Bone marrow stromal cells (BMSCs), which are also called mesenchymal stem cells (MSCs), are a promising resource for the treatment of spinal cord injury (SCI). Their contribution to neurological functional recovery has been previously reported. BMSCs, which are harvested from bone marrow, proliferate easily and quickly. They have the potential to differentiate into neurons, but their secretion of trophic factors and cytokines is thought to be very important for neuroprotection, axonal growth, and tissue repair within the injured spinal cord. Moreover, the homing ability of BMSCs toward the injured site has been noted. Therefore, the effectiveness of the intralesional, intra-arterial, intravenous, and intrathecal routes for the administration of BMSCs to the lesion site has been previously tested.

In clinical use, it is very important when selecting treatment routes to consider invasiveness and simplicity as
well as effectiveness. Recently, the intranasal administration of BMSCs into lesioned brains of rodents has been reported. After their administration by simple drops into the nostrils, BMSCs are thought to migrate into the brains through the olfactory nerve route or trigeminal ganglion route. From the point of view of minimizing invasiveness, the intranasal route is thought to be the least invasive of all the routes mentioned above.

To the best of our knowledge, this is the first report of an evaluation of intranasal BMSC administration to the injured spinal cord.

Methods

Animals

All animals were handled in accordance with the guidelines of the Laboratory Animals Care and Use Committee of the Osaka University Faculty of Medicine.

All rats were purchased from Japan SLC, Inc. The rats were provided food and water ad libitum and kept in a 12-hour light/dark cycle throughout the study.

BMSC Isolation, Culture, and Characterization

To prepare the BMSCs, two 8-week-old enhanced green fluorescent protein (EGFP) Sprague-Dawley male rats (Japan SLC, Inc.) were used.

After the rats were deeply anesthetized by an intraperitoneal overdose injection of medetomidine hydrochloride, midazolam, and butorphanol tartrate, the femurs and tibias were aseptically removed, and the bone marrow was flushed from each piece with 10 ml of Dulbecco modified Eagle medium (DMEM, Life Technologies Corp.) with a sterile syringe. After filtering with a 100-µm cell strainer (BD Falcon, BD Biosciences), the bone marrow was spun at 300 × g for 5 minutes at room temperature.

The collected cells were placed in 75-cm² culture flasks and cultured with DMEM supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin. The next day, the culture medium was changed to remove nonadherent cells. The culture medium was subsequently changed twice a week. The cells were passaged with antibodies against CD90 (+), CD11b (−), CD31 (−), and CD45 (−) (Fig. 1). The following antibodies were applied: a mouse monoclonal antibody against CD90 (1:100, 554892, BD Pharmingen, BD Biosciences), a mouse monoclonal antibody against CD11b (1:100, ab1211, abcam plc), a mouse monoclonal antibody against CD31 (1:100, ab24590, abcam plc), and a rabbit polyclonal antibody against CD45 (1:100, ab10558, abcam plc). For secondary antibodies, an Alexa Fluor goat anti–mouse 546-conjugated IgG (1:200, Life Technologies Corp.) and an Alexa Fluor donkey anti–rabbit 546-conjugated IgG (1:500, Life Technologies Corp.) were used.

Surgical Procedures

Forty-five 8-week-old male Sprague-Dawley rats were deeply anesthetized by the inhalation of 2% to 3% isoflurane in air with a flow of 2 L/minute. A laminectomy was performed to expose the T7–8 spinal cord segments, and a crushing injury was made at that level with an SCI device (180 kdyn, 5 seconds; IH impactor; Muromachi Kikai Co., Ltd.). The wound was closed in 2 layers. Flomoxef sodium (50 mg/kg; Shionogi & Co., Ltd.) was injected subcutaneously after the surgery to prevent urinary tract infections. Animals had their bladders manually emptied twice daily until the recovery of micturition, and they were carefully inspected for weight loss and dehydration.

Because 5 rats showed hind limb movements 6 hours after surgery, they were excluded from this experiment.

The day after surgery, all rats with a score of zero on the Basso-Beattie-Bresnahan (BBB) scale (n = 40) were divided into 5 groups (n = 8), and the treatments were performed.

For the BMSC treatment groups, an injured + intranasal (i.n.) BMSC–treated group and an injured + intrathecal (i.t.) BMSC–treated group were prepared. For the control groups, an injured-only group, an injured + i.n. vehicle–treated group, and an injured + i.t. vehicle–treated group were prepared.

Intranasal Administration of Cells/Vehicle

This procedure was done by inserting a 1-ml syringe and a 27-gauge blunt needle up to 5 mm into each nostril of each rat. After anesthetization by isoflurane inhalation, as previously mentioned, the rats were held by a hand grip to immobilize the skull.

Prior to the cell treatment, the rats were given 100 U of hyaluronidase (SERVA Electrophoresis GmbH) dissolved in 40 µl of sterile phosphate-buffered saline (PBS) in both nostrils in accordance with the literature to promote permeability. Thirty minutes later, the BMSC suspension (2 × 10⁶ BMSCs in 40 µl of sterile PBS) was applied twice to each nostril with an alternating application of 10-µl drops. The vehicle-treated group was given 40 µl of sterile PBS.

Intrathecal Administration of Cells/Vehicle

While under anesthetization with isoflurane inhalation as previously mentioned, the rats were positioned with their necks bent anteriorly. The cisterna magna was punctured with a 27-gauge needle. Proper placement of the needle was indicated by the presence of CSF in the needle hub. Then, the BMSC suspension (2 × 10⁶ BMSCs in 40 µl of sterile PBS) was slowly injected over 1 minute. The vehicle-treated group was administered 40 µl of sterile PBS.

Assessment of Hind Limb Locomotor Behavior

The BBB locomotor rating scale was used to assess hind limb locomotor behavior. The rats were observed walking freely in an open field for 10 minutes with video monitoring twice a week. Half of the rats were observed for 2 weeks, and the rest were observed for 4 weeks. Blinded observers assessed the scores.

Preparation of the SCI Sections

Half of the rats in each group were randomly selected and sacrificed 14 days postsurgery, and the rest were sacrificed 28 days postsurgery. They were killed by means...
of an intraperitoneal overdose injection of medetomidine hydrochloride, midazolam, and butorphanol tartrate. They were then perfused with PBS, followed by 4% paraformaldehyde. The spinal cords were rapidly dissected and cryoprotected in 4% paraformaldehyde for 24 hours, which was followed by 30% sucrose for 24 hours. Then, tissue blocks that were 8 mm long (4 mm rostrally and caudally from the epicenter) were taken. Transverse cryosections that were 10 μm thick were prepared.

Fluorescent Immunohistochemistry for the Quantification of the BMSCs

Every third section from the epicenter in the rostral and caudal directions was selected, and 20 sections were used in each of the animals. They were coated with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; VECTASHIELD, Vector Laboratories, Inc.). The total number of BMSCs was measured by counting the EGFP and DAPI double-positive cells that were visualized with fluorescence microscopy, and the mean BMSC counts of each group were compared statistically. To confirm whether EGFP and DAPI double-positive cells were really BMSCs, immunostaining with CD90 (a mouse monoclonal antibody against CD90, 1:100, 554892, BD Pharmingen, BD Biosciences) of the other 5 spinal cord sections of every animal in the i.n. BMSC–treated group was added. An Alexa Fluor goat anti–mouse 546–conjugated IgG (1:200, Life Technologies Corp.) was used as a secondary antibody, and the sections were observed with fluorescence microscopy.

Hematoxylin and Eosin Staining for Lesion Cavity Analysis

A total of 20 sections that included every third section that was collected from the epicenter in the rostral and caudal directions were used from 20 rats that were sacrificed 28 days postsurgery. We performed hematoxylin and eosin staining to analyze the extent of the cystic lesion. The mean cavity ratio of each rat was calculated by dividing the measured injured cavity area by the entire spinal cord slice manually with ImageJ software (version 1.47; http://rsbweb.nih.gov/ij/index.html). A mean ratio was calculated for each group, and between-groups differences in the ratios were analyzed for statistical significance.

The cavity ratios and final BBB scores of all 20 rats at 4 weeks were compared with a logistic regression analysis by using JMP Pro 10 (http://www.jmp.com/en_us/software/jmp-pro.html).

ELISA of the NGF and BDNF Levels in CSF

To evaluate the neurotrophic–productive ability of BMSCs, CSF samples (100 μl/animal) from 3 groups (injured + i.n. BMSC–treated group, injured + i.t. BMSC–treated group, and the injured-only group; n = 5) were carefully collected 2 weeks after the surgery by cisterna magna puncture so that the CSF was not mixed with blood. Centrifugation was done at 9000 × g for 15 minutes, and the supernatant was used. The NGF and BDNF concentrations were examined with ELISA kits (NGF Emax ImmunoAssay System and Promega/BDNF Emax ImmunoAssay System, Promega Corp.) according to the manufacturer’s protocol.
Statistical Analyses

The statistical analyses were performed with 1-way analysis of variance (ANOVA), which was followed by a Tukey multiple comparison test with JMP Pro 10; p values less than 0.05 were considered statistically significant. The results are expressed as the mean ± standard error of the mean (SEM).

Results

BMSC Migration Into Spinal Cord Lesions

The mean BMSC counts are shown in Fig. 2E. There were no DAPI and EGFP double-positive cells in the control groups. However, DAPI and EGFP double-positive cells were observed around the lesion cavities in the i.n. BMSC–treated group at both 2 weeks (80.00 ± 29.67) and 4 weeks (94.75 ± 26.75). But there were no DAPI and EGFP double-positive cells in sections obtained 4 mm from the SCI epicenter in either the rostral or caudal direction.

The highest number of BMSCs was detected in the i.t. BMSC–treated group 14 days postsurgery (328.25 ± 48.01); this mean value was significantly greater than the mean values in any other group. In particular, the cells penetrated deeply around the lesion cavities in this group (Fig. 2B). In addition, statistically significant differences in the number of BMSCs that had migrated at 4 weeks were seen in this group in comparison with the control groups.

There were DAPI and EGFP double-positive cells with CD90 positivity, and these cells were confirmed as BMSCs in the i.n. BMSC–treated group (Fig. 2D).

Hind Limb Locomotor Functional Recovery

The BBB scores of the groups that were observed at 2 weeks (n = 8) are shown in Fig. 3A. A statistically significant increase was observed in the i.n. BMSC–treated group (7.0 ± 0.68) and the i.t. BMSC–treated group (7.5 ± 1.32) at 7 days compared with the i.t. vehicle–treated group (1.75 ± 0.73). However, at 2 weeks, significant differences were observed only between the i.t. BMSC–treated group (9.25 ± 1.53) and the control groups (the injured-only group and the i.t. vehicle–treated group) (p < 0.05).

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4 weeks (n = 4) are shown in Fig. 3B. There was no statistically significant increase in the BBB score of the i.n. BMSC–treated group after 2 weeks. The final BBB scores in the i.n. BMSC–treated, the i.t. BMSC–treated, and the i.t. vehicle–treated groups were 11 ± 2.38, 9 ± 0.58, and 3.5 ± 1.89, respectively. Finally, a significant difference was found only between the i.t. BMSC–treated group and the i.t. vehicle–treated group (p < 0.05).

Spinal Cord Lesion Cavity Ratio

The results are shown in Fig. 4. The cavity ratios in the BMSC–treated groups were smaller than those in the control groups. However, a significant difference was observed only between the i.t. BMSC–treated group (21.86 ± 2.14%) and the i.n. vehicle–treated group (38.00 ± 5.67%) (p < 0.05) (Fig. 4D). A negative correlation was found between the final BBB scores at 4 weeks and the cavity ratio in the regression analysis (R² = 0.33, p = 0.0076) (Fig. 4E).

Axonal Regrowth

According to the anti-GAP-43 immunostaining, very little to no axonal regrowth was present in the control groups (data not shown). However, some axonal regrowth was observed near the migrated BMSCs in the BMSC–treated groups (Fig. 5A).

Quantification of NGF and BDNF Levels in the CSF

As shown in Fig. 5B, the NGF concentrations (ng/ml) in the CSF of the i.n. BMSC–treated group, the i.t. BMSC–
treated group, and the injured-only group were 10.55 ± 6.42, 9.33 ± 2.70, and 14.01 ± 5.29, respectively. However, the BDNF concentrations (pg/ml) in the CSF of the i.n. BMSC–treated group, the i.t. BMSC–treated group, and the injured-only group were 75.25 ± 33.65, 44.98 ± 20.12, and 41.53 ± 18.57, respectively. There were no significant differences among the 3 groups with respect to NGF or BDNF levels in the CSF.

Discussion

The Importance of Intranasal Cell Delivery

The intranasal delivery of cells to the brain was first reported by Danielyan et al. They demonstrated it following the intranasal application of fluorescent-labeled rat MSCs or human glioma cells to naïve mice and rats. They considered 3 delivery roots: the olfactory parenchymal route after crossing the cribriform plate, the olfactory CSF route after crossing the cribriform plate, and the trigeminal CSF route through the trigeminal ganglion.

Until now, the migration of intranasally administered MSCs to a lesion and the efficacy of this treatment have been reported for a cerebral infarction model in mice, an experimental autoimmune encephalomyelitis model in mice, and a Parkinson's disease model in rats. Surprisingly, the cells have been observed in the spinal cord as well as in the cortex, cerebellum, and brainstem 4 hours after the intranasal administration in the rat model of Parkinson's disease.

According to Danielyan et al., some factors, such as BDNF, tumor necrosis factor-alpha, and interferon-gamma, are thought to stimulate and regulate BMSC migration. In their review, Joyce et al. noted that injected MSCs have the ability to home to the site of injury, as they are attracted to areas of hypoxia, apoptosis, or inflammation.

Thus we undertook this study to clarify whether BMSCs travel to injured spinal cord in an adult rat model. Several studies on SCI models have reported that most of the administered cells disappear from the lesion site within 1 month. Quertainmont et al. found no intra-
venously grafted MSCs even in lesion sections examined 7 days postsurgery, although neurological improvements were seen after 1 month. Therefore, we killed half of our group of rats 2 weeks after surgery in order to detect intrasional cells. We killed the other half of the group 2 weeks later. Thus all the rats were killed within 1 month of surgery, which is relatively early, but we predicted that there would be some neurological recovery by this point if any were going to occur. The existence of BMSCs was confirmed in spinal cord sections obtained at both time points (2 and 4 weeks after surgery) in this study. We think that these findings are important because the intranasal approach is thought to be extremely favorable from the viewpoints of invasiveness and simplicity. BMSC migration was not confirmed in sections 4 mm from the injury epicenter, where the spinal cord appeared uninjured. Although we did not clarify whether BMSCs are transferred into the brain or CNS, our study suggests that, at least locally, BMSCs will move into an inflamed area.

**Cell Proportion, Limited Functional Recovery, and Possible Treatment Options With Intranasal Cell Delivery**

The proportion of intranasally administered cells delivered to the spinal cord lesion was estimated as follows. Overall, in the sections taken from rats killed 2 weeks after surgery, except for those used for cavity evaluation, there was an average of 80 BMSCs within a 570-μm-thick cross-section. Because the diameter of the impactor tip is 2500 μm, approximately 350 BMSCs (80 BMSCs × 2500 μm/570 μm) might exist in the lesion. This accounts for 1.75% of the administered cells.

The mean BBB score of the i.n. BMSC–treated group showed significant increases as early as 1 week after surgery. However, their functional recovery was limited after 2 weeks compared with that of the i.t. BMSC–treated group. In addition, the total numbers of migrated BMSCs at both 2 weeks and 4 weeks after surgery in the i.n. BMSC–treated group were less than those of the i.t. BMSC–treated group. These findings suggest that the intrathecal application route is better than the intranasal route as far as efficiency is concerned.

In order to proceed further toward the clinical use of i.n. BMSC treatment for SCI, the timing and dosage of BMSC administration must be considered. In the present study, BMSCs penetrated to the central lesion in the i.t. BMSC–treated group in the 2-weeks section, which might influence the functional recovery. Because the i.t. BMSC–treated group had approximately 4 times as many BMSCs within the spinal cord lesion when compared with the i.n. BMSC–treated group 2 weeks postsurgery, 8 × 10^4 or more cells are recommended to be administered via the intranasal route.

With respect to the timing of the injection, Cizkova et al. reported the interesting finding of significantly greater motor functional recovery after 3 daily injections of a total 1.5 × 10^5 MSCs via an intrathecal catheter than after a single injection of 5 × 10^3 MSCs in a rat model of contusive SCI. They attributed the greater recovery not only to an increase in the number of delivered MSCs, but also to the addition of fresh stem cells during the 3 daily applications. Furthermore, they showed different results by altering the timing of the repetitive injection.

Those finding suggest, together with our present results, that repetitive injections would be preferable, and the most effective timing should be further evaluated.

**The Role of BMSCs in Tissue Protection and Functional Recovery**

BMSCs are multipotent cells that have the ability to differentiate into neurons. However, the proportions of BMSCs that differentiate or survive in CNS lesions seems small. Mezey et al. have reported that no more than 5% of grafted stromal cells differentiate into neurons within a brain lesion. Others have shown the survival of less than 1% of grafted MSCs in injured spinal cord at 1 month after treatment, without any differentiation. Therefore, it is the neurotrophins and cytokines that are secreted from BMSCs that are believed to be much more important than the cells themselves for neuroprotection, axonal regrowth, and tissue repair; and neurotrophins and cytokines effect these outcomes via paracrine signaling.

For lesion cavity reduction and functional recovery after SCI, these mechanisms seem to act comprehensively. Neuroprotection, which is caused by reducing inflammation or preventing apoptosis, might occur in the acute stage after injury. Statistically significant BBB score improvements occurred in both the i.n. and i.t. BMSC–treated groups by 1 week in our study, and might suggest this action. In addition, axonal regrowth was shown within the GAP-43–immunostained lesion sections in the BMSC-treated groups at 4 weeks in our study. Some studies have previously reported that the tissue repair in SCI after intra-
cal administration of BMSCs was due to the proliferation of nonneural tissue, including extracellular matrices that are composed of collagen fibrils. In addition, others have demonstrated that the extracellular matrix that is produced by BMSCs supports neural cell growth.

In our experiment, there was a tendency toward smaller lesion cyst size with more efficient delivery of BMSCs (i.e., greater numbers of BMSCs delivered to the lesion). Furthermore, the regression analysis of the final BBB scores and the cavity ratio clearly showed that cavity reduction had great influence on neurological functional recovery after SCI. Therefore, there was no doubt that our results also supported a tissue-sparing effect of BMSCs and their contributions to functional recovery.

**Evaluations of NGF and BDNF**

Neurotrophins, such as NGF and BDNF, are known to play a significant role in paracrine effects. NGF expression by MSCs in vitro has been shown previously, and to a lesser extent, BDNF secretion has also been confirmed.

However, Quertainmont et al. have reported increased NGF levels, determined by ELISA, within the injured spinal cord tissue after a MSC graft and have described the contribution of NGF to lesion repair. Furthermore, according to Donega et al., BDNF and NGF mRNA expression from MSCs significantly increases after co-culture with hypoxic-ischemic brain extract.

In contrast, there have been very few reports of neurotrophic factor expression in CSF after BMSC administration for CNS disease. In one report, BMSC infusion into the CSF resulted in a significant increase in neuronal density and neurite length in cultured hippocampus neurons through its trophic effects. According to Wang et al., BDNF levels in CSF were significantly higher 15 days after the intraventricular administration of BMSCs and BDNF-overexpressing BMSCs in a rat model of brain injury.

In the present study, we did not find statistically significant differences in NGF and BDNF levels in the CSF among the injured-only group and the BMSC-treated groups at 14 days. It might be worth investigating the effects of neurotrophins in an earlier period in our model.

**Conclusions**

We present a new BMSC administration route for the treatment of acute SCI. The results of our study show, for the first time, BMSC migration to the spinal cord lesions in a rat model of acute SCI. However, in order to be a viable alternative to other treatment routes, this route needs further investigation. In addition, the mechanisms underlying the effectiveness of BMSCs in neurological functional recovery should be further clarified.

**References**


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**Author Contributions**

Conception and design: Ninomiya. Acquisition of data: Ninomiya. Analysis and interpretation of data: Ninomiya. Drafting the article: Ninomiya. Reviewed submitted version of manuscript: Ohnishi. Approved the final version of the manuscript on behalf of all authors: Iwatsuki. Statistical analysis: Ninomiya. Administrative/technical/material support: Ohkawa. Study supervision: Iwatsuki, Yoshimine.

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