Bone Marrow Mesenchymal Stem Cell Transplantation Enhances Nerve Regeneration in a Rat Model of Hindlimb Replantation

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Background: Successful limb replantation must be based not only on the viability of the amputated part but also on satisfactory long-term functional recovery. Once the vascular, skeletal, and soft-tissue problems have been taken care of, nerve recovery becomes the ultimate limiting factor. Unfortunately, nerve regeneration after limb replantation is impaired by several consequences. The authors tested the hypothesis that bone marrow mesenchymal stem cells could improve nerve regeneration outcomes in an experimental model of limb replantation.
Methods: Twenty rats underwent replantation after total hindlimb amputation. Animals were subdivided into two groups: a replanted but nontreated control group and a replanted and bone marrow mesenchymal stem cell–transplanted group. Three months after surgery, nerve regeneration was assessed using functional, electrophysiologic, histomorphologic, and immunohistochemical analyses.
Results: Bone marrow mesenchymal stem cell–treated animals showed significantly better sciatic functional index levels and higher compound muscle action potential amplitudes in comparison with the controls. Histomorphometric analysis revealed that the number of regenerating axons was approximately two-fold greater in the treated nerves. In addition, the mean g-ratio of these axons was within the optimal range. Immunohistochemical assessment revealed that expression of S-100 and myelin basic protein in the treated nerves was significantly higher than in controls. Correspondingly, the expression levels of anti–protein gene product 9.5 and vesicular acetylcholine transporter in motor endplates were also significantly higher. Finally, muscles in the bone marrow mesenchymal stem cell–transplanted group showed significantly larger average fiber areas.
Conclusion: The authors' findings demonstrate that it is possible to improve the degree of nerve regeneration after limb replantation by bone marrow mesenchymal stem cell transplantation. (*Plast. Reconstr. Surg.* 143: 758e, 2019.)

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Amputations have dramatic impacts on patients' lives, as loss of extremity will not only cause reduced autonomy but also hinder social interaction and work capacity.[1] It has been documented that the prevalence of traumatic amputations among Americans was more than 700,000 in 2005, and this prevalence will double by the year 2050.[2] The annual cost of traumatic amputations is estimated to be in the billions of dollars, likely because of long hospital stays and delayed return to work.[3] Fortunately, replantation techniques have evolved at a surprising speed, increasing the rate of salvaged limbs.[4] Certainly, the success of replantation must be based not only on the viability of the replanted part, but also on satisfactory long-term functional recovery.[5] In limb replantation, once the vascular, skeletal, and soft-tissue problems have been taken care of, recovery of the nerve ultimately becomes the limiting factor.[6]

Even with meticulous surgical technique, the outcomes of nerve repair are impaired by several consequences, including partially reversible axonal degeneration,[7] neural scarring,[8] depletion of neurotrophic support,[9] escape of axons into surrounding tissue,[10] axonal misdirection,[11] random innervation of inappropriate muscle,[12] and slow regeneration rates.[13] These limitations are amplified following limb replantation because of the extensive nature of amputation injuries. In these cases, distribution of tissue perfusion is one of the most important pathways underlying neural damage.[14] Besides the acute destruction of vascular structures by the traumatic impact itself, direct soft-tissue damage triggers an inflammatory response that often goes uncontrolled and results in excessive production of proinflammatory cytokines, leading to massive interstitial edema and consequently elevated tissue pressure and compromised perfusion.[15,16] Although the degree of ischemic damage is directly associated with the duration of ischemia, the cellular and biochemical interactions that emerge after the restoration of perfusion may aggravate the neural damage.[17] Although several mechanisms have been proposed to explain this phenomenon, oxidative stress and profound inflammatory response continue to receive much attention as critical factors in the genesis of reperfusion injury.[18]

Repair of the peripheral nerves may require certain combinations of therapies to support axonal regeneration. Cellular therapies, particularly mesenchymal stem cells, seem to be among the best remedies for the control of the local environment. Previous studies have documented the ability of bone marrow mesenchymal stem cells to promote peripheral nerve regeneration.[19–21] There is a consensus that these transplants can promote nerve regeneration not only by the differentiation into Schwann cell–like cells, but also by means of factors that enhance nerve regeneration and angiogenesis, and by decreasing inflammatory reaction and oxidative stress.[22–24] These unique properties of bone marrow mesenchymal stem cells support the hypothesis that these transplants could improve nerve regeneration outcomes in the setting of limb replantation.

MATERIALS AND METHODS

Animals

Our study was approved by Osmangazi University Ethical Committee for Experimental Research on Animals. Adult male Wistar rats weighing between 300 and 325 g were used. All procedures were performed under anesthesia with an intraperitoneal injection of xylazine and ketamine. All rats were euthanized by means of an anesthetic overdose injection at final evaluation.

Bone Marrow Mesenchymal Stem Cell Harvesting and Culture

Twelve-week-old Wistar rats were euthanized by overdose anesthesia. Both sides, femora and tibia, were removed and washed with phosphate-buffered saline (Biological Industries, Kibbutz Beit-HaEmek, Israel). Bone marrows were flushed with low-glucose Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Biological Industries). Cells were cultured in culture medium (low-glucose Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% stable glutamine) and incubated at 37°C in 5% carbon dioxide. The medium was refreshed on the fourth day and subsequently replaced every 3 days until the monolayer of adherent cells reached 85 to 90 percent confluence. Thereafter, cells were detached by 0.025% trypsin–ethylenediaminetetraacetic acid and replated at a rate of 1:4 for subculturing. Passage 3 bone marrow mesenchymal stem cells were used.

Characterization of Bone Marrow Mesenchymal Stem Cells by Flow Cytometry

The following monoclonal antibodies (BD Biosciences, San Jose, Calif.) were used for flow cytometric immunophenotyping of bone marrow mesenchymal stem cells: CD11b, CD29, CD44, CD45, CD73, and CD90. Flow cytometry was performed using a Navios Flow Cytometer (Beckman Coulter, Brea, Calif.), and the data were analyzed with Kaluza software (Beckman Coulter).

Characterization of Bone Marrow Mesenchymal Stem Cells by Quantitative Real-Time Polymerase Chain Reaction Assay

Total RNA was extracted with a High Pure RNA Tissue Kit (Roche Diagnostics, GmbH, Mannheim, Germany) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Mass.). A Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) was used to reverse transcribe the extracted RNA to complementary DNA. Quantitative real-time polymerase chain reaction assay was performed using the LightCycler 480 II real-time polymerase chain reaction system (Roche Diagnostics). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Results are expressed as a percentage relative to 1/ΔCt of the *GAPDH* gene. (**See Table, Supplemental Digital Content 1**, which shows primers used for quantitative real-time polymerase chain reaction analysis, ***http://links.lww.com/PRS/D357***.)

Count and Viability Assay

Viable cells were detected by using dual fluorescent probes of Count & Viability Assay kit (Merck Millipore, Burlington, Mass.) on a flow cytometer.

Bone Marrow Mesenchymal Stem Cell In Vitro Differentiation

The multipotent nature of the bone marrow mesenchymal stem cells was evaluated by ensuring their ability to differentiate along various lineages to confirm that the transplants meet the minimal criteria to characterize mesenchymal stem cells.[25] Bone marrow mesenchymal stem cells from the third passage were seeded onto 12-well plates. When they reached 90 to 100 percent confluence, the growth medium was aspirated off and one plate was given adipogenic induction and maintenance medium (Lonza, Walkersville, Md.) and the other plate received osteogenic differentiation medium (Lonza).

Surgical Procedure

Under anesthesia, the midthigh region of each animal was incised circumferentially. The sciatic nerve was exposed from the sciatic notch to the bifurcation point and sharply transected at 1 cm proximal to the knee. Thereafter, the femoral vessels and femoral nerve were isolated. Before division, microvascular clamps were applied proximally on the femoral vessels and this time point was noted as the beginning of ischemia. The muscles and femoral nerve were then cut to reach the femur. After subperiosteal dissection, the femur was cut and subsequently shortened by 0.5 cm to decrease repair tension. The amputated limb was wrapped in saline-moistened gauze and placed in a sterile container at room temperature. Replantation was begun 1 hour after the initiation of ischemia. First, the femur was fixed with an intramedullary 18-gauge needle. The formal vein and artery were then anastomosed in an interrupted fashion using 10-0 nylon. The timing of reperfusion was synchronized such that the total ischemia time was approximately 2 hours in all animals. The sciatic nerve was repaired with four interrupted epineural 10-0 nylon sutures. The muscles were repaired, and this was followed by skin closure. The postoperative analgesia was performed with buprenorphine, 0.05 mg/kg, subcutaneously, every 12 hours for 1 week. Neither systemic antibiotics nor anticoagulants were administered. An Elizabethan collar was used to prevent autocannibalization. The rats were caged separately and evaluated daily by clinical examination to assess limb viability.

Experimental Design

Twenty Wistar rats underwent replantation after total right lower limb amputation. Three rats were excluded from the study because of replantation failure but were replaced by others. Animals were subdivided into two groups according to the nature of injection; animals in the bone marrow mesenchymal stem cell group ($n = 10$) received 1 cc of phosphate-buffered saline mixed with 1×10^6 of bone marrow mesenchymal stem cells injected directly along the proximal and distal nerve stumps using a 33-gauge, blunt-tip needle immediately after nerve repair. Fibrin glue was used to stabilize bone marrow mesenchymal stem cells in the coaptation point. In contrast, animals in the control group ($n = 10$) received 1 cc of phosphate-buffered saline. Fibrin glue was applied also. All assessments were made 90 days after surgery.

Functional Assessment

Walking track assessment was performed every other week and the sciatic function index (SFI) value was calculated.[26] To this end, the hind paws of trained rats were dipped in ink, and these animals were allowed to walk down a corridor lined with white paper. The toe spread (TS), intermediary toe spread (ITS), and paw length (PL) were determined on both the normal (N) and the replanted (R) limbs:

$$SFI = -38.1 \times \frac{RPL - NPL}{NPL} + 109.5 \times \frac{RTS - NTS}{NTS} + 13.3 \times \frac{RITS - NITS}{NITS} - 8.8$$

The sciatic function index is a negative indicator of nerve dysfunction and varies from 0 to −100, with 0 meaning absence of dysfunction and −100 meaning complete dysfunction.

Electromyographic Assessment

Bipolar electrical stimulation was performed over the proximal stump of the sciatic nerve 90 days after replantation. The compound muscle action potential was recorded in the gastrocnemius muscle with a needle electrode and a reference cap electrode inserted distally into the same muscle tendon. The ground electrode was inserted into the tail skin. Amplitude and latency of compound muscle action potential were measured.

Histomorphometric Assessment

The extent of axonal regeneration was quantified 3 months after replantation by the evaluation of histomorphometric measures of nerve semithin and ultrathin sections. Segments from the distal stumps were removed and fixed in glutaraldehyde, postfixed with osmium tetroxide, dehydrated in ethanol, and embedded in plastic resin. For semithin sections, 1-μm-thick cross-sections at 0.5 cm distal to the anastomosis site were stained with toluidine blue and examined under light microscopy. At 1000× magnification, five randomly selected fascicle fields per nerve fiber were evaluated for myelinated axon count, the individual axon calibers, and the corresponding g-ratios (the ratio of the inner axonal diameter to the total outer diameter). At least 300 randomly chosen fibers per animal were evaluated. For ultrathin sections, sections 100 nm thick were cut, placed on copper grids, stained with lead citrate, and examined by transmission electron microscopy.

Immunohistochemical Assessment

Immunohistochemical stainings were performed 3 months after replantation using standard methods. Immunoreactivity was observed using a light microscope. The following antibodies were used: anti-S100 for axon regeneration, anti–myelin basic protein for peripheral myelin, anti–protein gene product 9.5 for axon innervations of the muscle, and anti–vesicular acetylcholine transporter antibodies as presynaptic markers. For anti-S100 and anti–myelin basic protein antibodies, the number of stained structures was determined at 40× magnification, and the mean value of 10 random fields per slide for each animal was used. For anti–protein gene product 9.5 and anti–vesicular acetylcholine transporter antibodies, a semiquantitative intensity scale ranging from 0 for no staining to 3+ for the most intense staining was used.

Muscle-Mass Ratio and Masson Trichrome Staining of the Muscle

Gastrocnemius muscles were dissected bilaterally and the muscle-mass ratio (wet weight of the right side/left side) was determined. Then, the midbellies of the right gastrocnemius muscles were embedded in paraffin and cut into 5-μm-thick transverse sections for Masson trichrome staining. After staining, muscle sections were digitally captured at 20× magnification. The circumferences of 20 randomly selected muscle fibers with intact cell membranes were outlined and fiber areas were calculated using ImageJ software (National Institutes of Health, Bethesda, Md.). Elongated fibers indicating oblique sections were not included.

Statistical Analysis

Data analyses were performed using IBM SPSS Version 21.0 (IBM Corp., Armonk, N.Y.). Samples with normal distribution were evaluated by independent-samples *t* test. Nonnormally distributed variables were compared across the groups by using the Mann-Whitney rank sum test.

RESULTS

Characterization of Bone Marrow Mesenchymal Stem Cells

Flow cytometric measurements showed that the cultured cells were positive for mesenchymal stem cell markers such as CD29, CD44, CD73, and CD90 and negative for the hematopoietic markers CD45 and CD11b. In line with these data, the quantitative real-time polymerase chain reaction assay analysis demonstrated high gene expression levels of the markers CD44, CD29, CD73, and CD90. Moreover, low expression levels were detected for the markers CD11b and CD45. [**See Figure, Supplemental**

Digital Content 2, which shows analysis of cell-surface markers in bone marrow mesenchymal stem cells at the third passage. (*Left*) Flow cytometric measurements demonstrated that all cells expressed mesenchymal stem cell markers including CD29, CD44, CD73, and CD90, but were negative for CD11b and CD45. (*Right*) Quantitative real-time polymerase chain reaction assay analysis demonstrated high gene expression levels of the markers CD29, CD44, CD73, and CD90. Low expression levels were detected for the markers CD11b and CD45. DCT, ΔCt, ***http://links.lww.com/PRS/D358***.]

Count and Viability Assay

Viability was quantified by flow cytometric analysis after staining with a counting and viability kit before application, and more than 90 percent live cells were detected.

Multilineage Differentiation Potential of Bone Marrow Mesenchymal Stem Cells

Bone marrow mesenchymal stem cells were assessed for adipogenic and osteogenic potential. On day 21 of differentiation, the presence of intracellular lipid droplets was confirmed by AdipoRed Assay Kit (Lonza). In contrast, the osteogenic potential was determined as calcium storage in differentiated cells using 1% alizarin red staining. [**See Figure, Supplemental Digital Content 3**, which shows multilineage differentiation capacities of bone marrow mesenchymal stem cells. (*Left*) The presence of intracellular lipid droplets was confirmed by AdipoRed staining. (*Right*) The osteogenic potential was determined as calcium storage using alizarin red staining (original magnification, × 10), ***http://links.lww.com/PRS/D359***.]

Limb Replantation

Replantation failed in two animals from the control group and one from the bone marrow mesenchymal stem cell group. In all three animals, after restoration of blood flow, the limbs became swollen, resulting in compression of the vein and artery. Ultimately, perfusion ceased after 1 to 5 days and replantation failed because of embolization in the artery. All animals were replaced by others.

Functional Assessment

Rats in the bone marrow mesenchymal stem cell and control groups showed time-dependent increases in sciatic function index values. During the first 6 weeks, the differences between sciatic function index values in both groups were not significant (all $p > 0.05$). However, the treatment group showed significantly better sciatic function index levels in comparison with the controls at weeks 8 ($p < 0.05$), 10 ($p < 0.05$), and 12 ($p < 0.01$) after replantation (Fig. 1).

Electromyographic Assessment

The mean compound muscle action potential amplitude of the bone marrow mesenchymal stem cell–treated group (12.7 ± 3 mV) was significantly higher than that of the control group (7.7 ± 2 mV) ($p < 0.01$). With respect to compound

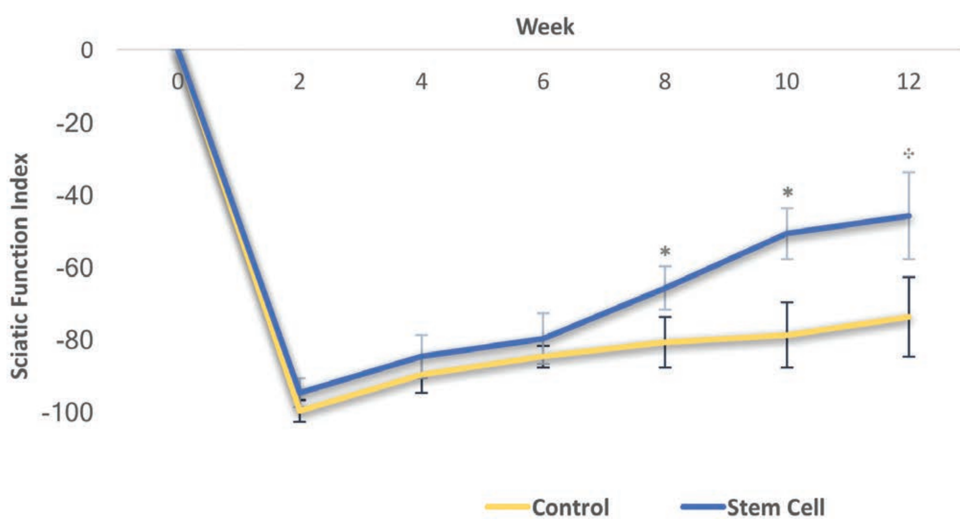

Fig. 1. Graph of the sciatic functional index. The bone marrow mesenchymal stem cell group showed significantly better levels in comparison with the controls at weeks 8, 10, and 12 after replantation. The sample size was $n = 10$ for each group. *$p < 0.05$ versus control; ✣$p < 0.01$ versus control.

muscle action potential latencies, there was no significant difference between the treated (2.1 ± 0.4 msec) and control (2.3 ± 0.3 msec) groups (p = 0.502). [**See Figure, Supplemental Digital Content 4**, which shows electrophysiologic assessment of sciatic nerve regeneration. (*Above*) The mean compound muscle action potential (*CMAP*) amplitude of the bone marrow mesenchymal stem cell group was significantly higher than that of the control group. (*Below*) There was no significant difference between compound muscle action potential latencies recorded in both groups. The sample size was n = 10 for each group. *$p < 0.001$ versus normal; ✣$p < 0.01$ versus control, ***http://links.lww.com/PRS/D360***.]

Histomorphometric Assessment

In the bone marrow mesenchymal stem cell–treated group, the number of axons just distal to the anastomosis site was approximately two-fold greater than that in the control group, and this difference was significant ($p < 0.001$). Morphometric analysis revealed significantly smaller axon diameters in treated animals ($p < 0.05$). A mean g-ratio of 0.61 ± 0.1, which lies within the optimal range for the sciatic nerve (0.55 to 0.68),[27] was obtained for nerve fibers of the bone marrow mesenchymal stem cell group. However, the mean g-ratio of the control group (0.52 ± 0.1) was below the optimal range. The difference between the two groups was significant ($p < 0.05$) (Fig. 2).

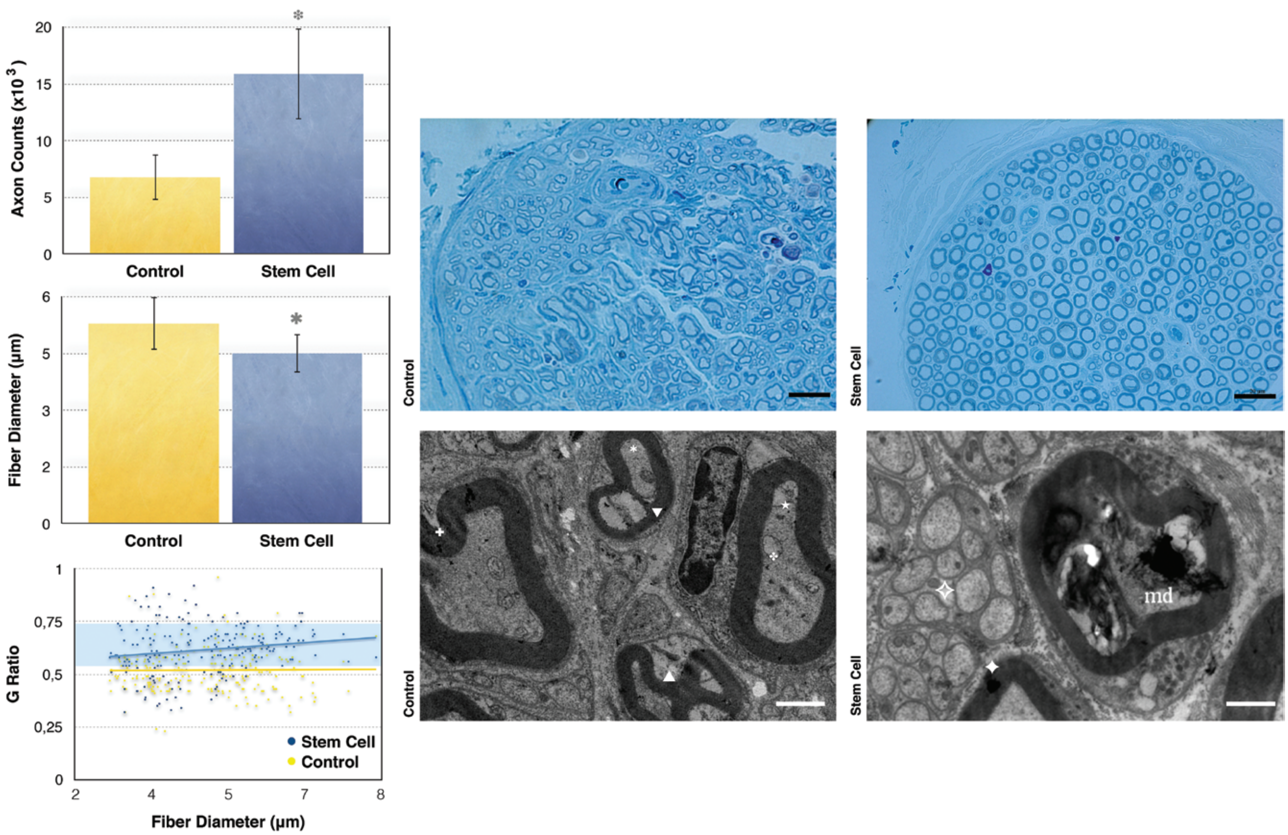

Fig. 2. Histomorphometric assessment of the nerve. (*Above*, *left*) The number of axons just distal to the anastomosis site was significantly higher in the bone marrow mesenchymal stem cell group. (*Center*, *left*) Morphometric analysis revealed significantly smaller axon diameters in bone marrow mesenchymal stem cell–treated animals. (*Below*, *left*) The mean g-ratio of the bone marrow mesenchymal stem cell–treated fibers was within the optimal range for the sciatic nerve. In contrast, the mean g-ratio of the control group was below the optimal range. The sample size was n = 10 for each group. *$p < 0.05$; ❋$p < 0.001$. (*Above*, *right*) Toluidine blue staining documented severe axonal demyelination and degeneration in animals of the control group. However, bone marrow mesenchymal stem cell–treated axons revealed nearly normal morphology with intact myelin sheath (original magnification, × 100). *Scale bar* = 20 µm. (*Below*, *right*) Transmission electron microscope images demonstrate evidence of invaginated myelin (✚) and compressed axoplasm (▲), cytoplasmic (✤) and membrane-bound vacuolization (★), mitochondrial swelling (✱), and axon-myelin separations (▼) in nerves obtained from the control group. In contrast, bone marrow mesenchymal stem cell transplantation markedly attenuated these signs of injury and stimulated the recovery of myelinated (✦) and unmyelinated (✧) axons. Despite local areas of degradation (*md*), the integrity of myelin sheath was maintained, and no signs of myelin-axon detachments were observed (original magnification, × 12,000). *Scale bar* = 1 µm.

Immunohistochemical Assessment

We evaluated axonal regeneration by S-100 immunohistochemistry. The expression of S-100 was found to be significantly higher in bone marrow mesenchymal stem cell–treated animals than in untreated controls ($p < 0.001$). Thereafter, we looked at the degree of myelination using antibodies against myelin basic protein. There appeared to be more myelinated fibers in the distal nerve in animals treated with bone marrow mesenchymal stem cells. The difference was significant ($p < 0.001$). Finally, the expression levels of protein gene product 9.5 and vesicular acetylcholine transporter in the motor end plates were assessed semiquantitatively. We found that the expression levels of protein gene product 9.5 and vesicular acetylcholine transporter were significantly higher in the bone marrow mesenchymal stem cell group ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 3).

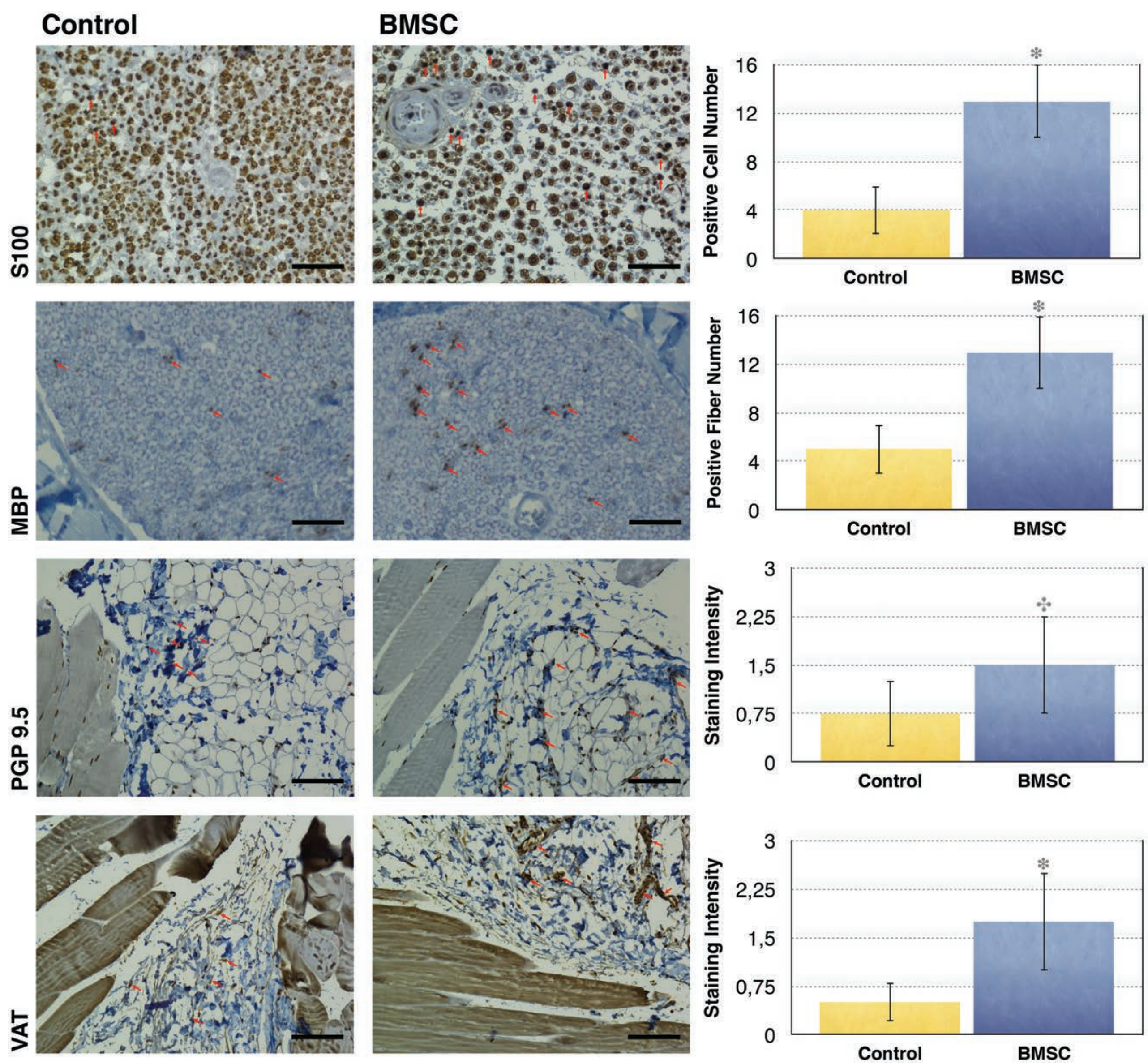

Fig. 3. Immunohistochemical assessment of the nerve. The expression of S-100 was significantly higher in bone marrow mesenchymal stem cell–treated animals. There appeared to be more myelinated fibers in animals treated with bone marrow mesenchymal stem cells. The expression levels of anti–protein gene product 9.5 and vesicular acetylcholine transporter were significantly higher in the motor end plates of the bone marrow mesenchymal stem cell group. The sample size was $n = 10$ for each group. ❆$p < 0.001$; ✣$p < 0.01$ (original magnification, × 40). *Scale bars* = 100 µm. *MBP*, myelin basic protein; *PGP 9.6*, protein gene product 9.5; *VAT*, vesicular acetylcholine transporter.

Muscle-Mass Ratio and Masson Trichrome Staining of the Muscle

The muscles in the bone marrow mesenchymal stem cell–treated group showed significantly larger average fiber area than the control group, indicating better recovery from muscle atrophy ($p < 0.01$). In contrast, there was no significant difference in the wet weight of the muscles between two groups ($p = 0.438$). [**See Figure, Supplemental Digital Content 5**, which shows evaluation of muscle atrophy. (*Left*) Masson trichrome staining of gastrocnemius muscles 3 months after replantation (original magnification, × 20). *Scale bars* = 50 μm. (*Above, right*) The graph represents the average ± SD of the fiber area. The muscles in the bone marrow mesenchymal stem cell (*BMSC*)–transplanted group showed a significantly larger average fiber area than the control group. (*Below, right*) The graph represents the wet weight ratios of gastrocnemius muscle (replanted side/healthy side) for both groups. The sample size was $n = 10$ for each group. *BMSC*, bone marrow mesenchymal stem cell. ✣ $p < 0.01$, ***http://links.lww.com/PRS/D361***.]

DISCUSSION

Although it is well known that mesenchymal stem cell application is an efficient method of overcoming the inherent limitations of nerve regeneration,[19] these limitations are amplified following limb replantation because of the extensive nature of amputation injuries. In addition to ischemic damage, reperfusion following replantation results in additional fiber degeneration through oxidative injury and profound inflammatory response.[28] Therefore, a logical strategy to promote nerve regeneration in such cases is to ameliorate these damage sources. From this point of view, mesenchymal stem cells are an attractive choice because of their ability to promote angiogenesis, modulate immune response, and reduce oxidative stress.[8]

Considering that the results of electrophysiologic and histomorphologic analyses do not necessarily correlate with the return of function,[29] functional recovery is the primary goal to be analyzed to document nerve regeneration.[30] Better functional recovery in the bone marrow mesenchymal stem cell–transplanted rats, as assessed using walking track analysis, is an important finding because it reflects the recovery of complex integrated motor, sensory, and proprioceptive functions.[31]

The compound muscle action potential amplitude is a complex function of three variables: the regenerated motor nerve fibers, their synchronization, and the size of reinnervated motor units.[32] As axonal regeneration proceeds, more muscle fibers are recruited, and their responses become increasingly more synchronized, resulting in an increase in compound muscle action potential amplitudes. Therefore, higher amplitudes after bone marrow mesenchymal stem cell transplantation represent an indirect measure of the number of regenerated motor nerve fibers. However, an important point that should be kept in mind is that compound muscle action potential amplitude may not measure the activity from all nerve fibers, but it does measure a subset of the total nerve fiber population.[33]

Axon counts from the distal stump of the sciatic nerve provided evidence that the transplantation of bone marrow mesenchymal stem cells significantly increased the number of regenerated axons. This suggests the possibility that bone marrow mesenchymal stem cells exerted their greatest effect on axonal sprouting and subsequent growth through the anastomosis site by either increasing the secretion of neurotrophic factors or decreasing inflammatory reaction and removing negative regulators of neurite outgrowth.[34–36] Our data suggest further that bone marrow mesenchymal stem cells may, in some way, inhibit the connective tissue reaction at the anastomosis site, and thereby allow more regenerated axons to breach this region. However, axon counts may not measure total nerve function because of improper end-organ reinnervation.[33] Excessive axons may be produced because of frustrated axonal growth.

The new regenerating fibers that invade the distal stump are at first thin, only later increasing in diameter as they contact their target cells.[37] Besides these regenerating axons, their parent fibers in the proximal stump also underwent a reduction in total fiber diameter.[38] It has been suggested that this reduction could be produced by the regenerated small fibers that had doubled back and grown up the proximal stump.[39] At the end of regeneration, which may take up to 6 months, both proximal and distal fibers regain their normal diameter.[38] In the bone marrow mesenchymal stem cell group, analysis of histologic sections documented an apparent predominance of small-diameter fibers. This can be explained by the occurrence of a sprouting of more than one growth cone from each severed axon, leading to an abundance of small regenerating axons that cross the anastomosis site.

The degree of myelination is an important marker of axonal maturation.[40] However, myelin thickness may not be an accurate indicator of maturity, because regenerating small axons do not have thick myelin layers. In contrast, the ratio of the inner axonal diameter to the total outer diameter, or g-ratio, is fairly constant in a normal nerve and is used as an index of optimal myelination.[41] In the bone marrow mesenchymal stem cell group, the myelin thickness is matched to axon diameter so that g-ratios fall within the optimal range. However, one should remember that myelination may not relate to nerve function because myelination generally occurs before the axons reach the end organ.[33]

Histologic evaluation of muscle atrophy is another parameter that could give information about the efficacy of reinnervation. The maintenance of muscle mass is controlled by a balance between protein synthesis and protein degradation pathways, which shifts toward degradation after denervation.[42] Under conditions of prolonged denervation, the recovery will be limited by atrophy and fibrosis of the muscle. In our study, histologic assessment of muscle specimens revealed less atrophy in the bone marrow mesenchymal stem cell–treated group. This suggests that bone marrow mesenchymal stem cells enhanced axonal regeneration, thereby preventing a condition of prolonged denervation and maintaining the muscle in a receptive status for reinnervation.

In line with previous studies, the extent of sciatic nerve regeneration was evaluated 3 months after the repair.[43–45] This timing was chosen taking into consideration the rate of axonal regeneration in rats, which is estimated to be 3 mm/day.[46] Therefore, all axotomized motoneurons would require approximately 20 days to regenerate along a distance of 50 mm in addition to a period of 1 month, which is needed for regenerating axons to cross a surgical suture site.[47] Moreover, it has been shown that rat ankle kinematics during gait seems still not to recover completely before the end of the third month.[48]

Of all the tissues found in the replanted limb, muscle tissue is the least tolerant of ischemia. Previous studies showed that irreversible muscle cell damage starts after 3 hours of warm ischemia.[49] Therefore, timing of reperfusion was synchronized such that the total ischemia time was approximately 2 hours in all animals.

Mesenchymal stem cells must be expanded in number over several weeks before transplantation, and this time-consuming process prevents autologous use in such acute cases.[50] Fortunately, the hypoimmunogenic properties of mesenchymal stem cells enables their transplantation over major histocompatibility complex barriers.[51] Therefore, ex vivo expanded cells from third-party donors is a promising approach to treat such emergency cases.

The translation of such rodent studies to human trials has been challenging, as many neuroregenerative strategies deemed to be effective in rodent models have been ineffective in humans. Although the translational barriers are still not fully understood, the physiologic and anatomical differences have been thought to be the potential reasons. Therefore, the development of primate models has the potential to bridge the translation of neuroregenerative therapies from rodent studies to human trials.

CONCLUSIONS

Our findings demonstrate that it is possible to improve the degree of nerve regeneration after limb replantation by bone marrow mesenchymal stem cell transplantation. However, the problems encountered after limb replantation are more complex, and important questions remain to be answered before these transplants become a clinically viable tool: What is the better cell source? What is the optimal dose? What is the best route of administration? What are the underlying protective mechanisms?

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