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RESEARCH

The effects of mesenchymal stem cells applied during the subacute period in peripheral neuropathy

Periferik nöropatide subakut dönemde uygulanan mezenkimal kök hücrelerin etkileri

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Abstract

Purpose: The study aims to investigate the effect of bone marrow-derived mesenchymal stem cells (BM-MSCs) administered subacute period to neuropathic mice on allodynia and nerve-muscle tissue functions during 24 weeks.

Materials and Methods: Peripheral neuropathy was induced by partial sciatic nerve ligation. Experiments were conducted in Control, Sham, Neuropathic, BM-MSC, and Neuropathic+BM-MSC groups. Allodynia was measured by cold plate test at the 2nd, 6th, and 24th weeks. Electrophysiological and histopathological examinations were performed on isolated nerve-muscle tissues at the end of the 24th week.

Results: Allodynia threshold increased in the Neuropathic+BM-MSC group (7.76±0.33 sec) from the 6th week and continued to increase along 24 weeks compared to the Neuropathic group (4.36±0.21 sec). Action potential (137.9±7.85 mV) and depolarization (0.74±0.01 msec) values of the Neuropathic+BM-MSC group exhibited partial improvement compared to the Neuropathic group (121.5±3.03 mV and 0.81±0.02 msec, respectively) at the 24th week. Muscle tissue's resting membrane potential values increased in the Neuropathic+BM-MSC group compared to the Neuropathic group (-73.4±0.2 and -87.7±0.2 mV, respectively). Histopathological examination of nerve tissue revealed loss of myelinated axons and significant fibrosis in the endoneurium in the Neuropathic group while Schwann cell proliferation and preservation of myelinated axons were observed in the Neuropathic+BM-MSC group. Muscle fiber atrophy, compensatory hypertrophic fibers, and increased central nuclei were seen

Öz

Amaç: Farelerde periferik sinir hasarı oluşturulduktan sonra subakut dönemde uygulanan kemik iliği kökenli mezenkimal kök hücrelerin (Kİ-MKH) 24 haftada nöropatik ağrı ve sinir-kas dokusu fonksiyonları üzerindeki etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Farelerde periferik nöropati, siyatik sinir parsiyel ligasyonu yapılarak oluşturuldu. Deneyler Kontrol, Sham, Nöropatik, Kİ-MKH, Nöropatik+Kİ-MKH gruplarında gerçekleştirildi. Allodini, 2.,6. ve 24. haftalarda soğuk plak latens testi ile ölçüldü. 24. haftanın sonunda tüm gruplardan izole edilen sinir-kas dokularında elektrofizyolojik ve histopatolojik incelemeler yapıldı.

Bulgular: Allodini eşiğinin Nöropati+Kİ-MKH grubunda (7.76 ±0.33 sn) nöropati grubuna (4.36±0.21 sn) göre 6. haftadan itibaren arttığı ve 24 hafta boyunca devam ettiği gösterildi. 24. haftada izole edilen sinir dokusunun elektrofizyolojik ölçümleri sonucu, Nöropati+Kİ-MKH grubunun aksiyon potansiyeli (137.9±7.85 mV) ve depolarizasyon (0.74 ± 0.01 ms) değerlerinin nöropati grubuna göre (sırasıyla; 121.5 ±3.03 mV ve 0.81 ± 0.02 ms) kısmi iyileşme gösterdiği belirlendi. Aynı şekilde kas dokusunun istirahat membran potansiyel değerlerinin Nöropati+ Kİ-MKH grubunda nöropati grubuna göre (sırasıyla; -73.4±0.2 ve -87.7±0.2 mV) artmış olduğu ölçüldü. Sinir dokusunun histopatolojik incelemesi sonu nöropati grubunda sinir liflerinin miyelinli aksonlarında kayıp ve endonöryumda belirgin fibrozis, Nöropati+Kİ-MKH grubunda ise Schwann hücre proliferasyonu, sinir liflerinin miyelinli aksonlarının korunduğu görüldü. Kas dokusunun histopatolojik incelemesinde Nöropati grubunda kas lifi atrofisi, kompansatuar hipertrofi lifleri ve

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in the Neuropathic group, while small atrophic muscle fiber groups were identified in the Neuropathic+BM-MSC group.
Conclusion: BM-MSC application in the subacute period is found to reduce allodynia and provide functional recovery in nerve-muscle tissue in experimental peripheral neuropathy.
Keywords:. Mesenchymal stem cell, peripheral neuropathy, pain

artmış iç çekirdekler görülürken, Nöropati+ Kİ-MKH grubunda küçük atrofik kas lifi grupları belirlendi.
Sonuç: Periferik nöropatide, subakut dönemde Kİ-MKH uygulamasının, nöropati grubuna göre hem nöropatik allodiniyi azaltma hem de sinir-kas dokusunda daha fazla fonksiyonel iyileşme sağladığı tespit edildi.

Anahtar kelimeler: Mezenkimal kök hücre, periferik nöropati, ağrı.

INTRODUCTION

Peripheral nerve damage is a significant clinical condition that can arise from various causes such as falls, vehicle accidents, cuts, tumors, and cystic formations[1,2]. Patients may experience persistent and severe symptoms including significant pain, motor function impairments, as well as signs such as muscle weakness, reflex changes, and numbness. The complete recovery of these symptoms depends on the type of nerve damage and the intervention time[2]. The manifestations resulting from peripheral nerve damage lead to a deterioration in the patient's quality of life, an inability to meet individual needs, and a weakening of social relationships[3]. Various palliative care practices, medications, and surgical methods are used to promote nerve regeneration, establish new connections between nerve fibers, and correct symptoms in peripheral nerve damage, but desired success is often elusive[4]. The prolonged duration of treatment methods, their side effects, and the inability to achieve the desired success prompt scientists to explore new treatment options.

In recent years, stem cell applications have provided hope for the treatment of various neurodegenerative diseases such as Parkinson's, stroke, amyotrophic lateral sclerosis, traumatic brain injury, and peripheral neuropathy[,6]. The application of stem cells in neurodegenerative diseases has been shown to be a powerful force in promoting regeneration in damaged tissue and restoring functions. Therefore, studies on stem cell applications for the treatment of peripheral neuropathy and neuropathic pain have gained importance. Mesenchymal stem cells are among the best options among stem cell groups to be applied in the treatment of neuropathic pain[6,7]. Mesenchymal stem cells are preferred in treatment due to their autotransplantation capability, easy obtainability from various sources such as bone marrow, adipose tissue, and umbilical cord, rapid and easy proliferation in *in vitro* and *in vivo* environments, the ability to differentiate into many tissue structures (blood, adipose tissue, osteocytes, connective tissues, chondrocytes, hepatocytes, myocytes, cardiomyocytes, and neurons), genetic stability, and non-teratogenic properties[6-8]. Additionally, mesenchymal stem cells are described as a "drugstore," releasing many factors that play a role in trophic and immunomodulatory functions. These released factors are considered important actors for the healing of damaged tissue 7[,9-11]. With all these characteristics, the ability of mesenchymal stem cells to differentiate into various neuron cells, promote synaptic transmission and regulate the microenvironment, induce angiogenesis, and prevent apoptosis makes them preferable in the treatment of peripheral neuropathy[7,11]. The results obtained from mesenchymal stem cell applications in peripheral neuropathy may depend on factors such as the source of the stem cell, the culture environment in which it is produced, the method of application, and the role of the applied cell quantity. Another crucial factor determining the regenerative effect of mesenchymal stem cells is the timing of their application after the development of the lesion[11].

In the conducted studies, it was determined that the regenerative effects of mesenchymal stem cells administered late after nerve injury were not sufficient. Specifically, it has been shown that treatment with stem cells administered within the first 4 days after injury yields better outcomes [11]. However, this application period for stem cells does not fully coincide with clinical applications. In clinical applications, it is seen that the process of obtaining and applying stem cells after nerve injury is usually more than 4 days.

This study aimed to investigate the effects of mesenchymal stem cells administered during the subacute period (two weeks after surgery) on nerve-muscle functions and tissue regeneration in mice with induced peripheral neuropathy, using electrophysiological and histopathological examinations. In addition, the pain threshold was

evaluated by cold plate latency test. This study provides a different perspective by evaluating the time-dependent changes in the positive effect of late-administered stem cells on nerve function recovery, which is consistent with clinical processes. Furthermore, it contributes to the elucidation of the mechanisms of action of late-administered mesenchymal stem cells in the literature.

MATERIALS AND METHODS

Experimental design

We carried out our study in the laboratory of Çukurova University Medical School, which was approved by 2011/17 Ethics Committee number. 35 adult male and 3 female Swiss Albino mice ranging between 25-30 gr were used for this experiment. Mice were brought a week before the experiment and were kept in a climate-controlled laboratory environment at 25^0C on a 12-hour light/dark cycle. Standard laboratory procedure and MSERAC ethical guidelines were followed, and mice had access to added libitum food and water.

Mice were divided into five groups; control (C, n=10), sham (S, n=10), peripheral neuropathy (NP, n=10), bone marrow mesenchymal stem cell, and peripheral neuropathy plus bone marrow mesenchymal stem cell (NP + BMMSC, n=15) groups. There was no surgical intervention for the control group. In the Sham group, an incision of about 1 cm was applied to the biceps femoris, and the incision site was sutured with 4.0 silk without the involvement of the sciatic nerve. Animals in the NP group received partial sciatic nerve ligation to create neuropathy, these animals were tested for allodynia utilizing the cold plate test after 2 weeks. Another group of animals received BMMSC application in the lesion area, a period of 4 weeks elapsed to allow the regenerative effect of BMMSC to take place. During this experiment, all mice were given cold-plate latency (CPL) tests in weeks 2, 6, and 24. On week 24, a total of 10 mice were sacrificed, and we used 5 mice for electrophysiological measurements and another 5 for histopathological studies.

Peripheral neuropathy surgery

Partial nerve tight ligation of the sciatic nerve was performed to produce neuropathy as described previously in rats by Seltzer et al. (1990)[12]. A partial sciatic nerve ligation (PSL) method was used to create neuropathic pain in mice. Mice were anesthetized prior to the procedure with an intramuscular injection of ketamine (80 mg/kg) and xylazine (2.5 mg/kg). Anesthetized mice under aseptic conditions received a 1cm (back of the sciatic nerve between ½ to ½ section) incision of the biceps femoris muscle at thigh level to dissect the muscle to expose the sciatic nerve. After this procedure tissue below the sciatic nerve was cleaned and stretched outside the body using inclined pliers and knotted with 4.0 silk suture according to the model provided. The incision was closed using a 4.0 silk suture.

Mesenchymal stem cell culture

Bone marrow was obtained from 3 male mice using tibia and femur bones utilizing density-gradient method. After this step, $1x10^5$ cells/mL cells were cultured in T25 flasks using MesenCult Expansion Kit (Stemcell Technologies, Vancouver, Canada) which is specified for mouse cells. When about 90% confluence, cells were trypsinized and recultured for expansion along three passages. After passage 3rd passage, the obtained mesenchymal cells were labeled with Vybrant Dil Cell-Labeling Solution (Invitrogen, USA). Before injection, cells in mice were tested for viability and suspended with $2x10^5$ live cells per milliliter. A number of cells were used for identification by flow cytometry current analysis and to show adipogenic/osteogenic differentiation. Cells were cultured with MesenCult Osteogenic Stimulatory Kit (Stemcell Technologies, Vancouver, Canada) for osteogenic differentiation, MesenCult Adipogenic Differentiation Kit for adipogenic differentiation. For cytometry, current cells were marked with CD34, CD45, CD73, CD105 during analysis. Oil Red-O staining was used for adipogenic differentiation detection; Alizarin Red-S staining was used for osteogenic differentiation[13].

Mesenchymal stem cell transplantation

Partial sciatic nerve ligation was conducted on test animals and a period of 2 weeks was allowed to elapse to attain peripheral neuropathy. After this period 15 mice were injected with $2x10^5$ MSC signed by *Vybrant Dil* in 100 µl of suspension around the damaged sciatic nerve. Five of the MSC group animals were killed by cervical dislocation 15 days after administration and sciatic nerve-muscle preparation was isolated and examined for migration of the MSC to the damaged region.

Assessment of allodynia

Allodynia is an important sign of neuropathy. Allodynia can lead to the triggering of a pain response from stimuli that do not normally provoke pain. Mechanical and thermal stimulants can be used to evaluate allodynia, the most common being cold stimuli to measure reactions in test animals. In our study, the cold-plate latency test (CPL) measured the chronic pain associated with cold allodynia[14]. The cold-plate test was carried out using a hot/cold-plate apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy). The temperature of the cold-plate was kept at 5 ± 0.5°C. Each mouse was placed on the metal surface of the apparatus. The latency to first withdrawal behavior (lifting, shaking, licking of one hind paw, or jumping) was measured and recorded as cold-plate latency (CPL). Each mouse was used for one experiment. A maximum cut off time of 120 seconds was used to prevent tissue damage at the lower temperatures.

Electrophysiological study

An intracellular microelectrode recording system was used in the electrophysiological study. In our experiments, the right leg soleus muscles were perfused with oxygenated Krebs solution (37 °C, gassed with 95% O_2 and 5% CO_2 at a constant flow rate of 4 mL/min). After 90 minutes of incubation, electrophysiological measurements were performed using borosilicate capillary glass microelectrodes (outer diameter 1.2 mm, internal diameter 0.69 mm) to ensure that the tissue samples were stabilized. The outer tip of the pulled microelectrode was measured as 0,5-0.6 μm (10-15 megaohms) with a microscope scale. They were filled with 3 M KCl (tip resistance, 10-15 MΩ), which were placed in a microelectrode holder. The microelectrode was coupled by an Ag/AgCl junction to a high-impedance capacitance neutralizing amplifier (Nihon Kohden Model MEZ-7200, Tokyo, Japan). An agar-bridge containing 3 M KCl was used as a reference electrode. The resting membrane potential was monitored continuously on a storage oscilloscope (VC-6045; Hitachi Denshi Ltd, Tokyo, Japan).

Histopathologic preparation

Soleus muscle examples from each group of mice were flash-frozen with liquid nitrogen and frozen samples were taken. Hematoxylin-eosin paint was used for routine histopathological inspection in samples. Special histochemical enzyme paints: 1-ATPase (pH 9,4 and 4,3) for determination of muscle fiber distribution, denervation, and reinnervation. 2-NADH-TR: for determination of myofibrillar. 3-Nonspecific esterase: to show enzyme increase. 4-Cytochrome oxidase and succinyl dehydrogenase: to view mitochondrial structure and distribution. Sciatic nerve samples from animals were identified in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0,1 M a cacodylate) during a 2-hour period at room temperature. Tissue samples were washed with %1 M cacodylate buffer and identified in %1'lik osmium tetroxide during a 2-hour period for a second time. Dehydration was conducted with an increasing degree of ethyl alcohol. Tissue was buried in Epon and blocks were prepared. 1-micron transverse samples were obtained with ultramicrotomy. The painting process was completed with a toluidine bath and tests were conducted with a light microscope.

Statistical analysis

The data were analyzed using GraphPad Prism 5 software. The one-way analysis of variance (ANOVA) was performed to determine potential differences among the groups. In addition, a *t*-test was used to determine the difference between the two groups. Significance was determined with a P-value of less than 0.05 ($P < 0.05$). The results were evaluated using mean and standard deviation values (X± SD).

RESULTS

Analysis by flow cytometry of bone marrow cell culture was utilized to define immunophenotypical BMMSC attained during the 3rd. passage. CD34 and CD45 expressions were negative, and CD73 and CD105 expressions were positive. Adipogenic differentiation of these cells was demonstrated by Oil Red-O and osteogenic differentiation was demonstrated by Alizarin Red-Thus, cells derived from cell culture were defined as BMMSC (Data not shown).

Following surgical procedures, a cold-plate latency test was performed in weeks 2, 6, and 24 to detect allodynia in control, sham, neuropathy (NP), and neuropathy + mesenchymal stem cells (NP + BMMSC) groups. The allodynia values in the control group, sham group, and the bone marrow mesenchymal cells (BMMSCs) group were similar.

Sensitivity to pain in the control group was measured at 9.44±0.39, 10.87±0.58, and 11.54±0.66 seconds respectively during these weeks. In the sham group was measured at 9.42±0.32, 9.87±0.58, and 10.14±0.66 seconds respectively. In the BM-MSCs group was measured at 9.40±0.39, 11.07±0.58, and 11.54±0.36. Measurements made in the NP group during weeks 2, 6, and 24 were 5.77±0.30, 4.36±0.21, and 4.14±0.2 seconds, respectively (Figure 1). According to these results, the sensitivity of the control, sham, and BMMSCs groups was similar to pain. The NP group demonstrated increased sensitivity to pain beginning in the 2nd week and this sensitivity continued throughout the experiment. In the NP + BMMSC group, the same procedure was followed with the cold plate latency test, and 5.39±0.27, 7.76 ±0.33, and 7,93±0,26 values were observed respectively. After the BMMSC application, the values observed in the NP + BMMSC groups show us that susceptibility to pain in the 2nd week did not change although, in the 6th week, there was a reduced degree of pain and lesser susceptibility to pain. Compared with the control group in the same process, it is seen that there is partial improvement in pain ($p<0.05$). BMMSC's effect on mice with neuropathy reveals that healing of pain is not temporary, but rather permanent, as it lasted up to the 24th week.

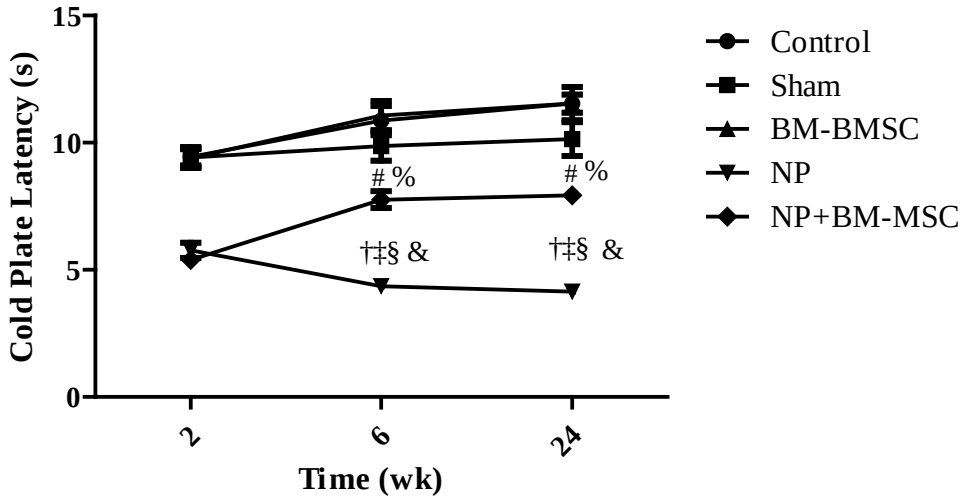

Figure 1. Effect of BM-MSC on neuropathic allodynia at time-dependent (n=10). Values represent mean ± SEM. Results were statistically compared by ANOVA, and Bonferroni as post hoc test: † p < 0.05 vs control; ‡ p<0.05 vs sham, § p<0.05 vs BM-MSC, & p<0.05 vs NP+BM-MSC, and # p < 0.05 vs control; % p<0.05 vs BM-MSC.

After the 24th week, sciatic nerve samples taken from sacrificed mice were electrophysiologically evaluated. The electrophysiological values in the control group and the sham group were similar. The peak-to-peak amplitude of single muscle fiber action potential (Vp-p) of control, BMMSCs, NP, and NP+BMMSC groups were 144.7±4.2, 143.6±3.9, 121.5 ±3.03, and 137.9±7.85 mV, respectively (Figure 2A). The peak amplitude of action potential from the NP group was found to decrease as compared with other groups ($p<0.05$). NP + BMMSC group Vp-p value recovered partly compared to the neuropathy group and reached a closer percentile (95%) value similar to the control group ($p<0.05$). Depolarization times (DT) of the same group were measured at 0.64 ± 0.01, 0.65 ± 0.04, 0.81 ± 0.02, and 0.74 ± 0.01 milliseconds, respectively (Figure 2B). Repolarization durations (RT) were measured at 1.65±0.03, 1.68±0.02, 2.33±0.07 and 2.28±0.03 milliseconds, respectively (Figure 2C).

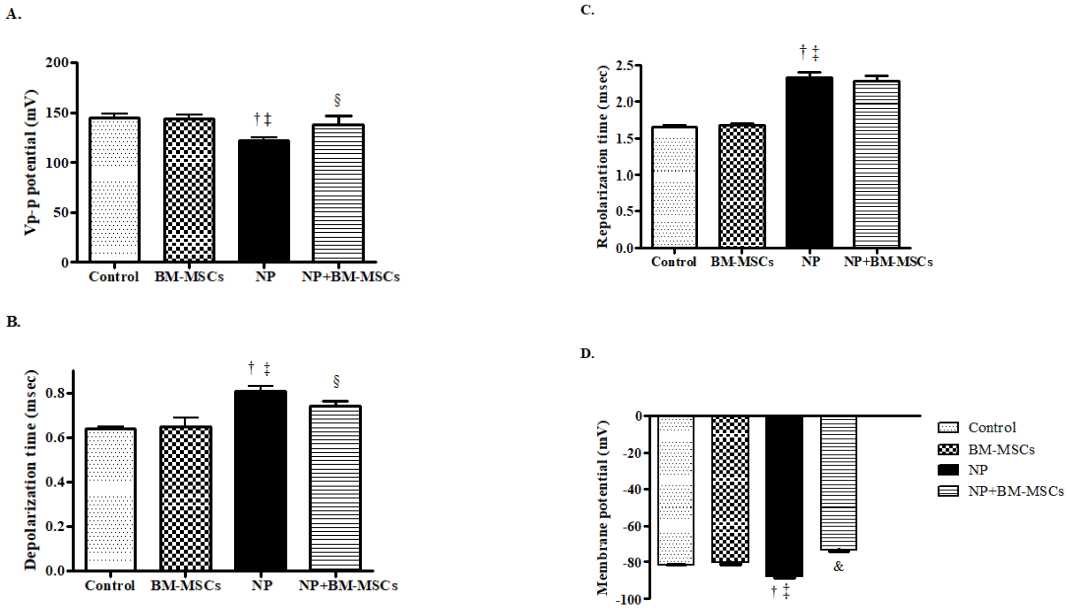

Figure 2. Electrophysiological evaluation of the effect of bone marrow mesenchymal stem cells transplant on soleus muscle tissue. Peak to peak potential (A), depolarization time (B), repolarization time (C), resting membrane potential (D) in soleus muscle. Five different donors were evaluated and the experiment was performed in triplicate.

Values represent mean ± SEM. Results were statistically compared by ANOVA, and Bonferroni as post hoc test: † $p < 0.05$ vs control; ‡ $p<0.05$ vs BM-MSC and § $p<0.05$ vs NP (A,B,C). † $p < 0.05$ vs control; ‡ $p<0.05$ vs Sham, § $p<0.05$ vs BM-MSC, & $p<0.05$ vs control (D).

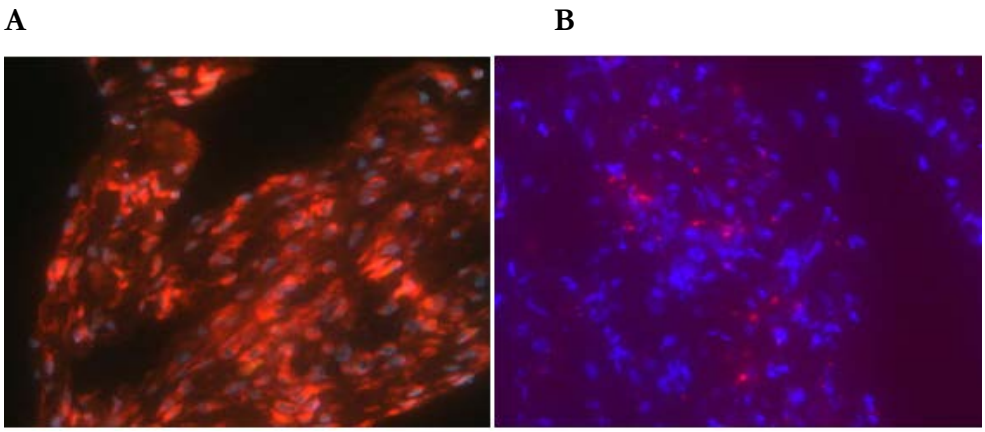

Figure 3. Evaluation of Dil-labeled bone marrow mesenchymal stem cell transplant on the sciatic nerve and soleus muscle tissue in neuropathic mice (n=5). Dil-labeled BM-MSCs emitted red-fluorescent in the degenerate middle part of nerve fiber and soleus muscle (X200 in A, B).

Both depolarization and repolarization times were prolonged in animals with NP ($p<0.05$). NP+BMMSC group depolarization time showed partial improvement compared to the NP group ($p<0.05$), but no improvement was observed in repolarization time.

At the end of the 24th week, the potential values of the soleus muscle membrane of sacrificed mice from NP and NP+BMMSC group were measured at -81.3±0.3, -80.2±0.2, -87.7±0.2 & -73.4±0.2 mV respectively (Figure 2D.). The potential value of the soleus muscle membrane in the NP group showed an increased value and hyperpolarization was evident. NP+BMMSC group potential value of the muscle membrane was in line with the control group determined value ($p<0.05$).

BMMSC was marked with *Vybrant Dil (*1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine*) Cell-Labeling Solution* (İnvitrogen, USA) and injected into the muscle in the lesional region of mice with sciatic nerve ligation. After 15 days, mice were sacrificed, and sciatic nerve and soleus muscle preparation were removed and analyzed by immunofluorescence microscopy. This examination revealed that marked BMMSC cell migration was concentrated in the central portion of the degenerated nerve fiber. Only a small number of cells were found in the nerve's end portion and muscle (Figure 3 A, B). These results indicate that the transplanted BMMSC cells intensely migrate and localize in the lesioned nerve area.fter the 24th week, the soleus muscle sample taken from sacrificed mice was histopathologically examined under a microscope. No significant pathology was observed in the endomysium and vein of striated muscle fibers of the control and BMMSCs group which was painted with HE and NADH-TP. In addition, it was observed through NADH-TP paint that the myofibrils were preserved. Due to intense enzyme activity, this process showed that dark stained fibers feature type 1 muscle fibers and lightly stained fibers feature type 2 muscle fibers (Figure 4 A, B). In the NP group, muscle sections painted with HE and ATPase showed a large group of muscle fiber atrophy and increased internal nuclei. Compensatory hypertrophy in atrophic fibers was detected in muscle fiber fascicle neighbors. In addition, muscle fiber type grouping was determined in ATPase paint. NADH-TP paint showed that dark stained fibers feature typed 2 muscle fibers and lightly stained fibers feature type 1 fibers. Atrophic muscle fibers were found to be characteristic of both type 1 and type 2 muscle fibers. (Figure 4 C). In the NP+BMMSC group striated muscle fibers painted with HE, NADH-TR showed atrophy in the sparsely scattered angular muscle fibers and small groups of atrophic muscle fibers. Small clusters of muscle fibers were detected in the ATPase (Figure 4 D). These findings show us that mice with neuropathy have degraded muscle structure and the treated group has partially healed muscle structure compared to mice with neuropathy.

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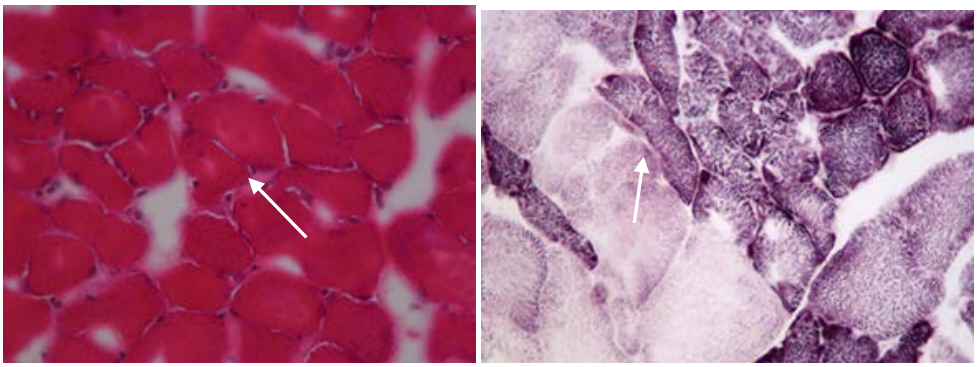

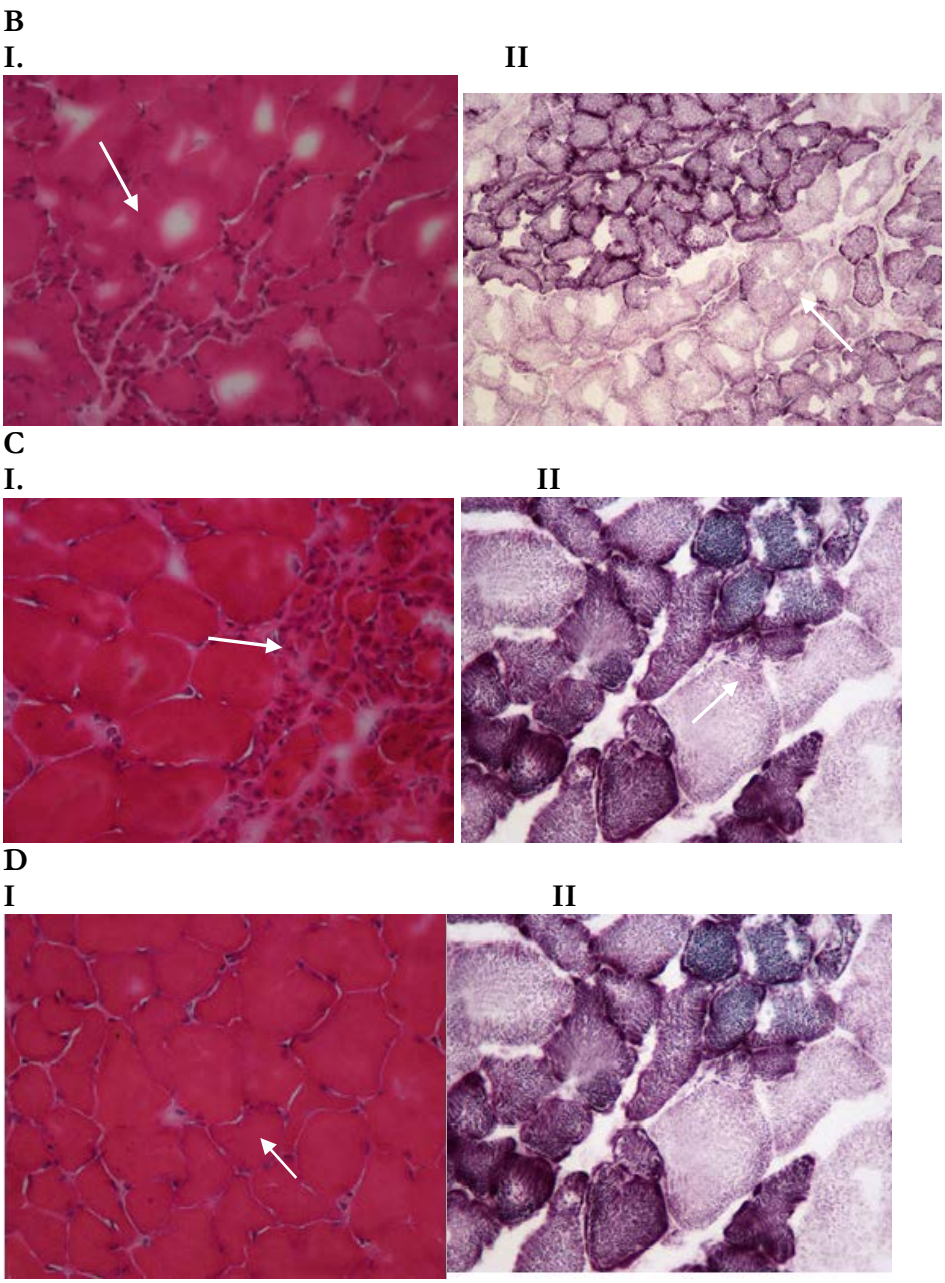

Figure 4. Histopathological evaluation of the effect of bone marrow mesenchymal stem cell transplant on soleus muscle tissue (n=5). No significant pathology was observed in endomysium and vein of striated muscle fibers of the control (A) and BM-MSCs (B) group which was painted with HE (X400). In the NP group (C), muscle sections painted with HE (X200) and NADH-TP (x200) showed a large group of muscle fiber atrophy and increased internal nuclei. Compensatory hypertrophy in atrophic fibers was detected in muscle fiber fascicles neighbors. NADH-TP paint showed that dark stained fibers feature typed 2 muscle fibers and lightly stained fibers feature type 1 fibers (X200). In the NP+BM-MSCs group (D) striated muscle fibers painted with HE (X400), and NADH-TR (X400) showed atrophy in the sparsely scattered angular muscle fibers and small groups of atrophic muscle fibers.

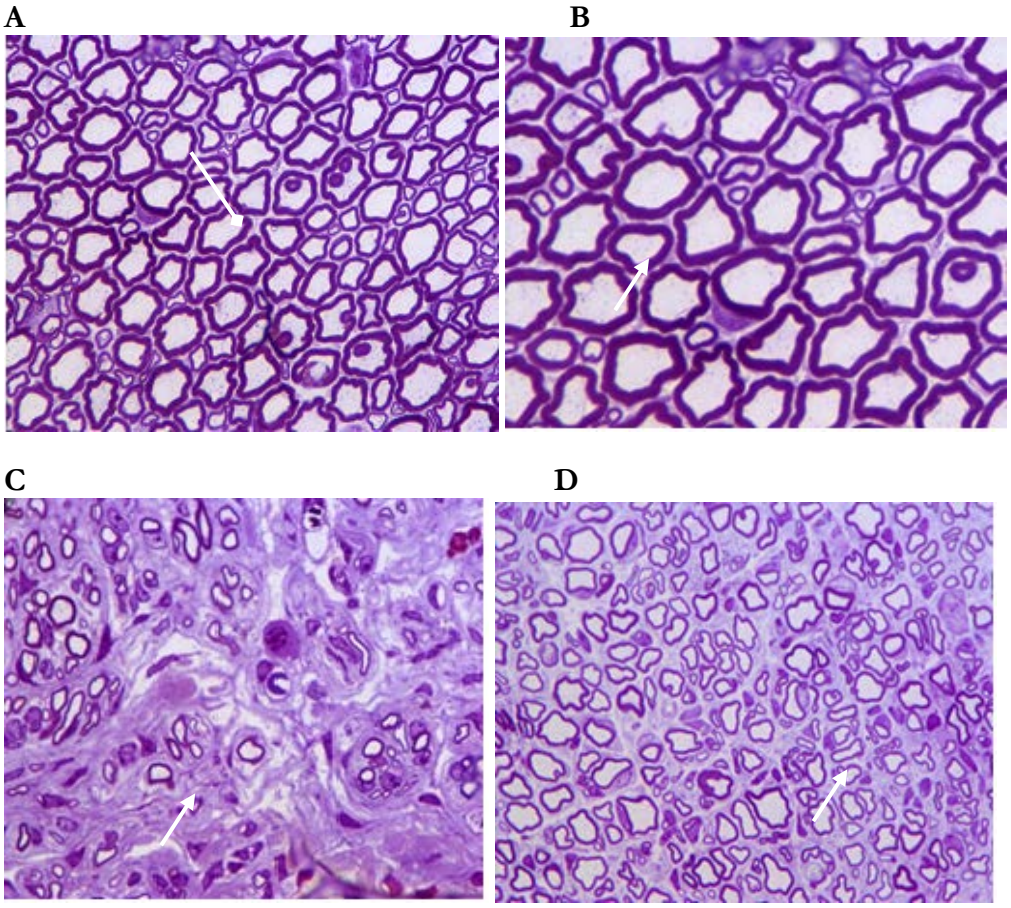

Figure 5. Histopathological evaluation of the effect of bone marrow mesenchymal stem cells transplant on sciatic nerve (n=5). Large and small myelinated axon distribution and normal endoneurium were observed during nerve preparations of the control (A) and BM-MSCs group (B) (X1000). In the NP group (C) loss of myelinated axons of nerve fibers, large degenerating axonal groups, and significant fibrosis in endoneurium were observed (X1000). In the NP+BM-MSCs group (D), large and small myelinated axons of nerve fibers were found to conserved. Schwann cell proliferation, regenerating axon clusters and an increase in mild connective tissue increase in endoneurium was observed (X1000).

After the 24th week, the sacrificed mice's sciatic nerves were isolated and stained with toluidine blue. Large and small myelinated axon distribution and normal endoneurium were observed during nerve preparations of the control, sham, and BMMSCs group (Figure 5 A, B). In the NP group loss of myelinated axons of nerve fibers, large degenerating axonal groups, and significant fibrosis in endoneurium were observed (Figure 5 C). In the NP+BMMSC group, large and small myelinated nerve fiber axons were found to be conserved. Schwann cell proliferation, regenerating axon clusters, and an increase in mild connective tissue increase in endoneurium were observed (Figure 5 D). These results show us that mice with neuropathy have degeneration of nerve tissue, but after BMMSC application there is partial recovery of nerve tissue.

DISCUSSION

In recent years, many studies have demonstrated that MSC has a protective effect on the damaged tissue region in neurodegenerative diseases. In many neurodegenerative diseases such as diabetic neuropathy[15], spinal cord injury[16], and peripheral nerve injury[17], MSC treatment has been shown to

improve nerve regeneration, improve neuropathic pain and also reduce gliosis. MSC has many biological properties, it has high regenerative potential and it is preferred in the treatment of neuronal damage[18]. The ability to easily obtain MSC cells from adult tissues proliferate quickly, transform into different cell types, perform autograft transplantation, produce no immunological reactions, synthesize many bioactive molecules, produce angiogenesis, provide anti-apoptosis feature, provide high genetic stability and no tumor development due to MSC are most important features[19]. However, studies have shown that there are differences between the effects of MSC in peripheral neuropathy. In some studies, it was found that neuropathic pain decreased after MSC application[16,19-21], while in some studies it was shown to be ineffective[22,23] and even increased pain[24]. These differences in nerve regeneration and pain treatment after MSC application in peripheral neuropathy have several implicating factors in the role of the animal species to which MSC is applied, the source from which the cells are obtained, the type and extent of nerve damage, the number of cells applied, the route of administration, the site of administration, and the time of cell application after nerve damage has occurred[11,24]. For this reason, it is necessary to pay attention to the parameters that change the efficacy to achieve the desired success in the treatments to be performed with BMMSC application. First of all, it is important to determine that the applied stem cells are located in the damaged nerve region. Because BMMSCs located in the damaged nerve region can synthesize various anti-inflammatory cytokines and growth factors in this region and become neuronal cells, as a result of these mechanisms regeneration and function of the nerve will improve[25]. Studies have shown that stem cells labeled with various markers, such as *Vybrant Dil* dye or PKH 26, and applied to the damaged nerve site shortly after peripheral nerve injury has been established, have been shown to be located in the damaged nerve region[25-27]. Our study shows that tongue-stained BMMSCs applied two weeks after the development of peripheral neuropathy, were densely located in the damaged sciatic nerve region and to a lesser extent in the nerve end and soleus muscle tissue. The concentration of these cells, especially in the damaged area, is important in terms of nerve regeneration and regulation of the microenvironment[25].

In stem cell research, the most important factor is the time to treatment after nerve damage occurs. After the neuronal damage occurs, if BMMSCs are given at the appropriate time - neuronal inflammation will be minimized, immune-mediated damage will be reduced and revascularization will be provided by an angiogenic effect. Nerve regeneration due to these effects has been shown to be much better[28]. In studies conducted with mesenchymal stem cells, neuropathic pain, and hyperalgesia have improved much better as a result of the application of MSC within the first 4 days after the occurrence of nerve damage[11]. However, considering the clinical situations, MSC applications cannot always be performed within this ideal time interval[29]. Therefore, it is important to determine the effects of the late application of MSC on nerve regeneration and recovery of nerve damage after nerve damage. However, there is a limited number of studies investigating the effect of late-onset MSC after nerve injury.

Neuhuber et al. have determined that there is no significant success in pain treatment when BMMSC taken from female rats is applied two weeks after the formation of spinal cord injury (there was no difference in axonal growth in neurons between BMMSC group and neuropathy group). It has been suggested that these findings may be due to the acquisition of BMMSC from different donors[30]. Vaquere et al. have shown that when BMRSC is applied to female rats 3 months after spinal cord injury, there is an increase in motor function and a decrease in pain sensitivity when a cold spray test is applied. Thus, it has been shown that BMMSC, which is applied late after nerve damage occurs, improves the function of damaged nerves to a great extent[16]. Kim et al found that there was significant clinical improvement, 6 weeks after damage to rats with chronic contusive spinal cord injury, via both methods of administration by intravenous and intralesional BMMSC. However, it has been shown that the number of engrafted cells and neurotrophic factor expression is better in the intralesional route[31]. It is seen that the studies related to late MSC applications after neuronal degeneration are generally performed in experimental models with spinal cord injury. However, there are not enough studies investigating the effects of late MSC administration and nerve regeneration on different types and models of peripheral neuropathy. Thus, it has been demonstrated that BMMSCs applied late in the period after the development of neuropathy have a significant effect on the treatment of neuropathic pain.

Another point of interest in studies investigating the effect of BMMSC on peripheral nerve injury is the evaluation of the effect after administration of BMMSC, usually within a short period of 8-13 weeks[11]. A longer study of the effect of BMMSC on degenerative nerve tissue is important to determine both the permanence of the effect and the degree of improvement in function. In our study, it was found that BMMSC significantly reduced neuropathic pain for a long time (24 weeks) in the peripheral neuropathy model induced by the partial sciatic nerve ligation model in mice. These results show that the effect of BMMSC on pain treatment increases with time and is long-term/permanent in peripheral nerve injury. Many characteristics of BMMSC play a role in the realization of neuronal regeneration and pain control. One of these mechanisms is the effect of BMMSC on inflammatory pathways. BMMSC enables the conversion of proinflammatory macrophages into antiinflammatory macrophages by contact activation between cells (Cell to cell). It also plays a role in the synthesis and release of many molecules as a result of communication with various cells. It has been shown that after migration of BMMSC to the damaged site, the level of pro-inflammatory cytokines such as IL1β and IL-17 decreases, and the level of anti-inflammatory cytokines and markers such as IL-6, IL-10 and CD206 have been shown to increase[26,32].

With a strong anti-inflammatory response produced by BMMSC, inflammation caused by nerve damage is prevented, myelin and cell debris are removed, and thus the nerve repair process begins[31]. BMMSC also controls long-term pain through opioid peptides, while creating a strong anti-inflammatory effect. BMMSC provides long-term control of pain by increasing the release of opioid peptides and activation of peripheral and central opioid receptors from immune cells such as granulocyte, T-cell, monocyte / macrophage migrating to the damaged nerve region[21]. The second important function of BMMSC in nerve regeneration is to increase the synthesis and release of neurotrophic factors such as CNTF, PDGF-alpha, TGf-beta1, beta-NGF, BDNF, nestin, Tubulin-β3, and other molecules[33,34]. As a result of this effect called Trophic activity; apoptosis is inhibited and nerve damage and injury are limited, fibrosis tissue and scar formation in the damaged area is prevented, tissue-specific and tissue-intrinsic progenitors such as neural stem cells have been shown to be stimulated. As a result of all these effects of BMMSC, it has been shown that damage to degenerative nerve tissue is limited and tissue regeneration is provided. The third important effect of BMMSC is that it synthesizes angiogenic factors such as vascular endothelial growth factor (VEGF) and causes neovascularization. It has been shown that neovascularization provides sufficient oxygen and nutrients to the damaged nerve tissue and neuroregeneration develops in that area[25,35]. All these mechanisms are thought to have a role in the regeneration of the nerve and pain control.

Electrophysiological measurements and histopathological examinations are of great importance in the functional evaluation of damaged nerve tissue. There is a close relationship between inflammation as a result of peripheral nerve injury and disruption of nerve myelinization. The concentration of inflammatory cytokines and phagocytosis cells in the damaged area leads to the disruption of myelin and increased nerve degeneration[36]. The structure, distribution, and function of the ion channels localized here are impaired as a result of damage to the myelin sheath in the nerves. Differences in the regulation of inwardly directed Na and Ca channels and outwardly directed K channels lead to changes in an action potential. While tetrodotoxin-sensitive Na (TTx Na) channels are upregulated, down-regulation is developing in Nav1.1, Nav1.2 Nav1.6 Nav1.7, Nav1.8, and Nav1.9 subtypes. This change in sodium channels also plays an important role in the formation of neuropathic pain[37]. Repair of nerve fibers and myelin sheath after damage to the peripheral nerves is important for improving nerve function. Peripheral nerve injury in rats 24 weeks after spontaneous regeneration of nerve fiber count increased, but axon count, nerve fiber diameter, and myelin sheath thickness were not fully developed[38]. Therefore, it is important to investigate the effect of BMMSC on nerve regeneration over a long period with electrophysiological measurements in peripheral nerve injury.

In the 24th week of our study; nerve action potential decreased in the neuropathy group compared to the control group, depolarization and repolarization times prolonged - this was observed by conducting microelectrode measurements from the sciatic nerve and soleus muscle tissue. In the BMMSC group, the action potential and depolarization time were partially improved and there was no improvement in the repolarization time compared to the neuropathy group. Some of the effects of BMMSC on nerve

tissue have an important role in this improvement measured in muscle action potential. One of the most important effects is the ability of BMMSC to transform into some neuronal cells, such as Schwann-like. Schwann cells play an integral role in nerve regeneration. It has been demonstrated that degenerate nerve fibers can germinate both in vitro and in vivo[39,40]. Schwann cell markers S-100, GFAP, and NGFR were detected in cells transformed from BMMSC. Conversion of BMMSC to Schwann cells leads to increased myelin synthesis, new axon sprouting, and regeneration of nerve tissue[41]. Another important effect is the formation of neurotrophic signaling and warning traffic. This effect appears to be a key factor in ion channel arrangement. Neuronal growth factors, such as brain-derived neurotrophic factor, neurotrophin-3, insulin-like growth factor, and glial cell line-derived neurotrophic factor, are important for modulating neuronal branching and synaptic plasticity[42]. BMMSC increases the synthesis and release of all neuronal growth factors, which leads to nerve regeneration and the corresponding action potential[11]. BMMSC provides regeneration of myelin sheath while regulating ion channels in this region. Studies show that the expression of sodium, potassium, and calcium channels changed depending on the cell cycle, but their physiological roles were not fully elucidated. These studies find that inward channels (INa.TTX and ICa.L) were more and faster upregulated than outward potassium channels (IKCa, Ito, and IKDR or heag1)[43,44]. Our study endorses the idea that all these mechanisms may play a role in the improvement of the action potential and depolarization time in the BMMSC group. Since no improvement in repolarization time can be detected, the delay in the regulation of K channels due to the cell cycle might play a role. In the study, the membrane potential of the soleus muscle was decreased in the neuropathy group compared to the control group, whereas it was found that there was an increase in the BMMSC group compared to the neuropathy group. Studies have shown that the strength of the muscle membrane potential depends on the number of nerve fibers, the diameter of the axon, the thickness of the myelin sheath, and the length of the internodes[45]. Damage to the peripheral nerve leads to deterioration of these parameters, decreased/diminished muscle excitability, and membrane potential[46]. BMMSC increases the release of neurotrophic and antiinflammatory factors, resulting in myelin sheath formation, and presynaptic Ca channel upregulation in damaged nerve fibers, resulting in increased acetylcholine release[44]. In our study, the improvement in muscle membrane potential is thought to be due to increased excitability of nerve-muscle junction as a result of all these effects of BMMSC.

In this study, sciatic nerve-soleus muscle tissue was examined histopathologically in all groups to investigate the functional functions of nerve-muscle tissue. As a result of the examination of the sciatic nerve; In the neuropathy group, it was shown that large degenerated axon fibers, myelinization of nerve fibers, and fibrosis in the endoneurium developed. In the BMMSC group, Schwann cell proliferation, increased myelinization, axon regeneration, and increased connective tissue were detected. These results were consistent with the findings detected by histopathological examination of peripheral nerve injury[47,48]. Soleus muscle tissue examination; Atrophic and hypertrophic muscle fibers were increased in the neuropathy group and the internal nucleus was increased in the atrophic fibers. Small atrophic muscle fibers, decreased internal nuclei and decreased hypertrophy areas were detected in the BMMSC group. Development of significant regenerative effects in both nerve and muscle tissue in the neuropathy group treated with BMMSC, increased release of specific proteins by stimuli stemming from the environment in which the stem cells are located, regulation of extracellular matrix stores, collagen synthesis, fibroblast proliferation, platelet activation, fibroblast proliferation, platelet activation, fibrinolysis, angiogenesis properties such as increased release of anti-inflammatory agents and decreased apoptosis are thought to play a role[28].

The limitation of the study is that changes in nerve-muscle tissue have not been supported by electron microscopic examinations and histomorphometric methods. Additionally, the analysis of biomarkers that play significant roles in nerve regeneration could provide important insights. In future studies, measuring the number and diameters of nerve axons, and investigating the levels of biomarkers such as nestin, neuronal growth factor, and vascular endothelial growth factor would be beneficial.

In summary, these results showed that the effect of BMMSC on neuropathic pain started late after administration of peripheral nerve injury with partial nerve ligation method (2 weeks), started to increase after a short period of time, and sustained for a long time. Although there was no complete improvement

in the histopathological examination and electrophysiological measurements of the damaged nerve and stimulated muscle tissue, partial improvement was found. The lack of functional improvement in neuromuscular tissue but a significant decrease in neuropathic pain suggests that BMMSC may affect these two parameters by some independent mechanisms. It is thought that BMMSC can produce this effect by increasing the synthesis and release of various growth factors, triggering angiogenesis, and limiting apoptosis by immunomodulation. However, in order to better understand the effects of late BMMSC, various studies are needed at molecular level. This study is valuable to demonstrate the positive effect of late BMMSC administration on neuropathic pain and its long-term effect in patients with peripheral nerve injury in accordance with clinical practice. It also adds a distinct value in terms of showing better functional recovery in damaged tissues. Perhaps in this way, it will be possible to protect the patients who have missed the treatment period from long-term drug use of multiple drugs, their high doses and side effects. Perhaps, as a result of more detailed studies, the application of late MSC will be a light for improving the quality of life of patients with peripheral nerve injury.

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