SCIENTIFIC ARTICLE

Marrow Stromal Stem Cell Autologous Transplantation in Denervated Fracture Healing: an Experimental Study in Rats

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Objective: To investigate the influence of bone marrow stromal stem cell (BMSCs) transplantation on healing of fractures combined with central nerve injuries in rats.

Methods: Forty-eight healthy adult SD male rats were randomly divided into the following three groups (16 rats in each group): group A, simple (left) tibial fracture; group B, tibial fracture combined with T₁₀ spinal cord transection (SCT); group C, tibial fracture combined with T₁₀ SCT and BMSCs transplantation. The tibial fractures were stabilized with modular intramedullary nails and all operated hind limbs were further immobilized in plaster casts to prevent unequal load bearing. BMSCs were labeled with bromodeoxyuridine and implanted into the fractures of C group rats 2 days after creation of the model. The animals in B and C groups were evaluated by postoperative Tarlov scores. The fractured tibiae were evaluated separately radiographically (X-ray and CT) and immunohistochemically 1, 2, 3 and 4 weeks after injury to assess fracture healing. In addition, the wet weights of the left tibiae were measured.

Results: All Tarlov score of the B and C group animals reached the requirements of the experiment. One, 2 and 3 weeks after surgery, the tibial callus widths in B and C group animals were significantly greater than those of group A rats ($P < 0.05$). At 4 weeks the tibial callus width in group C animals had decreased, but still differed significantly from that in group A rats ($P < 0.05$). One, 2, 3 and 4 weeks after surgery, the wet weights of B and C group tibiae were significantly greater than those of group A ($P < 0.05$). Hematoxylin–eosin-stained sections showed bony union and increased bone trabecula in B and C groups and areas with particles positive for alkaline phosphatase staining were more abundant in groups B and C, especially in group C.

Conclusion: Neural regulation plays an important role in fracture healing. Treatment with BMSCs has a positive effect on defective callus in rats that have been subjected to SCT.

Key words: Bone marrow stromal stem cells; Fracture; Rat; Spinal cord injury; Transplantation

Introduction

Innervation of bone has been delineated in normal rats and those with pathological conditions^{1–4}, several studies having shown that periosteum and bone are richly innervated by sensory and sympathetic fibers^{5–9}. Several neuropeptides have been implicated as local modulators of bone metabolism: they

may influence periosteal and medullary blood flow, angiogenesis, and nociception, in addition to having direct effects on osteoblasts and osteoclasts^{10–13}.

In our clinical work, we have noted that the healing process tends to be slower when fractures occur in combination with peripheral nerve injuries. In contrast, fractures

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combined with spinal and cerebral cortical injuries have accelerated healing. In children with myelomeningoceles who need orthopaedic or surgical correction in the lower extremities, we have frequently noted an anomalous type of repair (fast and with abundant callus production) of fractures of the femur and tibia^{14,15}.

This phenomenon is found not only in children with myelomeningoceles, but also in people with paraplegia of other causes, such as subdural hematoma, spinal fractures associated with cord lesion, lumbosacral root avulsion, transverse myelitis and cord tumors.

The effects of nerve lesions on callus formation at fracture sites are not completely understood. The purpose of the present study was to assess callus formation in tibial fractures of rats with concomitant central nerve injuries and the influence of such neural injuries on fracture healing. In addition, we investigated changes in callus when we transplanted bone marrow stromal stem cells (BMSCs) into tibial fractures of rats with central nerve injuries.

Materials and Methods

Equipment and Reagents

Sources of equipment and reagents used are as follows: 5'-bromo-2'-deoxyuridine (BrdU) (Sigma, St Louis, MO, USA); α -minimum essential medium (α -MEM; Gibco, Gaithersburg, MD, USA); 10% fetal bovine serum (Hyclone, Logan, UT, USA); 0.25% trypsin and 1 mmol/L ethylenediaminetetraacetic acid (EDTA; Gibco); fluorescein isothiocyanate (FITC)-conjugated cluster of differentiation (CD)45, phycoerythrin (PE)-conjugated CD34, CD44 antibodies (Sigma); mouse anti-BrdU (Sigma); FITC conjugated anti-mouse IgG antibody (Sigma); fluorescent microscope (Olympus, Tokyo, Japan); CO₂ incubator (Heraeus, Hanau, Germany); IX71 inverted phase contrast microscope (Olympus) and flow cytometer (BD, Franklin Lakes, NJ, USA).

Animals

Experimental Animals

Sprague–Dawley male rats were obtained from the Experimental Animal Center of Lanzhou University (Gansu, China). All animals were housed in air-filtered, humidity- and temperature controlled rooms with equal 12 h light–12 h dark cycles. All animal protocols were approved by the Lanzhou University Committee on Ethics in the Care and Use of Laboratory Animals and were carried out in accordance with international standards on animal welfare and the China Animal Welfare Legislation. Efforts were made to minimize the number of animals used and their suffering.

Animal Models

Forty-eight healthy adult SD rats (male, 220–250 g) were allocated to three groups of 16 rats each depending on the injuries inflicted. Group A rats had a simple (left) tibial fracture, group B a left tibial fracture combined with T₁₀ spinal

cord transection (SCT) and group C a left tibial fracture combined with T₁₀ SCT plus autologous mesenchymal stem cell transplantation. Surgery was performed under sterile conditions. The rats were anaesthetized with 5% ketamine hydrochloride (2.03 mL/100 g) by i.p. injection. The left tibiae of the anesthetized animals were subjected to a standardized midshaft tibial fracture using a specially designed fracture forceps and the fracture fixed with a Kirschner wire. In Groups B and C a median incision was made in the back of each rat, the thoracic vertebrae exposed and about 2 mm of spinal cord at the T_{9–11} level resected simultaneously to create a model of left tibial fracture combined with T_{9–11} spinal cord transection injury. All fractures were stable at the end of surgery and rotation was checked by comparing the alignment of the foot and thigh. Postoperatively, the rats were cared for in different cages according to their groups; supplied with sufficient food and water and the bladders of the rats with spinal cord injury were massaged every 6 h.

Neurological Assessment

The hind-limb motor function of the B and C group animals was assessed by two blinded observers using the following modified Tarlov scale¹⁶: 0, paraplegic with no evident lower extremity motor function; 1, poor lower extremity motor function, weak antigravity movement only; 2, moderate lower extremity function with good antigravity strength but inability to draw legs under body; 3, excellent motor function with the ability to draw legs under body and hop, but not normally; and 4, normal motor function.

Cast Immobilization

The fractured hindlimbs of all animals were immobilized in plaster of Paris casts with the foot in extension and the knee in 90° of flexion¹⁷. The casts were extended 2 cm below the foot, making weight bearing impossible. They were sprayed with pyrooleum animale crudum to prevent destruction by gnawing and changed under anesthesia when necessary.

Bone Marrow Stromal Stem Cells

Culture and Proliferation of Bone Marrow Stromal Stem Cells

Bone marrow stromal stem cells from young Sprague–Dawley rats were retrieved using established techniques^{18,19}. Briefly, bone marrow was taken by femoral puncture 15 days before model establishment. Bone marrow was then flushed out with a growth medium containing α -MEM (Gibco), supplemented with 10% fetal bovine serum (Hyclone), and 1% (v/v) penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were then seeded into culture flasks and grown in the growth medium in a humidified atmosphere of 5% CO₂ at 37°C. After culturing for 24 h, the cultures were washed with phosphate buffered saline (PBS) to remove non-adherent cells. The medium was changed every 3 days. The adherent cells were allowed to reach approximately 80% confluence (7–9 days for the first passage). The cells were then passaged in sterile culture flasks, passage three cells being used for the experiments.

Identification of Bone Marrow Stromal Stem Cells

Passage three cells were harvested by incubation with 0.25% trypsin and 1 mmol/L EDTA (Gibco) at 37° for 3–4 min and replated at a density of 1×10^5 cells/mL. The cells were then washed in PBS for 5 min and incubated for 30 min at 4°C in the dark with mouse monoclonal antibodies. The antibodies used include CD34, CD44 and CD45. The cells were then examined by flow cytometry (BD) to identify the BMSCs.

Bromodeoxyuridine Labeling and Detection

Passage three cells were harvested and replated in 96-well plates at 1×10^5 cells/well, then the purified BMSCs were incubated with BrdU at different concentrations (5, 10, and 15 μ mol/L; Sigma) for incubation times of 24, 48 and 72 h post-seeding. After washing with PBS, the cultures were fixed in ice-cold 70% ethanol for 10 min and permeabilized with 2 mol/L HCl for 30 min at 37°C. After washing in PBS and blocking in 5% FBS plus 1% bovine serum albumin, the cultures were incubated with mouse anti-BrdU (Sigma) for 1 h at 37°C and then incubated with FITC conjugated anti-mouse IgG antibody (Sigma) for 1 h at 37°C. BrdU incorporation was examined under high magnification and proliferation assessed as the percentage of cells positive for BrdU incorporation relative to the total number of nuclei.

Transplantation of Bone Marrow Stromal Stem Cells

Finally, approximately, 1×10^6 BMSCs labeled with BrdU in 0.5 mL Dulbecco modified Eagle medium (DMEM) were slowly injected over 5 min into the fractures of group C rats 2 days after the models had been created. The control group received i.v. DMEM similarly to the transplanted group, but without BMSCs. Immunosuppressive therapy was not used in any animals.

Tissue Processing

After 28 days of healing, all animals were weighed and then killed with a phenobarbital overdose. Prior to 28 days, two animals died during recovery from anesthesia and three were killed because of decubital skin injuries of their feet. The tibiae were resected and all soft tissues and intramedullary nails carefully removed, care being taken to leave the callus of the tibiae intact. The bones were weighed to the nearest 0.1 mg (wet weight), then frozen wet at –20°C before further testing was carried out. During all later handling, the tibiae were kept moist with Ringer solution. The fractured tibiae were radiographically and immunohistochemically evaluated separately.

Radiographic Examination

Radiographs were taken of the healing tibiae and scored blindly and independently for the presence of callus by two radiologists with long experience in orthopedics. Each radiograph was rated from 0 to 3 (0, no callus; 1, just visible callus; 2, normal amount of callus; and 3, excessive callus) and the average of the frontal and side views calculated. A score of less than 1 was taken as “no callus formation”.

Statistical Analysis

All experiments were performed at least twice with similar results. The numbers of BMSCs were counted from ten randomly selected fields ($\times 100$). The proportion of positive BMSCs to total cell population was calculated. Data are presented as mean $\pm$ SD and analyzed by SPSS 13.0. Statistical analysis was performed using Student *t*-test, a value of $P < 0.05$ being accepted as statistical significance.

Results

Separation, Culture and Proliferation of Bone Marrow Stromal Stem Cells

In our study, separation of BMSCs was confirmed by morphological observation through a phase contrast microscope (Olympus). BMSCs were adherent in the primary culture after 24 h of plating. In primary BMSC cultures, the cells were heterogeneous, with morphologies ranging from spindle shapes to large polygons; they then proliferated and formed colonies at post-culture 7–10 days. When they reached 80% confluence, adherent cells were passaged. After three passages, the cells became homogeneous, elongated and spindle-shaped (Fig. 1).

Flow Cytometry Detected the Surface Markers of Bone Marrow Stromal Stem Cells

Flow cytometry analysis showed that 81.27% of BMSCs were positive for CD44 (Fig. 2a), whereas only 2.32% were positive for CD34 (Fig. 2b) and 0.35% for CD45 (Fig. 2c). The morphological characteristics and cell-surface antigen profiles of the BMSCs that were cultured were consistent with previous reports^{20,21}. These results indicate that BMSCs were successfully isolated and cultured.

Rate of Bromodeoxyuridine Staining

Immunofluorescent staining with BrdU was performed on the BMSCs. Passage three cells showed BrdU positive immunostaining in the nuclei after being incubated with BrdU at concentrations of 5, 10 and 15 μ mol/L, whereas no positive cells were seen in the the negative up and blank control groups. Over 95% of BMSC nuclei were positively labeled with 10 μ mol/L of BrdU. There was no significant difference in the rate of BrdU staining between the groups incubated with 10 μ mol/L and 15 μ mol/L ($P > 0.05$); however, the group incubated with 5 μ mol/L was 60% positive for BrdU staining ($P < 0.05$ compared with 10 μ mol/L). Over 95% of BMSC nuclei were positively labeled with BrdU after incubating for 48 h. Extending the labeling time did not result in an increase in the number of staining-positive cells. There was no significant difference between incubating for 48 h and 72 h ($P > 0.05$); however, there was between the groups incubated for 48 h and 24 h (60%; $P < 0.05$). A concentration of 10 μ mol/L and incubating time 48 h were therefore chosen as optimal for staining of BMSCs with BrdU (Fig. 1e). According to phase contrast microscopy assessment, there were no significant differences in morphology, growth and proliferation of BMSCs between this and the control group.

Fig. 1 Morphologic observation of BMSCs by phase contrast microscopy. In primary BMSC cultures, the cells were heterogeneous, with morphologies ranging from spindle shapes to large polygons; they then proliferated and formed colonies at post-culture 7–10 days. (A) BMSCs cultured for 48 h ($\times 100$). (B) BMSCs cultured for 3 days ($\times 100$). (C) BMSCs cultured for 5 days ($\times 100$). (D) BMSCs cultured for 9 days ($\times 100$). (E) BMSCs labeled with 10 $\mu\text{mol/L}$ BrdU for 48 h ($\times 100$). (F) BMSCs labeled with BrdU in tissue 4 weeks after transplantation ($\times 100$).

Neurological Assessment

The postoperative individual neurologic scores of B and C group rats are shown in Table 1.

Observation according to X-ray films and Computed Tomography

The morphology of the fractures is shown in Figs 3 and 4: we found less bony callus formation in group A rats than in the denervated fractures groups' rats 4 weeks after injury. The B and C group rats with spinal injuries had not only developed more bony callus than the simple fracture group rats, they also evidenced more numerous and regular bone trabeculae, occlusion of the medullary cavity and solid bony connections.

The characteristics of bone callus in the various groups are shown in Figs 3 and 4. The size, height and width of fracture callus were all bigger in the spinal injury B and C groups than in group A rats at 1, 2 and 3 weeks ($P < 0.05$). In the fourth week, the size, height and width of callus in group C rats were smaller than in group B ($P < 0.05$); however, group C calluses were still bigger than in group A ($P < 0.05$).

Wet Weights of Tibiae

The wet weights of tibiae in the various groups are shown in Fig. 5. The wet weights of tibiae were greater in the spinal injury B and C groups than in group A in the first week ($P < 0.05$). Later on, the weights of tibiae in group C were

Fig. 2 Detection of antigen profiles on the cell surface of rat BMSCs by flow cytometry analysis: 81.27% of BMSCs were positive for CD44 (A), whereas only 2.32% were positive for CD34 (B) and 0.35% for CD45 (C).

TABLE 1 Tarlov scores

Groups	0	1	2	3	4
B	13	3	—	—	—
C	14	2	—	—	—

decreased in the second week and in all groups in the third week; however, B and C group tibiae were still heavier than those in group A ($P < 0.05$).

Hematoxylin–eosin Staining of Bony Callus

As Fig. 6 shows, in hematoxylin–eosin (HE) stained sections from group A rats' tibiae in the fourth postoperative week,

Fig. 3 X-ray films and bar charts showing the fracture healing characteristics in the three groups in the fourth week. The size, height and width of fracture callus were all bigger in the spinal injury B and C groups than in group A rats at 1, 2 and 3 weeks ($P < 0.05$). In the fourth week, the size, height and width of callus in group C rats were smaller than in group B ($P < 0.05$); however, group C calluses were still bigger than in group A ($P < 0.05$).

Fig. 4 CT images showing fracture healing characteristics in the three groups in the fourth week: the size, height and width of callus in group C rats were smaller than in group B; however, group C calluses were still bigger than in group A ($P < 0.05$).

Fig. 5 The wet weights of the tibiae by postoperative week and group. The wet weights of tibiae were greater in the spinal injury B and C groups than in group A in the first week ($P < 0.05$). Later on, the weights of tibiae in group C were decreased in the second week and in all groups in the third week; however, B and C group tibiae were still heavier than those in group A ($P < 0.05$).

fracture hematoma and fibro-cartilaginous metaplasia were usually found to have been replaced by abundant bony bridges and areas with ossification fronts and osseous-cartilaginous metaplasia callus tissue (Fig. 6a). In B and C groups, bony union was evident, together with more numerous and regular bone trabecula, occlusion of the medullary cavity and solid bony connections (Fig. 6b,c).

Alkaline Phosphatase (ALP) Staining

Alkaline phosphatase (ALP) staining showed abundant fibroblasts and some chondrocytes in the fractures one week post-operatively and small ALP-positive particles between the fractures. The ALP-positive particles were much more numerous in the second week. In the fourth week, as Fig 7b,c shows, the area positive for ALP staining are more abundant in groups B and group C, when dense bone callus and increased irregular bone trabecula were seen at the fracture sites. Subsequently, the number of ALP-positive particles decreased significantly.

Discussion

In our clinical work, we have noted that bone fractures of paralyzed limbs in patients with central neural injuries may be accompanied by rapid formation of excessive callus and

heterotopic ossification²². We could not draw definite conclusions as to the cause because of the influence of many factors, including delay in recognition of fractures (due to lack of sensation) and variations in levels of fractures and ages of patients. In an attempt to clarify the contributions of these factors, we devised an experimental model. For this, we chose the rat because it is easy to obtain animals of the same sex, age and weight, and because this model had already been used to study the repair of fractures in denervated limbs.

To prevent differences in load bearing between the spinal injury and control groups after fracture, fixation of the tibial fractures was performed percutaneously with modular intramedullary nails, which have about the same stiffness and strength as intact tibiae of the rats used in the present experiment. The fractures were made with fracture forceps, which causes minimal soft tissue damage. The combination of intramedullary nailing and casting was designed to prevent uncontrolled and unequal loading of the fractured tibiae, thereby ensuring an equal degree of disuse osteoporosis and providing very stable fixation in each group.

The effect of spinal cord injury on tibial fracture healing in rats was studied 28 days post-trauma. In an attempt to determine the effects of spinal injury on fracture healing,

Fig. 6 HE staining to display characteristics of bone callus condition in the fourth week. In A group, fracture hematoma and fibro-cartilaginous metaplasia were usually found to have been replaced by abundant bony bridges and areas with ossification fronts and osseous-cartilaginous metaplasia callus tissue. In B and C groups, bony union was evident, together with more numerous and regular bone trabecula, occlusion of the medullary cavity and solid bony connections.

Fig. 7 ALP staining to display characteristics of bone callus condition in the fourth week: the area positive for ALP staining are more abundant in groups B and group C.

radiographic, histomorphometric and chemical methods were used to evaluate callus formation in the tibial fractures of the experimental rats.

We found that the different types of injury resulted in different speeds of fracture healing. Healing was accelerated in rats with a combination of fracture and spinal injury, formation of external callus being more rapid in the spinal injury group than in controls. In groups B and C, we saw bony union with numerous bone trabeculae, abundant bony bridges, areas with ossification fronts and osseous-cartilaginous metaplasia and finally, solid bony connections. However, mechanical strength in three-point cantilever bending is reportedly not better in rats with spinal injury, implying defective tissue organization²³. In the last decade, some researchers have demonstrated and confirmed that neural factors can affect the healing processes of fractures; however, the mechanism(s) remains unclear.

Calcitonin gene-related peptide (CGRP) is one of several biologically active neuropeptides identified by Rosenfeld *et al.* through DNA recombination and molecular biology techniques in 1983²⁴. CGRP is coded by the calcitonin gene, which gene is composed of 2800 bp and contains a five piece intervening sequence and six piece exons. At present, CGRP is the strongest vasodilator substance known; its regulatory effect on bone metabolism is becoming clearer with further investigation²⁵.

Aoki *et al.* demonstrated that CGRP is abundantly distributed in bone via sensory nerves, especially in epiphyseal trabecular bones²⁶. CGRP is reportedly expressed endogenously by osteoblasts. Transgenic mice with osteoblasts that over-express CGRP are characterized by an increased rate of bone formation and enhanced bone volume, suggesting that CGRP indeed acts on bone metabolism not only via a neural route, but also via an autocrine loop. Calcitonin gene-related peptide has an osteogenic stimulating effect, either by stimulating stem cell mitosis or osteoprogenitor cell differentiation (or both) *in vitro*²⁷. CGRP also potentially inhibits bone resorption²⁸.

In our experiment, the speed of fracture healing in the spinal cord injury group was faster than in the control group and there was more abundant local bony callus in the former group. However, the mechanical properties of the healing tibiae are reportedly defective, suggesting that both the quan-

tity and quality of callus formation are influenced by the nervous system. Takahashi *et al.* found that spinal transection increased tibial bone blood flow in the rat after 6 h, 4 or 12 weeks²⁹. Some researchers have found that spinal cord injury can accelerate the speed of healing through participation of massive amounts of CGRP synthesized and secreted by neurons and perhaps via some autocrine substances released by the injured bone itself. It is possible that in the post-traumatic state both osteoblasts and osteoclasts receive regulatory signals via nerves. Spinal cord injury may change the neural influences on both osteoblasts and osteoclasts.

Based on the above, we have explored the effect of the nervous system on the pathogenesis of denervated fracture. Our investigations suggest that normal innervation is important for the response of bony tissue to injury and that the nervous system modulates the development of fracture healing after tibial fracture. Recently, BMSCs have been extensively studied as good candidates for tissue engineering³⁰ and used to treat a variety of diseases, such as osteogenesis imperfecta and mucopolysaccharidoses^{31,32}. They can be induced to differentiate into neuron- and glia-like cells³³, osteoblastic lineage³⁴, chondrocytes³⁵ and so on and have little immunogenic potential³⁶. Consequently, in this study, we aimed to explore some techniques for treating denervated fractures using BMSCs.

Postnatal bone marrow has traditionally been seen as an organ composed of two main systems rooted in distinct lineages: the hematopoietic tissue proper and the associated supporting stromal cells. Unlike hematopoietic stem cells, whose role in the treatment of hematopoietic diseases has been known for a long time, BMSCs were originally examined only because of their critical role in the formation of the hematopoietic microenvironment. More recent data came with the recognition that BMSCs are stem/progenitor cells of ectodermal, mesodermal and endodermal tissues^{37,38}. Their potential to differentiate into nonhematopoietic organ cells granted them membership in the family of somatic stem cells. The use of BMSCs in cell therapies may have some advantages over the use of cells from other sources: they are relatively easy to isolate from bone marrow, grow well in tissue cultures, can be used in autologous transplantation protocols and bone marrow as a source of cells has been already approved for the treatment of hematopoietic diseases^{39,40}.

Since 1966, when Friedenstein *et al.* first isolated and cultured BMSCs from bone marrow stromal, scientists have proposed many different techniques for isolation BMSCs⁴¹. Currently, BMSCs are frequently isolated using density gradient centrifugation⁴², an adherence method⁴³, flow cytometry or magnetic cell sorting⁴⁴. Because the latter two methods are expensive and limited by many factors, they are not widely used. The technique of density gradient centrifugation reported by Pittenger *et al.* has been considered classical; however, density gradient centrifugation is complex and it takes a long time²⁰. Moreover, because stem cells' capability to adhere is lost during this isolation process, cell injury and contamination can easily occur⁴⁵. Adherence methods are based on the ability of stem cells to adhere: it entails removal of non-adherent cells, including hematopoietic cells and endothelial cells, and highly purified BMSCs are obtained after multiple passages. Consequently, in this study, we used an adherence method to isolate and culture BMSCs.

Because no specific molecular marker of BMSCs has yet been found, BMSCs are mainly evaluated by their morphological features and surface markers. In this study, according to flow cytometry the cultured cells expressed CD44 but not CD34 and CD45, in accordance with the characteristics of BMSCs, suggesting that the cultured cells were not hemopoietic stem cells (which express CD34, CD45)⁴⁶. In addition to expression of CD44, the morphology of our passage three BMSCs was consistent with that previously reported: they were homogeneous, elongated and spindle-shaped, further suggesting that we had successfully isolated, cultured and amplified BMSCs in this study.

Human medical and experimental research would benefit from the labeling of implanted stem cells and the availability of noninvasive methods to image the labeled cells post-implantation. In our study, we showed that BMSCs can be labeled with BrdU.

5'-bromo-2'-deoxyuridine can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle) by substituting for thymidine during DNA replication: this makes "new" DNA fluoresce green. BrdU labeling and detection with a high labeling rate and without radioactive contamination is an ideal method for tracking dynamic changes in transplanted cells⁴⁷. Because cells labeled with BrdU cannot be observed directly, it is necessary to use immunohistochemical methods to identify these cells. Moreover, the method of BrdU labeling is cytotoxic⁴⁸. In this study, we found that incubation of BMSCs with BrdU at a concentration of 10 µmol/L and incubating time 48 h achieved the highest positive labeling rate (>95%). In 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide and BrdU cell proliferation

assays, there is a similar growth pattern in labeled and non-labeled control groups. These findings show that the basic characteristics of BMSCs do not change significantly during proliferation after staining with BrdU. In addition, that BrdU reportedly improves BMSCs multi-lineage differentiation potential⁴⁹.

In our experimental studies, we found that the spinal injury group developed more abundant bony callus than the simple fracture group. However, the callus has defective organization because of the lack of appropriate neural influence. Consequently, autologous BMC implantation has been used in experimental rats with transversal spinal cord lesions. In our study, we found the tibial callus width in the B and C group rats was significantly greater than in group A rats during the first, second and third postoperative weeks ($P < 0.05$). However, the tibial callus width in group C decreased in the fourth weeks. Implantation of autologous BMSCs can accelerate fracture healing and pathological changes in the callus healing process may explain this. Areas staining ALP-positive were more abundant in groups B and group C, this being particularly obvious in group C. We concluded that treatment with bone marrow cell populations has a positive effect on defective bony callus after SCT in rats. BMSCs transplantation is a potentially powerful treatment method for spinal cord injury; however, current experiments with stem cells have not given us information about the behavior of the transplanted cells in the host organism *in vivo*, especially regarding their migration and fate within the target structures and their potential neoplastic growth⁵⁰. The lack of these data may create serious obstacles to the therapeutic use of such cells.

Although our experimental studies and those of others in animals and pre-clinical human studies have demonstrated the effectiveness and safety of BMSCs therapy, there are still many questions to be answered regarding the mechanisms of engraftment, homing, inter-cellular interactions, immunological profiles in *in vivo* differentiation and long-term safety. Together, these studies support further investigation of BMSCs as a potential strategy for repair of denervated fractures.

In conclusion, this study shows that neural regulation as well as the known osteotropic, systemic factors (such as osteogenic growth peptide⁵¹, bone morphogenetic protein⁵², growth factors Y and prostaglandins^{53,54} released as a consequence of the fracture. We believe that these nervous system influences may be especially important in the process of bone formation and resorption according to specific demands on shape, strength, and size of bone based on motility and movement. The detailed mechanisms for these effects of the peripheral nervous system have yet to be clarified.

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