The mesenchymal stem cell–derived microvesicles enhance sciatic nerve regeneration in rat: A novel approach in peripheral nerve cell therapy

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BACKGROUND: The accomplishment for desired functional peripheral nerve regeneration is still challenging despite various materials and methods. The effects of local application of omental adipose mesenchymal stromal cell–derived microvesicles (MVs) on peripheral nerve regeneration were studied using a rat sciatic nerve transection model.

METHODS: A 10-mm gap of sciatic nerve was bridged with a chitosan conduit. The rats were divided into five experimental groups randomly as follows: cultured undifferentiated omental adipose-derived stromal cells, rest mesenchymal stem cell–derived MVs (c-MVs), anti-inflammatory mesenchymal stem cell–derived MVs (anti-MVs), proinflammatory mesenchymal stem cell–derived MVs (pro-MVs), and negative control (Chit).

RESULTS: The functional assessment of nerve regeneration (walking track analyses), electrophysiologic measurements, muscle mass measurements, as well as histomorphometrical and immunohistochemical indices showed drastic improvement in nerve regeneration in c-MVs and anti-MVs animals compared with pro-MVs animals ($p < 0.05$).

CONCLUSION: The anti-inflammatory stem cell–derived MVs can be used as an alternative for the improvement of rat sciatic nerve regeneration.
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KEY WORDS: Stem cell–derived microvesicles; sciatic nerve regeneration; omental adipose-derived stromal cells; rat.

Peripheral nerve injuries are common in clinical practice owing to trauma or deliberate surgical resection.[1] Autologous nerve graft is widely accepted as the most effective procedure for repairing a neural gap; however, the availability of donor nerves and donor site morbidity are major concerns.[2] Therefore, numerous surgical methods such as bioabsorbable or nonbioabsorbable conduits are being used for bridging nerve defects.[3–5] It has been approved that cellular elements are needed to provide the neurotropic and neurotrophic support for axonal regrowth.[5]

Cell transplantation, autologous Schwann cells, and stem cell therapy have been successfully used for the improvement of peripheral nerve regeneration.[6] The benefit of stromal vascular fraction[7] and cultured or uncultured undifferentiated multipotent mesenchymal stem cells (MSCs) for the treatment of peripheral nerve injuries have been identified.[8–11]

Microvesicles (MVs) are nano-sized circular membrane fragments that are produced from cells and act as shuttles for selective pattern of ligands, receptors, enzymes, cytokines, transcription factors, messenger RNA, and microRNA into target cells. After the attachment of MVs into the target cells or their internalization, various epigenetic reprogramming and phenotypic changes ensue.[12–14] Furthermore, MSC-derived MVs mostly bear receptor repertoire and donor ligand cells.[15]

Recent studies have shown that MSC-derived MVs contribute in the treatment of the injured organs or tissues.[16–18] Therapeutic application of MSCs bears some limitations.[17–21] The objective of the present study was to evaluate effectiveness of local application of omental adipose mesenchymal stromal cells (s)–derived MVs on peripheral nerve regeneration in a rat sciatic nerve transection model.

MATERIALS AND METHODS

Experimental Design

Ninety male White Wistar rats weighing approximately 220 g were randomly divided into six groups (n = 15), including the sham operation group (sham) and five treatment groups as follows: negative control conduit group (Chit), cultured undifferentiated omental adipose-derived stromal cells group (OADSCs group as a positive control) , rest MSC-derived MVs (c-MVs) group, anti-inflammatory MSC-derived MVs (anti-MVs) group, and proinflammatory MSC-derived MVs (pro-MVs) group. Each group was further subdivided into three subgroups of five animals each.

The animals were treated under the standard condition during entire experiment. All procedures were carried out in accordance with the guidelines of the ethics committee,[22] and the University Research Council approved all experiments.

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TLR Priming Protocol

Lipopolysaccharide (10 ng/mL; Sigma-Aldrich, St. Louis, MO) and poly I.C (1 μg/mL; Sigma-Aldrich) were added to OADSCs culture growth medium at 60% to 70% confluency as agonists for TLR4 and TLR3, respectively. After 1-hour incubation at 37°C and 5% CO_2 atmosphere, growth medium was refreshed without the addition of TLR agonists, and MVs were isolated from conditioned medium during next 12 hours. Short incubation and minimal concentration of TLR agonists exposure were used according to Waterman et al.,[23] to mimic the gradient of danger signals.

Isolation of MVs

Isolation of MVs was based on a modification protocol of Thery et al.[24] (2006). Briefly, 12 hours after TLR priming, collected OADSCs culture supernatant at second passage were centrifuged at 2,000 G for 20 minutes and 100,000 G for 1 hour. The resultant pellet was suspended in an acidic buffer (140-mM NaCl, 10-mM citrate, pH 4) to remove residual receptor-bound agonists. After 60 seconds, phosphate-buffered saline (PBS) was added to MVs suspension and then was centrifuged again at 100,000 G for 1 hour. The final MVs pellet was suspended in PBS. Bradford assay was used to quantify protein content of resultant MVs.[15]

Electron Microscopy

Ten microliters of MV suspension (10 μg/100 μL) was loaded on a formvar-coated copper grid. Negative staining was performed by the addition of 10 μL of neutral 1% aqueous phosphotungestic acid. The grids were examined under transmission electron microscope (Philips Bio Twin, CM100, the Netherlands) at 75 kV, and electromicrographs were taken and subjected to analyses. Analyses of MVs by electron microscope showed the presence of nano-sized, MVs (Fig. 1).

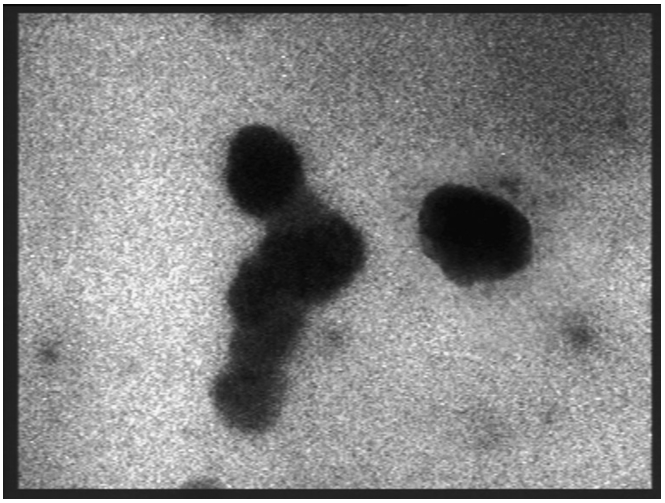

Figure 1. Structural analysis of omental adipose stromal cell–derived MVs. MVs were observed by a transmission electron microscope (Philips Bio Twin, CM100) at 75 kV. MVs are seen as nano-sized vesicles in electron micrograph.

Grafting Procedure

Animals were anesthetized using ketamine hydrochloride 90 mg/kg (Ketaset 5%, Alfasan, Woerden, the Netherlands) and xylazine hydrochloride 5 mg/kg (Rompun 2%, Bayer, Leverkusen, Germany) intraperitoneally. In the sham group, the left sciatic nerve was exposed through a gluteal muscle splitting, and following exposure of sciatic nerve, the splitted muscle was closed using 4/0 Vicryl (Ethicon, Norderstedt), and the skin using 3/0 nylon (Dafilon, B/Braun, Germany). In the treatment groups, the left sciatic nerve was exposed as mentioned before and transected proximal to the tibioperoneal bifurcation. An 8-mm segment was excised, and a gap approximately 10 mm was made because of the retraction of the nerve ends. The created stumps were each inserted 2 mm into a 14-mm chitosan conduit, and two 10/0 nylon sutures were placed at each end to fix the conduit in place. In the treatment groups, the conduit was filled with 1×10^5 OADSCs, 45-μg c-MVs, 45-μg anti-MVs, and 45-μg pro-MVs, respectively, all prepared up to 30 μL with PBS solution, accordingly. In the negative control group, the conduit was filled with 30-μL PBS solution.

The collection of omental adipose tissue and preparation of cultured undifferentiated omental adipose-derived stromal cells were based on techniques described in our pervious study.[11] The efficacy of the conduit on peripheral nerve regeneration in a rat model has been described in other previous study.[25]

At the end of the experiment, animals were anesthetized as mentioned before and were perfused via the left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) at 4, 8, and 12 weeks after surgery.

Functional Assessment of Nerve Regeneration

We adopted a walking track analysis at 4, 8, and 12 weeks after surgery based on a method described by others.[26] In brief, the lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the operated side (E) and the contralateral unoperated side (N) in each rat. The sciatic function index (SFI) in each animal was calculated using the following formula:

$$\begin{aligned} \mathrm{SFI} = & -38.3 \times (\mathrm{EPL} - \mathrm{NPL})/\mathrm{NPL} + 109.5 \\ & \times (\mathrm{ETS} - \mathrm{NTS})/\mathrm{NTS} + 13.3 \\ & \times (\mathrm{EIT} - \mathrm{NIT})/\mathrm{NIT} - 8.8 \end{aligned}$$

Overall, the SFI approximately 0 was considered for normal nerve function and around −100 SFI for total dysfunction. The SFI was assessed based on the sham group, and the normal level was considered as 0.

Electrophysiologic Measurement

After 12 weeks, the animals were subjected to electrophysiologic studies using Nacro bio system 320-3760 A trace 80. Under general anesthesia (discussed earlier), left sciatic nerve was reexposed. Single electrical pulses (at supramaximal intensity) were delivered via bipolar electrodes placed in turn at the proximal and distal trunk of the regenerated nerve cable and electromyography (EMG) was recorded by inserting an electrode into the belly of gastrocnemius muscle. The latency and amplitude of EMG were obtained. Difference in latency of EMG

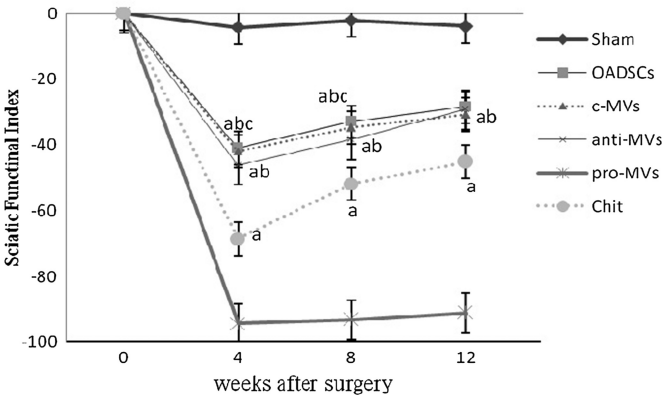

Figure 2. The functional parameters were calculated in each group (n = 5 for each group) after surgery as described in the Materials and Methods section. Data are shown as mean (SD). *A*, $p < 0.05$ versus pro-MVs group. *B*, $p < 0.05$ versus Chit group. *C*, $p < 0.05$ versus anti-MVs group.

was measured, and the distance between the proximal and distal sites of stimulation was measured to calculate the conduction velocity across regenerated nerve. On the uninjured side of each animal, similar measurements were made for the determination of conduction velocity. The conduction velocity of bridged nerve was expressed as a percentage of that on the intact side of each animal to cancel off variations between animals (%CVR).[27] The recovery index of EMG amplitude in all groups was calculated by the following formula:

Recovery index = peak amplitude of the operated side / peak amplitude of the intact side.[28]

Muscle Weight Measurement

The animals were sacrificed, and the gastrocnemius muscles were collected. The muscles were weighed, and the muscle wet weight ratio was determined by the following equation: wet weight ratio = experiment site muscle wet weight / contralateral normal site muscle wet weight × 100%.

Histologic Preparation and Morphometric Studies

Graft middle cable of sham, OADSCs, c-MVs, anti-MVs, and pro-MVs groups was harvested and fixed in 2.5% glutaraldehyde. The grafts were then embedded in paraplast paraffin, cut in 5 μm, and were then stained with toluidine blue. Morphometric analysis was performed using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD). Equal opportunity, systematic random sampling, and two-dimensional dissector rules were followed to cope with sampling-related, fiber location–related, and fiber size–related biases.

Immunohistochemical Analysis

Anti-S-100 (1:200, DAKO North America, Inc. 6392 Via Real, Carpinteria, CA) was used as a marker for myelin sheath. Specimens before immunohistochemistry were post fixed with 4% paraformaldehyde for 2 hours and embedded in paraffin. After nonspecific immunoreactions were blocked, the sections were incubated in S-100 protein antibody solution for 1 hour at room temperature. They were washed three times with PBS and incubated in biotynilated antimouse rabbit IgG solution for 1 hour. Horseradish peroxidase–labeled secondary antibody was developed by the diaminobenzidine method. The results of immunohistochemistry were examined under a light microscope.

Statistical Analysis

Experimental results were expressed as mean (SD). All data were analyzed by one-way analysis of variance to assess statistical significance between experimental groups (SPSS 17.0 for Windows, Chicago, IL). Dunnett's test for pairwise comparisons was used to examine the effect of time and treatments. The differences were considered significant when $p < 0.05$.

RESULTS

Functional Assessment of Nerve Regeneration

SFI values are presented in Figure 2. Before surgery, SFI values in all groups were near zero. After sciatic nerve transection, the mean SFI decreased to −100 because of the complete loss of sciatic nerve function in all animals. During experiment, SFI improved significantly in the c-MVs and anti-MVs groups compared with that of the pro-MVs group. This improvement was better in the c-MVs group than in the anti-MVs group at 4 weeks and 8 weeks after surgery; however, the recorded values were the same at the end of 12 weeks after surgery.

Electrophysiologic Measurement

Figure 3 shows the mean percentage of conduction velocity at the end of the experiment. The mean conduction velocity along the c-MVs– and anti-MVs–regenerated sciatic nerves was 72% CVR and 70.7% CVR of the intact right side, respectively. These were significantly higher than those of the pro-MVs group, which was 15% CVR ($p < 0.001$).

The recovery index of EMG amplitude in all groups calculated based on Suzuki et al. (1999).[27] The difference in EMG amplitude was not statistically significant between the c-MVs and anti-MVs groups, whereas EMG amplitude of these groups were significantly higher than that of the pro-MVs group ($p < 0.001$) (Fig. 4).

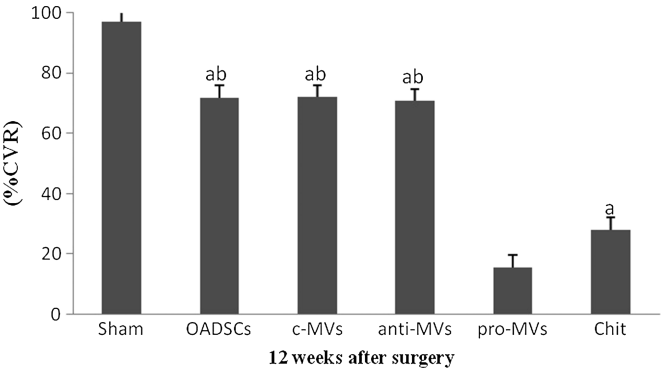

Figure 3. Percentage recovery of conduction velocity in experimental groups. Data are presented as mean (SD). *A*, $p < 0.001$ versus pro-MVs group. *B*, $p < 0.05$ versus Chit group.

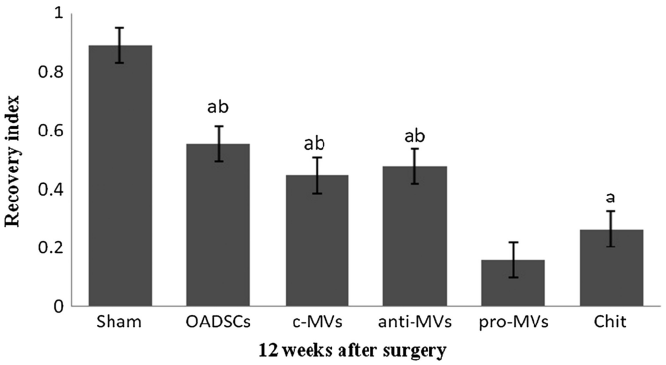

Figure 4. Bar graph shows the recovery index of EMG amplitude in experimental groups. Data are presented as mean (SD). *A*, $p < 0.001$ versus pro-MVs group. *B*, $p < 0.05$ versus Chit group.

Muscle Mass Measurement

Wet weight ratio in the sham, OADSCs, c-MVs, anti-MVs, pro-MVs and Chit groups were 97.86%, 57.58%, 41.46%, 54.89%, 23.51%, and 33.33%, respectively. There was a statistically significant difference between the muscle weight ratios of he anti-MVs group and the c-MVs and pro-MVs groups ($p < 0.05$). Moreover, the muscle weight ratio of the c-MVs group was significantly better than that of the pro-MVs group ($p < 0.05$).

Histologic Preparation and Morphometric Studies

Statistical analysis showed that 4, 8, and 12 weeks after surgery, the c-MVs and the anti-MVs groups presented significantly greater number of fibers, diameter of fibers, axon diameter, and myelin sheath thickness compared with those of the pro-MVs animals ($p < 0.005$). The number of fibers and the diameter of fibers in the anti-MVs group were significantly higher than those in the c-MVs group throughout the experiment ($p < 0.01$). Axon diameter was greater in the anti-MVs group than that of the c-MVs group; this value was significantly different at the end of 4 weeks and 12 weeks after surgery (Table 1).

Immunohistochemical Analysis

Immunoreactivity to S-100 protein was extensively observed in the cross-sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression, indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 5). In the anti-MVs and c-MVs groups, the structure and function of regenerated axons and myelin sheath were far more similar to those of the normal nerve compared with those of pro-MVs group. In the pro-MVs group, the expression of S-100 was dispersed, and the findings resembled those of the histologic evaluations.

DISCUSSION

The effect of OADSCs-derived MVs on sciatic nerve regeneration in rat sciatic nerve transection model was studied. The results showed drastic improvement of the nerve regeneration

TABLE 1. Morphometric Analyses of the Sciatic Regenerated Nerves for Each of the Experimental Groups

Weeks / Group	No. Fibers			Diameters of Fibers, μm			Diameter of Axon, μm			Myelin Thickness, μm		
	4	8	12	4	8	12	4	8	12	4	8	12
Sham	8,024 (404)	8,379 (446)	8,124 (385)	12.01 (0.01)	11.93 (0.17)	12.06 (0.23)	7.03 (0.02)	6.97 (0.39)	7.06 (0.46)	2.56 (0.01)	2.48 (0.02)	2.53 (0.01)
OADSCs	3,164 (115)***	3,870 (233)***†	5,895 (177)***†	7.95 (0.43)***	9.21 (0.28)***	10. 29 (0.59)***	3.28 (0.28)***	5. 22 (0.31)***	6.24 (0.24)***†	1.83 (0.29)***	1.73 (0.22)***	1.82 (0.19)***
c-MVs	2,987 (155)***	3,492 (247)***	5,291 (193)***	7.29 (0.79)***	8.94 (0.43)***	9. 82 (0.39)***	2.87 (0.34)***	4. 91 (0.28)***	5.69 (0.24)***	1.71 (0.30)***	1.74 (0.21)***	1.81 (0.24)***
Anti-MVs	3,675 (167)***†‡	4,589 (261)***†‡	6,732 (158)***†‡	8.34 (0.19)***†	9.45 (0.21)***†	10.37 (0.25)***†	3.39 (0.24)***†	5.24 (0.25)***	6.47 (0.22)***†	1.87 (0.12)***	1.91 (0.14)***	1.96 (0.11)***
Pro-MVs	865 (141)	1,156 (236)	1,275 (119)	2.23 (0.15)	2.43 (0.18)	2.36 (0.24)	2.02 (0.22)	2.14 (0.22)	2.17 (0.25)	0.11 (0.02)	0.14 (0.02)	0.12 (0.04)
Chit	1,378 (176)*	2,673 (215)*	3,024 (198)*	2.83 (0.18)*	4.51 (0.24)*	4.86 (0.19)*	2.12 (0.22	3.74 (0.23)*	3.77 (0.21)*	0.41 (0.02)*	0.43 (0.03)*	0.63 (0.03)*

*$p < 0.05$ versus pro-MVs group.
**$p < 0.05$ versus Chit group.
†$p < 0.05$ versus c-MVs group.
‡$p < 0.05$ versus OADSCs group.
Values are given as mean (SD).

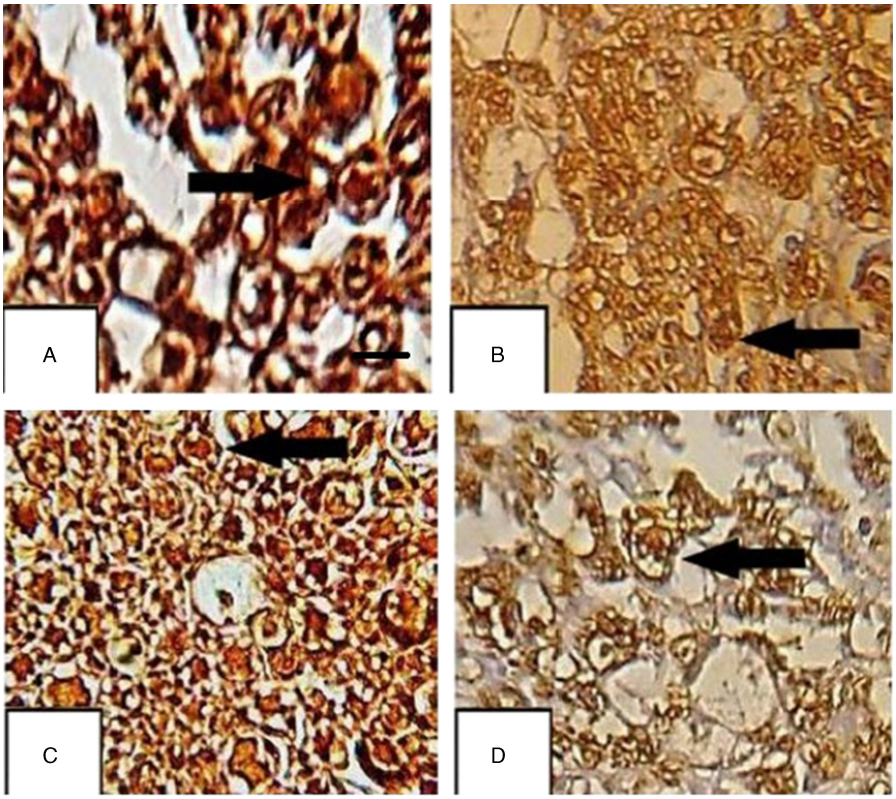

Figure 5. Immunohistochemical assessment of the nerves 12 weeks after the operation from midpoint of OADSCs (*A*), c-MVs (*B*), anti-MVs (*C*), and pro-MVs (*D*) . Positive staining of the myelin sheath–associated protein S-100 (*arrows*) around the nerve fiber is shown. This indicates a well-organized structural nerve reconstruction in c-MV– and anti-MV–treated nerve compared with that of the pro-MVs. Scale bar, 10 μm.

in the c-MVs and anti-MVs groups compared with that of the pro-MVs group.

Walking track analysis is a comprehensive test that has been used widely for evaluating the recovery of motor function as a result of posttraumatic regeneration of peripheral nerve in rats.[29] The results of the present study showed that the rest, anti-inflammatory phenotype of MVs and OADSCs when loaded in a chitosan conduit ended up having a faster and significant improvement in the functional recovery of the sciatic nerve in the course of time.

The conduction velocity is an objective and reliable index for the evaluation of the conduction of action potential in peripheral nerves.[5] Only when enough regenerated nerve fibers grow across the nerve gap to innervate the distal target muscle can compound muscle action potential be measured. Moreover, the conduction velocity depends on the diameter of axons, the thickness of the myelin sheath, and the length of internodes.[30] The amplitude of the EMG is directly proportional to the number of nerve fibers innervating the muscle that allows the conduction velocity of the motor nerve to be calculated. EMG examinations offer an essential index for the conduction function of the peripheral nerve.[31] In the present study, the recovery index of EMG amplitude and the mean percentage of conduction velocity showed that animals in c-MVs, anti-MVs and OADSCs groups had substantial improvement in nerve regeneration. However, the indices were lower in the pro-MVs group. In fact, the maximum recovery index (0.55) and conduction velocity rate (72%) in this group might be correlated with multiple factors such as the smaller diameter of the regenerating axons, thinner myelin sheaths with shorter internodes, and immaturity of myelinated nerve fibers as a whole,[30,31] which was consistent with the morphometric findings.

As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation.[32] In the present study, greater mean gastrocnemius muscle weight ratios in OADSCs, c-MVs, and anti-MVs groups indicated indirect evidence of successful end-organ reinnervation. The mean greater muscle weight ratio in the anti-MVs group may suggest the better effect of the anti-MVs in the treatment of the injury.

In general, the significantly higher mean number of the regenerated nerve fibers in the anti-MVs group compared with the other treatment groups indicated an additional beneficial effect of the anti-MVs in the nerve regeneration compared with those of the OADSCs and c-MVs group. The mean myelin sheath thickness in the anti-MVs, OADSCs, and c-MVs groups was higher than that of the pro-MVs group. Meanwhile, the mean myelin sheath thickness showed no significant difference among the anti-MVs, OADSCs and c-MVs groups and was in favor of the anti-MVs group. The mean axon and fiber diameter in the anti-MVs, OADSCs, and c-MVs groups was greater than

that of the pro-MVs group. Meanwhile, there was a significant difference between the anti-MVs and the c-MVs groups, and this value was in favor of the anti-MVs group compared with the OADSCs group. It seems that the anti-MVs achieved faster improvement in the regeneration of the injured nerve.

In immunohistochemistry, the expression of axon and myelin sheath special proteins was evident in all groups, which indicated the normal histologic structure. The location of reaction to S-100 in the OADSCs, c-MVs, and anti-MVs groups was clearly more positive than in the pro-MVs group, further implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of myelination and the structural recovery of regenerated nerve fibers.

The beneficial effect of MSC transplantation in the regeneration of peripheral nerve injuries have been shown in several studies.[10,11] Recently, as a main mechanism involved in regeneration properties, the ability of these cells to secrete a wide variety of trophic molecules (IGF, HGF, EGF, VEGF, LIF, etc.) has been more highlighted than transdifferentiated cells.[13,19]

The early death of transplanted cells, functional frustration, risks of aneuploidy or teratoma formation, and immune rejection following in vivo allogeneic administration are described to be the main causes of defaulted and limited MSC-based therapies.[17,20,21]

The maintenance and enhancement of MSC reparative potency via the therapeutic application of MSC-derived MVs and TLR-primed MSC-derived MVs can be considered as an alternative approach to override the current defaults in stem cell therapy.[17,23] The regenerative efficacy of the resting MSCs and their derived MVs has been shown. These MVs have been used to reverse fulminant hepatic failures,[16] to protect against acute tubular injury[17] and to reduce myocardial ischemia/reperfusion injury.[18] Two phenotypes of MSCs, MSC-1 and MSC-2, were found based on the different micro environmental signals. Accordingly, MSC-1, the reparative/anti-inflammatory phenotype, may contribute to early tissue injury responses while MSC-2, proinflammatory phenotype, may contribute to late tissue resolution responses and wound healing.[23] Among a series of TLRs that may be expressed on MSCs, TLR3 and TLR4 have been shown to be potent modulators of stem cell phenotypes. Polarization of MSCs into different phenotypes can result from different concentration and times of ligands/agonists exposure to the MSCs.[23] In the present study, we used lipopolysaccharide and poly I.C as agonists of TLR4 and TLR3 for the isolation of the MVs.

It has been proposed that the composition and function of MVs depend on the cells from which they originate. MVs facilitate cell-to-cell communications and, thus, can alter cell activities in target cells.[17] Recent studies have demonstrated that the vesicles released from cells may be implicated in cell-derived trophic effects.[18,33] MVs act as shuttles for selective pattern of enzymes, cytokines, and trophic molecules at both protein and messenger RNA levels. Their attachment or fusion in peripheral cell membrane may cause cell regeneration or genetic reprogramming, inhibition of inflammation, and enhancement of angiogenesis.[18,34] Reportedly, MVs participate in myelin formation[35] and in neurite outgrowth and neuronal survival.[36] MVs can transfer bioactive molecules and deposition of packaged bioactive effectors such as specific genes, small organelles, or a cocktail of cytokines from MSCs to the injured tissue.[17] The direct and indirect role of anti-inflammatory cytokines in nerve regeneration with different effects are also reported.[37]

The results of the present study showed further improvement of nerve regeneration of animals in the c-MVs and anti-MVs groups compared with the pro-MVs group. Meanwhile, faster improvement of nerve regeneration occurred in animals that received MVs derived from polarized cells by TLR3 agonist (anti-inflammatory phenotype). It seems that c-MVs, anti-MVs, and pro-MVs by attaching to or fusing on a Schwann cell membrane caused alteration in the cell biologic function, which resulted in the improvement or impeding of the nerve regeneration. The authors have not provided the histologic and molecular evidences for the exact mechanisms of the MVs achievement, which may be considered as a limitation of this study. Meanwhile, OADSC-derived MVs, as an effective and safe cell-free therapeutic approach, could have clear advantages of stimulating cell proliferation in regenerative medicine. It is possible to prepare them from allogeneic donors in advance, preselected and screened with large quantity and controlled quality, which is able to reproduce the beneficial effects of their cellular counterparts in tissue regeneration.[38]

In conclusion, the results of the present study indicated that the anti-inflammatory stem cell–derived MVs can be used as an alternative to omental adipose-derived MSCs for the improvement of rat sciatic nerve regeneration.

AUTHORSHIP

A.R. performed the surgical procedure and data collection. S.A. designed this study and wrote the manuscript. N.D. conducted the immunohistochemical analysis. B.H. performed the electrophysiologic study. A.A.F. performed the electron microscopic study. K.A. performed the data analysis.

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DISCLOSURE

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