in the treatment group and the other 12 numerical values increased to varying degrees. Although plasma ACTH levels of rats in the model and treatment groups declined over time, they remained significantly higher than that in the normal control group. Although plasma NE and E content in the model and treatment groups also decreased over time, they continued to be obviously higher than the control group. This indicated that sympathetic nerves in the bodies were in a highly excitatory state. It appears that the stress state continues to exist following SCI. However, serum CORT content in the treatment group at 28 days was significantly lower compared with that in the model group, indicating that the stress state was already beginning to be relieved after BMSC transplantation.

At 14 and 28 days after BMSC transplantation, numbers of GluR1 positive cells in each hippocampal region (except CA3) in the treatment group were decreased compared those in the control group and model group. Most of these differences were statistically significant. This suggests that, after BMSC transplantation, BMSCs were involved in regulating expression of GluR1 receptors.

In conclusion, following transplantation, BMSCs can promote motor function recovery of hind limbs in SCI rats. Furthermore, it may exert a preventive role of chronic stress by downregulating GluR1 expression in the central nervous AMPA channel and inhibiting secretion of stress-related hormones. These results indicate a potential anti-chronic mechanism.

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Disclosure of conflict of interest

None.

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