



## Research Article

# Stem cell, Granulocyte-Colony Stimulating Factor and/or Dihexa to promote limb function recovery in a rat sciatic nerve damage-repair model: Experimental animal studies

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## ABSTRACT

**Background:** Optimizing nerve regeneration and re-innervation of target muscle/s is the key for improved functional recovery following peripheral nerve damage. We investigated whether administration of mesenchymal stem cell (MSC), Granulocyte-Colony Stimulating Factor (G-CSF) and/or Dihexa can improve recovery of limb function following peripheral nerve damage in rat sciatic nerve transection-repair model.

**Materials and methods:** There were 10 experimental groups (n = 6–8 rats/group). Bone marrow derived syngeneic MSCs ( $2 \times 10^6$ ; passage $\leq 6$ ), G-CSF (200–400  $\mu\text{g}/\text{kg}$  b.wt.), Dihexa (2–4 mg/kg b.wt.) and/or Vehicle were administered to male Lewis rats locally via hydrogel at the site of nerve repair, systemically (i.v./i.p.), and/or to gastrocnemius muscle. The limb sensory and motor functions were assessed at 1–2 week intervals post nerve repair until the study endpoint (16 weeks).

**Results:** The sensory function in all nerve boundaries (peroneal, tibial, sural) returned to nearly normal by 8 weeks (Grade 2.7 on a scale of Grade 0–3 [0 = No function; 3 = Normal function]) in all groups combined. The peroneal nerve function recovered quickly with return of function at one week (~2.0) while sural nerve function recovered rather slowly at four weeks (~1.0). Motor function at 8–16 weeks post-nerve repair as determined by walking foot print grades significantly ( $P < 0.05$ ) improved with MSC + G-CSF or MSC + Dihexa administrations into gastrocnemius muscle and mitigated foot flexion contractures.

**Conclusions:** These findings demonstrate MSC, G-CSF and Dihexa are promising candidates for adjunct therapies to promote limb functional recovery after surgical nerve repair, and have implications in peripheral nerve injury and limb transplantation. IACUC No.215064.

## 1. Introduction

Battlefield blast trauma to extremities such as hands and legs is a common occurrence that leads to devastating injuries and amputations. Injuries to peripheral nerves such as sciatic, femoral, and facial can cause respective regional paralysis. About 2.8% of trauma patients are affected by peripheral nerve injuries. The only reliable method of treatment in complete nerve transection is surgical re-anastomosis. Sciatic nerve injury is a common peripheral neuropathy that results in persistent and severe pain, numbness, muscle weakness and motor

dysfunction leading to long-term disability [18]. Specifically recovery of muscle function is compromised due to slow axon growth and delayed muscle re-innervation [28]. Functional recovery is critical for limb salvage in the management of sciatic nerve injuries.

Advances in peripheral nerve repairs such as end-to-end neuro-orrhaphy, nerve grafting, nerve transfer and nerve guidance conduits have improved clinical outcome, yet remain suboptimal [59]. Poor functional recovery is partly due to incomplete regeneration of the damaged nerve components and re-innervation of target muscle groups [31,72,82]. There are several alternative approaches emerging to

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promote peripheral nerve repair including stem cell administration [9, 11, 13, 25, 26, 28, 40, 45, 56, 57, 59, 60, 69, 70, 86]. However, the role of stem cells on peripheral nerve regeneration is not completely understood; a combination of trophic factor production, extracellular matrix synthesis, axon guidance, remyelination, microenvironmental stabilization, and/or immune modulation support mediated by stem cells appear to promote peripheral nerve regeneration and function [5, 8, 10, 12, 26, 42, 44, 47, 55, 56, 59, 61]. Mesenchymal stem cells (MSCs) have been shown to improve functional recovery in animal models of nervous system diseases (Parkinson's disease, multiple sclerosis, stroke, traumatic brain injury, spinal cord injury) including peripheral nerve damage [5, 14, 19, 36, 71]. The paracrine factors (VEGF, IGF-1, HGF, and Bcl-2, HSP20, HO-1, SCF, SDF, TGF- $\beta$ 1, NO etc.) produced by MSCs have the potential to induce myogenesis, angiogenesis, anti-apoptotic activity, and regeneration leading to improvement in organ function [76, 87]. In addition, MSCs migrate preferentially to the areas of tissue injury, and augment tissue repair [27, 58, 85].

To further enrich the microenvironment that both supports axon survival and target innervation and activates critical neurotrophic factor (NF) systems, we sought to administer Granulocyte-Colony Stimulating Factor (G-CSF) [56] and Dihexa [48] which currently have received less attention in peripheral nerve damage repair. Studies of spinal cord injuries have shown that G-CSF administration enhanced motor recovery [20, 51] and provided neuroprotection in experimental models of neurological disorders [49]. Furthermore, treatment with G-CSF enhances muscle regeneration via the proliferation of satellite cells, reducing the number of apoptotic cells, and increasing the number of myocytes [30, 68]. Dihexa, a novel small molecule activator of the endogenous HGF/c-Met system supports synaptic plasticity and reverses nervous system deficits [4]. Furthermore, Dihexa has neurotrophic activity seven times greater than brain-derived neurotrophic factor (BDNF) [4]. It appears Dihexa is an excellent novel candidate molecule to augment peripheral nerve regeneration and functional recovery.

Delivery of cells and drugs to the injury site and their long-term maintenance at the site is critical for accelerated target organ repair and functional recovery. Biomaterial-based delivery systems (hydrogels) have been shown to be excellent carriers [24] that enhance cell/drug localization, survival and therapeutic efficacy in organ injury repair [3, 32, 38, 53]. In the present study, we used hydrogel to deliver MSCs and growth factors.

Optimal functional recovery after peripheral nerve injury requires the regenerating axons form functional connections with their original muscle fibers as well as the restoration of the number and size of the motor units in these muscles [43]. Although stem cells have previously been used for repair at nerve lesion sites, little attention has been paid to enhancing the recovery of the denervated target muscles; even with enhanced nerve regeneration aided by the stem cells, muscle recovery remains sub-optimal [62]. Axotomy of the peripheral nerve leads to a rapid decline in muscle mass, which can be reversible if there is good quality muscle re-innervation. Sustained denervation leads to progressive atrophy, myocyte death, and fibrosis with irreversible muscle impairment. In this study, we sought to enhance functional recovery of the target muscle by injecting mesenchymal stem cells and growth factors (G-CSF and Dihexa) directly into the target muscles after surgical nerve repair.

The objectives of this study were two-fold: 1) To determine whether MSC, G-CSF, and Dihexa alone or in combination therapy can improve limb functional recovery in a sciatic nerve transection-repair model; and 2) To determine whether MSC, G-CSF, and Dihexa administration into target muscle (gastrocnemius) mitigates muscle atrophy and enhances limb functional outcome.

## 2. Material and methods

### 2.1. Animals

Ten to 12-week-old inbred male Lewis (RT1<sup>l</sup>) rats, weighing ~300 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Male rats were preferred to avoid hormonal influence that varies in female rats with reproductive cycle. Rats were used, cared, and maintained according to the 'Guide for the Care and Use of Laboratory Animals' published by the National Research Council/Institute of Laboratory Animal Research (ILAR). Animal housing, husbandry and experiments were conducted as per the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) following approval by the Institutional Animal Care and Use Committee (IACUC), as per protocol (No.215064) and institutional guidelines similar to previously published reports [5]. The report presented is as per the ARRIVE (Animal Research: Reporting In Vivo Experiments) protocols [37].

We had ten experimental groups (n = 6–8/group) including treatments and appropriate controls: Group 1, Saline i.v. (Vehicle control); Group 2. Saline i.v. + Hydrogel local (Vehicle control); Group 3. MSC i.v. + local Hydrogel; Group 4. MSC i.v + Local MSC via Hydrogel; Group 5. MSC i.v. + Local MSC & G-CSF via Hydrogel; Group 6. MSC i.v. + Local MSC & Dihexa via Hydrogel; Group 7. MSC i.v. + Local MSC & G-CSF via Hydrogel + Gastrocnemius injection with MSC & G-CSF via saline; 8. MSC i.v. + Local MSC & Dihexa via Hydrogel + Gastrocnemius injection with MSC via saline & Dihexa via DMSO; Group 9. MSC i.v + Local MSC and DMSO (vehicle control for Dihexa in Group 6) via hydrogel; and Group 10. MSC i.v + Local MSC & DMSO via hydrogel + Gastrocnemius injection with MSC & DMSO (vehicle control for Dihexa in Group 8). Animals were randomly assigned to control and treatment groups. Experimental design is shown in Fig. 1.

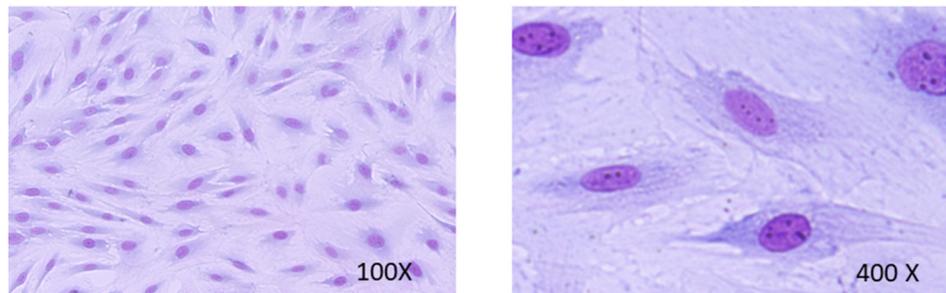
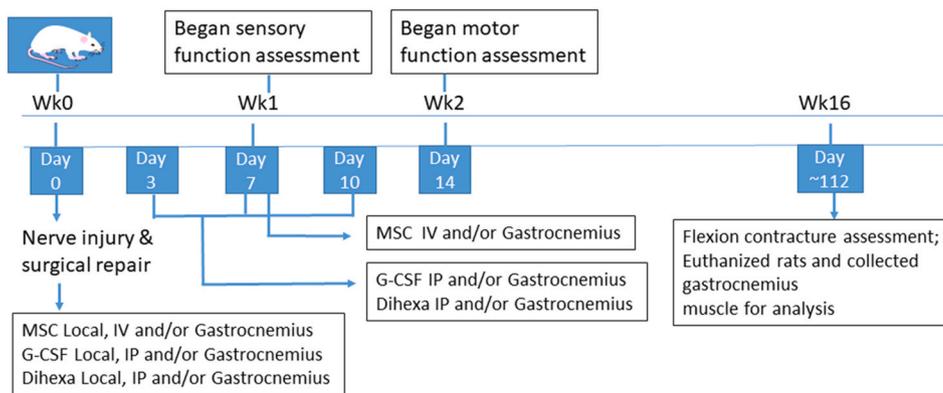
We used a sciatic nerve repair (SNR) model that involved transection of the main sciatic nerve just above the trifurcation into tibial, sural, and peroneal branches as described previously [5]. SNR was performed on the right hind limb, and the left naïve limb served as a non-transected nerve control. Starting about  $\geq 1$  week after SNR the rats were administered manual physiotherapy to the right hind limb for about 5 min, 1–2 times per week as described previously [5, 23]. The primary outcome measures were limb sensory and motor functions. Limb sensory function was determined at weekly intervals starting at 1-week post SNR and limb motor function was determined at two week interval starting at 2-week post SNR; the functional assessments continued until about 16 weeks post SNR. Secondary outcome measures were foot-flexion contracture assessment at the study endpoint (~16 weeks) prior to animal euthanasia and gastrocnemius mass (right and left) following animal euthanasia. The evaluators were blinded to the treatment and control experimental groups.

### 2.2. MSC, G-CSF, and Dihexa preparation and administration

#### 2.2.1. Mesenchymal stem cells

Bone marrow-derived MSCs (Fig. 2) were isolated and administered as previously described [5, 23]. Briefly, Lewis rats were euthanized and long bones (tibia, femur) harvested aseptically. Bone marrow cells (BMCs) harvested from long bones were cultured at a density of  $5\text{--}10 \times 10^7$  cells/ml in MSC complete medium. The complete medium was prepared using low glucose DMEM (IDulbecco's Modified Eagle's medium) containing Glutamax and Pyruvate, 10% fetal bovine serum, penicillin [100 units/ml], and streptomycin [100  $\mu$ g/ml] as described previously [5]. BMCs were plated at a density of  $0.5 \times 10^6$  cells/cm<sup>2</sup> in 75 or 175 cm<sup>2</sup> flasks and cultured at 37 °C with 5% CO<sub>2</sub>. At about 72 h of culture, the supernatant containing non-adherent cells was removed and fresh complete medium was added. The adherent cells further cultured until they reached about 70–80% confluent and were sub-cultured at 1:3. *Ex vivo* expanded MSCs (passage  $\leq 3$ ) were harvested and stored at

## Experimental Design



**Fig. 2.** Ex vivo expanded Mesenchymal Stem Cells (MSCs) stained with Wright's stain (100X and 400X). Freshly cultured cells (passage  $\leq 6$ ) were injected into rats.

–150 °C. The freezing medium used was RPMI 1640 containing 10% dimethyl sulfoxide (DMSO), 30% fetal bovine serum, penicillin at 100U/ml and streptomycin at 100  $\mu\text{g}/\text{ml}$  [5]. About one to two weeks prior to MSC injection, frozen cells were removed and expanded in cultures as described previously [5].

Following sciatic nerve surgical repair, MSCs ( $2 \times 10^6/\text{rat}$ ) in  $\sim 0.4$  ml of 1x hydrogel (Hyaluronan-Heparin based pre-made hydrogel, ESI Bio. Inc., USA) or via saline (control group) was infused locally at nerve repair site and along the nerve before muscle approximation and skin closure. Immediately after surgery, 1.0–1.5 ml of MSCs ( $2 \times 10^6/\text{rat}$  in saline) or saline (vehicle) was injected intravenously (IV) via the dorsal penile vein slowly over 2–3 min. Intravenous MSC ( $2 \times 10^6$ ) or saline injection was repeated on Day 7 post SNR. We administered MSCs both locally and systemically to ensure high numbers at lesion site. The MSC dose used was consistent with previous reports in SNR and limb transplant models [5,23,47,83].

### 2.2.2. Granulocyte-Colony Stimulating Factor

We obtained G-CSF (Neupogen/Filgrastim) from Amgen Inc., Thousand Oaks, CA. G-CSF was administered once locally via hydrogel (100  $\mu\text{g}/\text{rat}$   $\sim 250$ – $300$  g) along and around the nerve repair site on Day 0 (day of surgery). G-CSF was administered at 60  $\mu\text{g}/\text{rat}$  ( $\sim 250$ – $300$  g) systemically via intraperitoneal (IP) injection and/or into the gastrocnemius muscle (depending on the experimental group) on Days 0, 4, 7, and 10 post SNR as described previously [56].

### 2.2.3. Dihexa

Our collaborator Professor Joseph Harding (Washington State University, Pullman, WA), provided Dihexa. The Dihexa was administered once locally via hydrogel (1 mg/rat) along and around the nerve repair site on Day 0 (day of surgery). It was administered at 0.5mg/rat

**Fig. 1.** Schematic showing experimental design. Rats received MSCs ( $2 \times 10^6/\text{rat}$ ) locally (Day 0), intravenous (Days 0 & 7) and/or gastrocnemius injections (Days 0 & 7). G-CSF (100  $\mu\text{g}/\text{rat}$ ) or Dihexa (1 mg/rat) was administered locally (Day 0), intraperitoneal (Days 0, 4, 7 & 10) and/or gastrocnemius injections (Days 0, 4, 7, & 10). MSC, G-CSF or Dihexa was administered locally at the nerve repair site via hydrogel. MSCs were administered intravenously and to gastrocnemius via saline. G-CSF was administered intraperitoneal and to gastrocnemius via saline. Dihexa dissolved in DMSO (dimethyl sulfoxide) and diluted in saline ( $\leq 5\%$  DMSO) was administered locally via hydrogel, and intraperitoneal and gastrocnemius via DMSO-saline. Sensory function was evaluated at weekly interval starting one-week after sciatic nerve repair (SNR), and motor function evaluated every two-weeks starting two-weeks after SNR. Study end point was 16 weeks post SNR.

systemically via IP injection and/or 0.5mg/rat to the gastrocnemius muscle (depending on the experimental group) on Days 0, 4, 7, and 10 post SNR as described previously [48]; approximate rat weight was 250–300 g. We dissolved Dihexa in dimethyl sulfoxide (DMSO) and final DMSO concentration in the preparation was  $\leq 5\%$ .

### 2.3. Surgical procedures

We used a sciatic nerve repair model as described previously [5,6,23,84]. Briefly, the rat was anesthetized with intraperitoneal injections of Ketamine (40–80 mg/kg b.wt.) and Xylazine (5–10 mg/kg b.wt.); we used inhalant 1–2% isoflurane to maintain anesthesia. Rats were treated with cefazolin (25 mg/kg b.wt. SQ) a preoperative antibiotic, and Vidisic (ophthalmic ointment) was applied to the eyes to prevent corneal drying. We prepared the surgical site by clipping hair and sterilizing with 10% chlorhexidine and 70% alcohol. The animal was placed on a thermos regulated warming pad to maintain body temperature at about 38 °C. A circumferential skin incision around the right mid-thigh region was made. The biceps femoris was exposed and divided near the distal attachments to the stifle and tibia; the muscle was then reflected to expose the sciatic nerve. The sciatic nerve dissected out proximally to the point of emergence from below the gluteus muscle. Tag sutures of 10–0 nylon were placed on proximal and distal ends of the sciatic nerve, and the nerve was transected proximal to the trifurcation of sciatic nerve into tibial, peroneal, and sural nerves as described previously [5]. For anti-coagulation, we administered 300  $\mu\text{l}$  (50U) heparin via the tail vein. The animal was then monitored under anesthesia for an hour without any further manipulation to mimic a traumatic peripheral nerve injury/damage situation with transport time to a facility for treatment. Neuroorrhaphy (sciatic nerve proximal and distal end approximation) performed with 10–0 nylon sutures, followed by biceps femoris repair

and skin closure. The SNR surgical procedure is shown in Fig. 3.

#### 2.4. Postsurgical management

The rats received postsurgical care and physiotherapy as described previously [5,23,84].

**Post-operative care:** Briefly, we administered Lactated Ringers solution (5 cc, SQ) to prevent dehydration; buprenorphine (0.02–0.05 mg/kg, SQ) was administered every 12 h as needed for analgesia; and cefazolin (20 mg/kg, SQ) every 12 h for 3 days as an antibiotic. We monitored animals closely for signs of pain or distress and changes in body weights; data recorded daily/weekly. Physiotherapy was administered 1–2 weeks post-surgery for 1–2 times per week that included gentle and repeated manipulation of the nerve repaired limb through the normal range of motion under manual restraint as described previously [5,84]. Each physiotherapy session lasted as long as the animal tolerated it well and for a maximum of 5 min. We provided additional physiotherapy by housing animals in wire mesh floor cage for about 8 h a day; the cage was equipped with access for solid floor space (Fig. 4).

#### 2.5. Limb function assessment

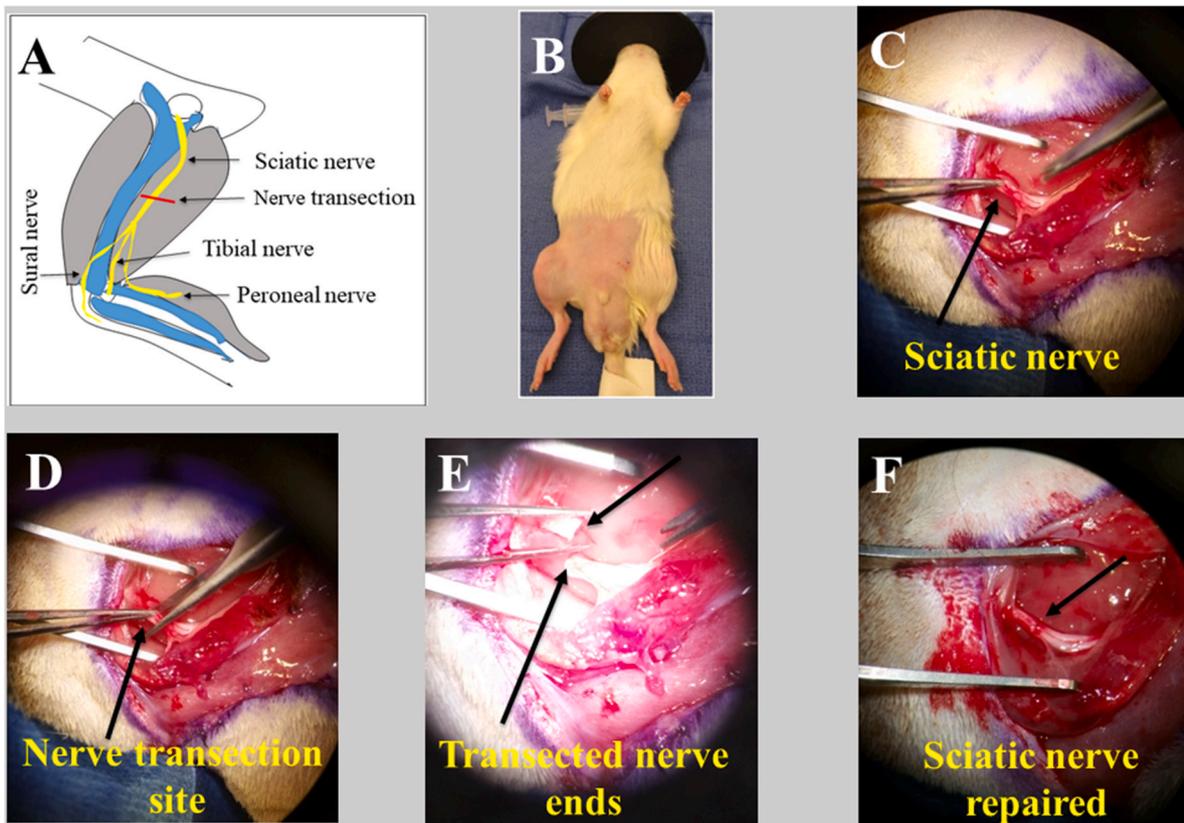
##### 2.5.1. Sensory function

We used cutaneous pain reaction test (*the flexor “withdrawal”* spinal reflex) to assess sensory function as previously described [5,23,84]. Animals were tested for sensory function at weekly intervals starting one-week post SNR. Briefly, rats were handheld with the hind-limbs in suspension. Using atraumatic forceps the stimulus was applied momentarily by pinching the areas of tibial, peroneal, sural and saphenous nerve boundaries (Fig. 5A) as described previously [5,23,80,84]. Normal innervation results in an immediate withdrawal response,

with or without vocalization. The normal response to the stimulus was first determined in the naive left hind limb. The stimulus was applied in the same nerve boundary of the SNR right hind limb. The response was graded in comparison to the normal (naïve) left limb response. We graded the withdrawal reflex (0–3) as described previously [67]: 0, No response; 1, Mild response; 2, Moderate response; 3, Strong response (normal). Animals were not under sedation or anesthesia for this analysis.

##### 2.5.2. Motor function

The motor function was assessed using walking track analysis as described previously [2,5,7,23,73,74]. Briefly, rat was made to walk in a confined walkway (10 cm wide x 10 cm high x 70 cm long) lined with white paper and led into a dark shelter as shown in Fig. 5B. We applied water-soluble black ink to the plantar surfaces of the right and left hind feet of the animal and immediately allowed to walk down the walkway from its entrance into the shelter. Note animals were conditioned by walkway practice trials 3–5 days prior to sciatic nerve repair surgery. The motor function was assessed starting two weeks following surgery at two-week intervals until the study endpoint. Due to poor quality of toe to toe print separation we were unable to calculate the sciatic function index (SFI), a measure to assess hind limb motor function by conventional method based on foot print characteristics (toe to toe, and toe to heel distances) as described previously [2]. However, we used the recently described alternate method [23] of motor function assessment using toe and heel foot print characteristics. Briefly, the prints were graded on a scale of 0–4 (0 = no print; non-functional); (4 = complete print [nearly normal function]).

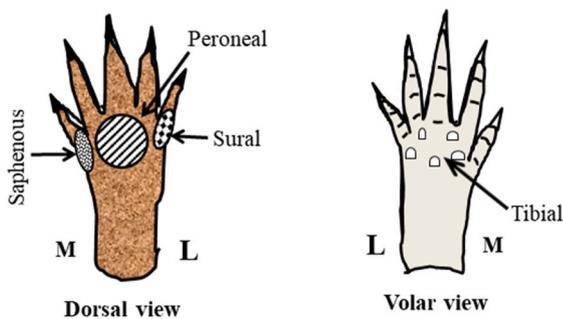


**Fig. 3.** Sciatic nerve transection and repair surgeries: A, sciatic nerve transection site schematic; B, animal prepared for right sciatic nerve transection and repair surgeries; C, lateral dissection exposing sciatic nerve; D, sciatic nerve transected site; E, transected nerve ends; and F, sciatic nerve repair (SNR) with interrupted sutures. Following SNR muscles were approximated and skin incision was closed.



**Fig. 4.** Rat physiotherapy. Animals were group housed for 6–8 h a day on wire mesh floors with easy access to solid platform, food and water to enhance physiotherapy in addition to manual limb exercise (5 min 1–2 times/week).

### A. Sensory function assessment



### B. Motor function assessment



**Fig. 5.** Limb functional analysis. A. Sensory function assessment utilizing cutaneous pain reaction test: manual pinch technique was used to stimulate the territories of tibial (T), peroneal (P), sural (Sur), and saphenous (S) nerves as described, previously [28,53,56,57]. Withdrawal/vocal response of the nerve repaired limb was scored in comparison to the naïve contralateral limb (0 = no response, 1 = slight, 2 = moderate, 3 = normal). L, Lateral; M, Medial. B, Motor function assessment utilizing walking track/foot print analysis method: Progression of walking track of naïve (normal) and nerve transected/repaired limb foot-prints were obtained on a paper in a rat walking apparatus as described previously [28,53,59–62]. Foot prints were graded 0–4 as described previously [53]: Grade 0, no print; Grade 1, Heel print only; Grade 2, Heel print and one toe print; grade 3, Heel print and 2–3 toe prints; and Grade 4, Heel print and 4–5 toe prints.

### 2.6. Gastrocnemius muscle mass

At study endpoint animals were euthanized, and left and right gastrocnemius muscles carefully dissected, removed and weighed. The mean gastrocnemius muscle mass (g) was compared between normal (left) and nerve repaired (right) limbs in each experimental group. We calculated percent decrease in right gastrocnemius muscle mass for each group and compared among all groups.

### 2.7. Foot flexion contractures

Rat foot-flexion contracture was assessed and graded 0–4 as described previously [23]: 0, no contracture; 1, 0–30°; 2, 31–60°, 3, 61–90°, and 4, >90°.

### 2.8. Statistical analysis

The data was analyzed using statistical software SPSS version PASW Statistics18 (SPSS Inc., Chicago, IL). We compared the data between the two groups using Student *t*-test, and more than two groups by ANOVA with Bonferroni correction. All P-values were two-tailed, and values ≤ 0.05 considered statistically significant.

## 3. Results

### 3.1. Mesenchymal stem cell characterization

MSCs expanded *ex vivo* (passage ≤6) >90% expressed CD29 and CD90 (MSC positive markers) and <10% expressed CD31, CD34, and CD45 (MSC negative markers; HSC positive markers). MSCs were pluripotent based on their differentiation potential into osteocytes, adipocytes and chondrocytes [23].

### 3.2. Limb functional recovery

#### 3.2.1. Sensory function

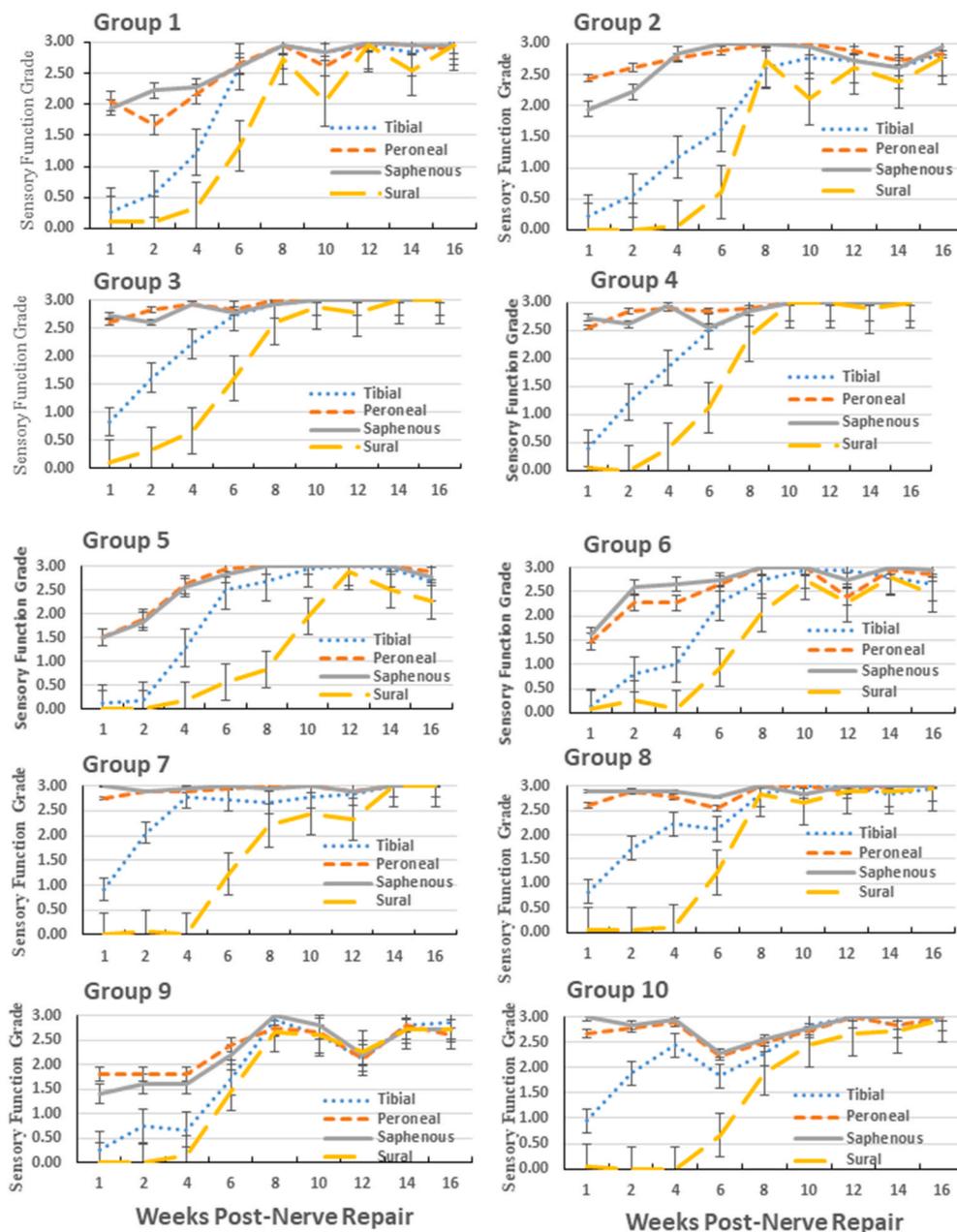
The mean sensory function scores for all experimental groups up to 16 weeks post SNR are presented in Table 1 and Fig. 6. The sensory function in the peroneal nerve boundary recovered first (~1 week), followed by tibial and sural boundaries in all experimental groups studied (Fig. 6). Saphenous nerve boundary sensory function recovery was early and higher, similar to peroneal nerve; saphenous nerve is not a branch of sciatic nerve, therefore, sciatic nerve transection was not expected to affect saphenous nerve innervation, and response to stimulus was considered normal. Total sensory function (average response of all four nerve boundaries taken together) at two weeks post SNR, in all experimental groups (with or without treatment) ranged from 1.0 to 2.0 on a scale of Grade 0–3 (0 = No function; 3 = Normal) (Table 1). However, in Groups 7 and 8 where animals received G-CSF and Dihexa, respectively, via gastrocnemius injections, the sensory function at 2 weeks was more pronounced (Grade 1.9–2.0). Overall, the sensory function gradually improved in all groups and by Week-10 it reached nearly normal (2.6–3.00) (Table 1; Fig. 6). Total sensory function recovery (Groups 2, 4–8) is shown in Fig. 7. G-CSF (Group 7) and Dihexa (Group 8) administration to gastrocnemius muscle showed early onset of total sensory function recovery (Grade ~2 by two weeks) and sustained high levels of function compared to saline control Group 2 (Fig. 7).

**Table 1**

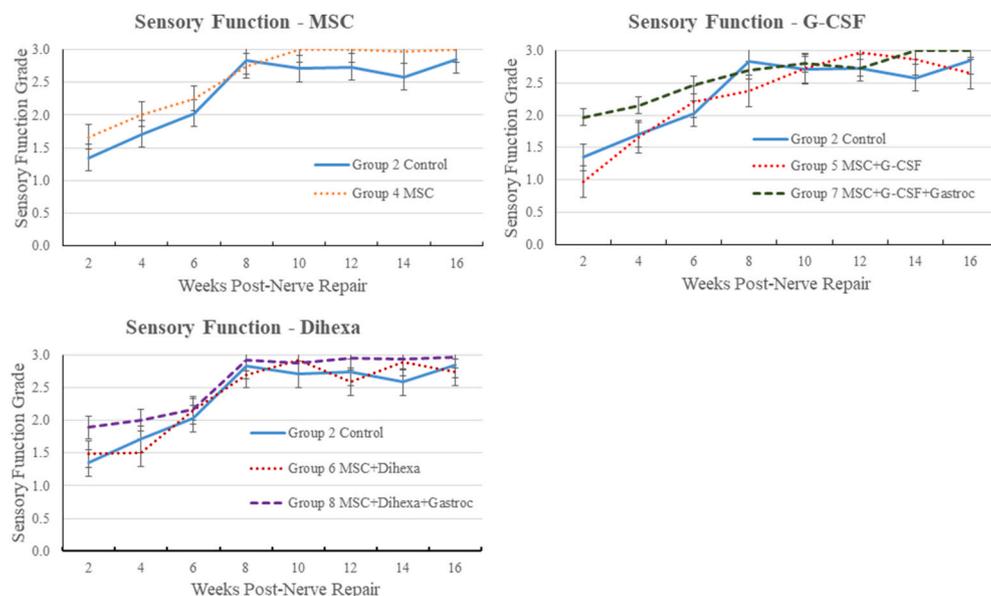
Total sensory function (Mean ± SD) in the paws of rats up to sixteen weeks post-sciatic nerve injury.

Expt.	Week 2		Week 4		Week 6		Week 8		Week 10		Week 12		Week 14		Week 16	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	1.1	0.7	1.5	0.7	2.3	0.2	2.9	0.1	2.6	0.4	3.0	0.0	2.8	0.3	2.9	0.1
2	1.3	0.4	1.7	0.3	2.0	0.4	2.8	0.2	2.7	0.2	2.7	0.3	2.6	0.3	2.8	0.2
3	1.8	0.4	2.2	0.3	2.5	0.2	2.9	0.2	3.0	0.1	2.9	0.1	3.0	0.0	3.0	0.0
4	1.7	0.3	2.0	0.2	2.3	0.2	2.8	0.1	3.0	0.0	3.0	0.0	3.0	0.1	3.0	0.0
5	1.0	0.5	1.7	0.4	2.2	0.3	2.4	0.2	2.7	0.2	3.0	0.1	2.9	0.2	2.7	0.4
6	1.5	0.1	1.5	0.4	2.2	0.4	2.7	0.2	2.9	0.1	2.6	0.2	2.9	0.1	2.7	0.3
7	2.0	0.2	2.2	0.1	2.5	0.2	2.7	0.1	2.8	0.2	2.7	0.3	3.0	0.0	3.0	0.0
8	1.9	0.2	2.0	0.2	2.2	0.4	2.9	0.1	2.9	0.2	2.9	0.1	2.9	0.1	3.0	0.0
9	1.0	1.0	1.1	0.8	1.9	0.9	2.8	0.2	2.7	0.5	2.2	0.6	2.8	0.4	2.7	0.2
10	1.9	0.2	2.1	0.2	1.7	0.7	2.3	0.6	2.7	0.2	2.9	0.1	2.8	0.1	3.0	0.0

Sensory function was determined by cutaneous pain reaction test and graded 0–3.0 as described, previously [23,28]. Sensory function in the tibial, peroneal, sural and saphenous nerve boundaries were assessed individually and averaged to obtain total sensory function. Total sensory function steadily increased with time in all experimental groups studied; however, recovery was more profound in animals that were administered with stem cells and G-CSF or Dihexa directly into the target muscle gastrocnemius (Groups 7 and 8). N = 6 rats per group.



**Fig. 6.** Sensory Function Assessment in individual nerve boundaries: Sensory function was assessed by pinch technique in the territories of the tibial, peroneal, sural and saphenous nerves as described, previously [28,53]. Withdrawal/vocal response was scored in comparison to the naive contralateral limb (0 = no response, 1 = slight, 2 = moderate, 3 = normal). The sensory score was assessed in experimental groups 1–10 up to 16 weeks post sciatic nerve repair. Peroneal nerve function recovered first followed by tibial and sural nerve functions; the order of sensory function recovery in nerve boundaries was consistent in all experimental groups. However, saphenous nerve function was normal and not affected due to sciatic nerve transection because it is not a branch of sciatic nerve. By about 10 weeks the sensory functional recovery in nerve boundaries tested was nearly normal in all experimental groups (n = 6/group).



**Fig. 7.** Total sensory function recovery in MSC, G-CSF, and Dihexa administered groups. Sensory function was assessed by pinch technique in the territories of the tibial, peroneal, sural and saphenous nerves and graded as described, previously [28,53]. Total sensory function was calculated by averaging the mean responses of individual nerve boundaries in each experimental group  $([\text{peroneal} + \text{tibial} + \text{sural} + \text{saphenous}] \div 4)$ . Total sensory function recovery onset was relatively early and improved in Groups 7 and 8 where G-CSF or Dihexa was administered to the gastrocnemius muscle compared to control group 2 ( $n = 6/\text{group}$ ).

**3.2.2. Motor function**

The mean value of walking track foot prints graded on a scale of 0–4 as previously described [23] are presented in Table 2. At two weeks post SNR in all experimental groups, taken together the motor function ranged from Grade 2.3 to 4.00. However, with the passage of time, the motor function deteriorated in most of the groups (Grade 1.2 to 2.3 at 16 weeks post SNR) with the exception of Groups 7 and 8 (Grade 3.0) that received G-CSF and Dihexa, respectively, to the gastrocnemius muscle (Table 2). Motor function recovery with MSC (Group 4), MSC + G-GCF (Groups 5 & 7) and MSC + Dihexa (Groups 6 & 8) with or without gastrocnemius injection in comparison with saline control is shown in Fig. 8. Motor function was significantly ( $P < 0.05$ ) improved with MSC + G-CSF or MSC + Dihexa administration into the gastrocnemius muscle by 16 weeks post SNR. However, when G-CSF or Dihexa was administered locally and systemically (IP) without gastrocnemius administration there was no improvement in the motor function.

**3.3. Gastrocnemius muscle mass**

There was a significant decrease ( $P < 0.05$ ) in the gastrocnemius muscle weight in the sciatic nerve repaired limb (Right) compared to contralateral normal (Left) limb in all experimental groups studied (Table 3; Fig. 9). It ranged 32–45% reduction in gastrocnemius mass in the nerve-repaired limb compared to contralateral naïve limb. Furthermore, none of the treatment groups showed a significant reduction in

**Table 2**  
Limb motor function up to sixteen weeks post-sciatic nerve repair in rats.

Exptl. Group	Week 2		Week 4		Week 6		Week 8		Week 10		Week 12		Week 14		Week 16	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	2.3	1.5	2.5	1.2	1.8	1.0	2.2	1.5	1.8	1.2	1.5	1.2	1.5	1.4	1.7	1.2
2	3.3	1.2	4.0	0.0	2.2	1.5	1.7	1.2	1.3	1.4	1.3	0.8	1.7	1.2	1.5	1.2
3	2.8	1.2	2.7	1.2	2.3	1.5	2.2	1.5	2.2	1.5	2.2	1.0	2.5	1.0	2.3	1.0
4	2.7	1.5	3.8	0.4	3.3	0.8	2.8	1.5	2.2	1.5	2.5	1.2	2.7	0.8	1.7	0.5
5	3.5	0.8	3.3	1.2	2.3	1.4	1.7	1.4	1.8	1.5	1.7	1.4	1.7	1.0	1.5	1.4
6	4.0	0.0	3.4	1.3	1.6	1.5	1.2	0.8	1.2	0.8	1.4	0.9	1.0	0.7	1.2	0.8
7	3.8	0.4	3.5	0.8	3.2	1.2	3.2	1.0	3.2	1.0	2.8	1.2	3.2	0.8	3.0	0.9
8	3.7	0.5	3.8	0.4	2.7	1.5	2.5	1.4	2.5	1.2	2.5	1.2	2.7	1.2	3.0	0.9
9	4.0	0.0	2.6	1.5	2.6	1.5	2.2	1.6	1.8	1.1	1.4	1.5	2.0	1.9	2.2	0.8
10	4.0	0.0	2.7	0.8	1.5	0.5	1.8	1.2	1.8	0.8	1.7	0.5	2.0	1.1	2.0	0.6

Limb motor function (Mean  $\pm$  SD) was assessed by walking track analysis and graded (0–4) as described previously [53]. Animals administered with MSC and G-CSF or Dihexa (Groups 7 & 8) in to the target muscle (gastrocnemius) showed significant improvement by 16 weeks post sciatic nerve repair compared to controls (Groups 1, 2, 10) and other treatment groups (3–6, 9).  $N = 6$  rats/group.

loss of gastrocnemius muscle mass at study endpoint (~16 weeks post SNR).

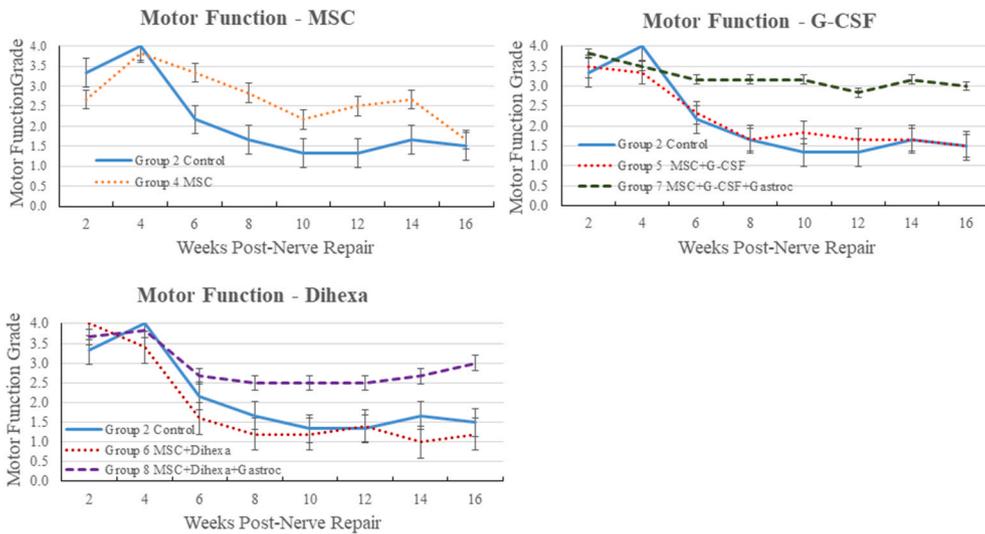
**3.4. Foot-flexion contractures**

Flexion contractures graded 0–4 in Groups 7, 8 and 10 are shown in Fig. 10. Flexion contracture was significantly ( $P < 0.01$ ) reduced in Group 7 (Grade 1.2) that received G-CSF + MSC administration into gastrocnemius muscle and in Group 8 (Grade 1.8) that received Dihexa + MSC administration in to gastrocnemius muscle when compared to vehicle control group 10 (Grade 3.2).

**4. Discussion**

The present study focused on identifying novel biologic therapies and their delivery systems to promote limb functional recovery in a rat sciatic nerve transection-repair model. Several experimental groups were included to identify the efficacy of novel agents as monotherapies and combination therapies in conjunction with mesenchymal stem cells. We demonstrated for the first time a significant improvement in sensory and motor limb function recovery, and reduced flexion contractures in SNR model particularly with the administration of G-CSF or Dihexa along with MSCs into the gastrocnemius muscle. Cellular and molecular analyses were beyond the scope of this study.

MSC monotherapy (Group 4) showed slight improvement in sensory



**Fig. 8.** Motor function recovery in MSC, G-CSF, and Dihexa administered groups. Walking track foot prints of normal and sciatic nerve transected/repared limb were obtained and graded based on heel and toe prints, as described previously [53]. There was a significant ( $P < 0.05$ ) improvement in motor function in Group 7 and 8 where G-CSF or Dihexa was administered to the gastrocnemius muscle compared to control Group 2. Increased flexion contractures observed in control Group 2 versus Group 7 or 8 (Fig. 10) resulted in poor foot prints/functional recovery ( $n = 6/\text{group}$ ).

**Table 3**  
Mean Gastrocnemius muscle weights at experimental endpoint.

Exp. Group	Left Gastrocnemius (g)	Right Gastrocnemius (g)	% Weight decrease in Right Gastrocnemius Mean
	Mean $\pm$ SD	Mean $\pm$ SD	
1	2.28 $\pm$ 0.17	1.40 $\pm$ 0.21	38.39 $\pm$ 9.37
2	2.07 $\pm$ 0.08	1.39 $\pm$ 0.19	32.97 $\pm$ 7.93
3	2.15 $\pm$ 0.17	1.45 $\pm$ 0.15	32.20 $\pm$ 10.10
4	2.29 $\pm$ 0.13	1.50 $\pm$ 0.09	34.21 $\pm$ 5.69
5	2.45 $\pm$ 0.28	1.61 $\pm$ 0.07	33.52 $\pm$ 8.16
6	2.46 $\pm$ 0.13	1.63 $\pm$ 0.20	33.32 $\pm$ 8.93
7	2.26 $\pm$ 0.11	1.44 $\pm$ 0.16	36.30 $\pm$ 6.25
8	2.18 $\pm$ 0.12	1.35 $\pm$ 0.16	37.80 $\pm$ 7.10
9	2.15 $\pm$ 0.07	1.41 $\pm$ 0.11	34.07 $\pm$ 5.37
10	2.17 $\pm$ 0.09	1.17 $\pm$ 0.16	45.84 $\pm$ 9.64

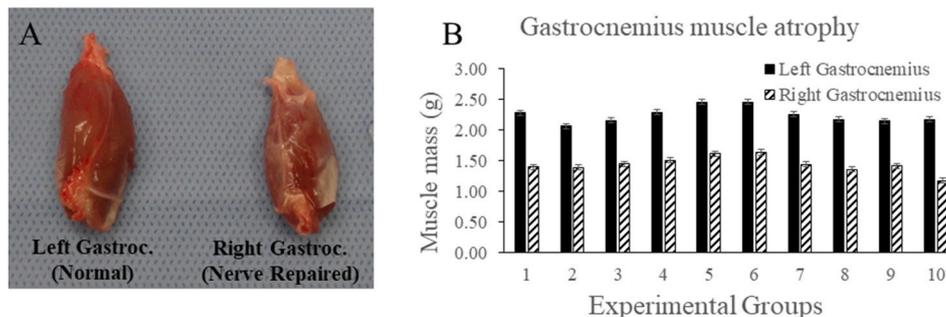
There was a significant decrease ( $P < 0.05$ ) in the gastrocnemius muscle weight in the sciatic nerve repaired limb (right) compared to contralateral naïve/normal (left) limb in all experimental groups studied. However, none of the treatment groups showed significant reduction in loss of gastrocnemius muscle mass at experimental end point ( $\geq 16$  weeks post-nerve repair) compared to contralateral limb.  $N = 6$  rats per group.

function recovery in our SNR model when compared to saline control (Group 2; Fig. 7) similar to previous report [5]. Song et al. have shown improved sensory function with MSC administration in a rat hind-limb transplant model [66]. The sensory function recovery in the peroneal, tibial, saphenous and sural nerve boundaries was evident by 1–6 weeks with further gradual progress until study endpoint (16 weeks) post SNR in all groups and is in agreement with previous reports where a rat sciatic nerve crush injury or transection model was used [5,21,46,74,

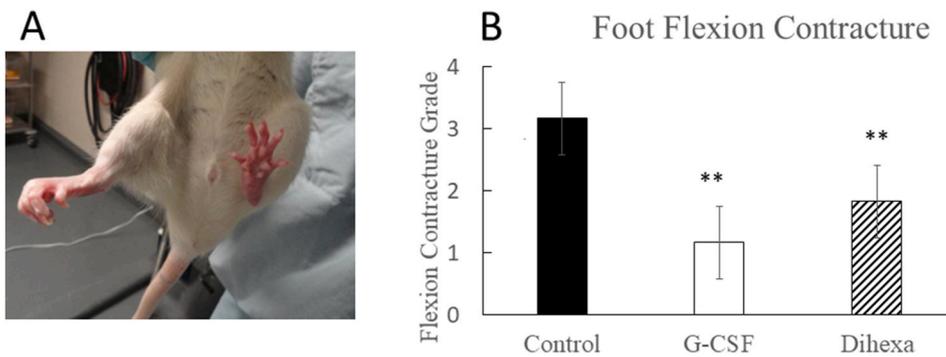
75]. Interestingly, Goel et al. [26] found enhanced nerve regeneration with the transplantation of bone marrow-derived mononuclear cells in their sciatic nerve transection model, and was attributed to stem cell trophic factors. Bone marrow MSCs have been shown to differentiate into Schwann cell-like cells both *in vivo* and *in vitro* and induce myelination of regenerated nerve fibers after sciatic nerve injury [10,15,63]. Improved sensory function in the present study was perhaps due to combination of Schwann cell formation and production of trophic factors by the MSCs administered.

Limb motor function was markedly improved with MSC therapy (Group 4) when compared to saline control (Group 2) which is in agreement with our previous report in SNR model [5]. The motor function results are in agreement with the findings of Wei et al. [78] in a partially transected nerve site wrapped with a scaffold containing adipose derived stem cells. Human amniotic fluid stem cells [56] and adipose derived MSC [47] administrations have been shown to improve limb motor function significantly in a sciatic nerve crush injury model. On the contrary, Song et al. [66] observed no improvement in the motor function (SFI) recovery with bone marrow-derived MSC administration. In the present study, suboptimal functional recovery was perhaps due to insufficient axon growth, improper alignment of the nerve fascicles following nerve transection-repair and other unknown factors [17,26,29,34,57,63,77].

Novel growth factors G-CSF and Dihexa in conjunction with MSC administration markedly improved sensory and motor function recovery (Figs. 7 and 8). It was the delivery of these novel agents and stem cells to the target muscle gastrocnemius that made a significant impact on improved functional recovery. The G-CSF related neuroprotection is mediated by cell mobilization, anti-inflammatory or anti-apoptotic



**Fig. 9.** A. Gastrocnemius muscle mass comparison between naïve (left) and sciatic nerve transected/repared limb (right). B. Gastrocnemius muscle atrophy was significant ( $P \leq 0.05$ ) in all sciatic nerve transected/repared limb in all experimental groups (Table 3;  $n = 6/\text{group}$ ).



**Fig. 10.** Foot flexion contracture in G-CSF and Dihexa treated rats. A. Foot flexion contracture was graded 0–4 as described previously [53]: 0, no contractures; 1, 0–30°; 2, 30–60°, 3, 60–90°, and 4, >90°. All animals developed some degree of foot contracture by 16 weeks post sciatic nerve repair. G-CSF (Group 7) and Dihexa (Group 8) administered to gastrocnemius muscle animals showed significant ( $P < 0.01$ ) reduction in flexion contractures compared to control animals (Group 10) that received vehicle ( $n = 6/\text{group}$ ).

activity [51,81]. Furthermore, treatment with G-CSF enhances muscle regeneration via the proliferation of satellite cells and by reducing the number of apoptotic cells and increasing the number of myocytes [30, 68]. Simoes et al. [64] observed G-CSF treatment to promote a favorable microenvironment for axonal regeneration and functional recovery in their sciatic nerve crush injury model in MDX mice. We believe the improved functional recovery with G-CSF administration in the present study was possibly due to a combination of above processes.

Dihexa and MSC combination therapy delivered to the gastrocnemius muscle in the present study showed significant improvement in limb motor function (Fig. 8). The improved motor function with Dihexa treatment in the present study could be due to the activation of HGF/c-Met system with subsequent promotion of muscle nerve innervation. Activation of the HGF/c-Met system has been shown to cause neuroprotective/neurorestorative activity in multiple disease states to include amyotrophic lateral sclerosis [35], Parkinson's disease [41], spinal cord trauma [39], and multiple sclerosis [1]. We believe that Dihexa can serve as an excellent candidate molecule to augment limb function following peripheral nerve damage. However, to our knowledge, there are no similar reports to compare our Dihexa findings, and we believe this is the first report with Dihexa treatment in peripheral nerve damage repair.

Significant limb muscular (gastrocnemius) atrophy observed in all experimental groups with SNR was probably due to poor nerve regeneration and re-innervation of the target muscles is in agreement with previous reports [31,50,72]. Administration of MSCs, G-CSF or Dihexa in our study did not mitigate the muscular atrophy in our SNR model similar to Song and co-workers [66] study in a rat hind-limb transplant model. In contrast, Chen et al. observed increased gastrocnemius mass and motor function recovery with bone marrow-derived stromal cell administration but it was in a sciatic nerve conduit model [10]. The results appear to vary with the models used. Nonetheless, in our study, motor function improved significantly with MSC and G-CSF or Dihexa administration into gastrocnemius muscle compared to controls that did not receive injections into gastrocnemius.

In the present study, progressive foot flexion-contractions developed over time following SNR and resulted in poor foot prints in walking track analysis. The flexion contracture development is a common occurrence following limb transplants and sciatic nerve repair due to poor peripheral nerve regeneration and muscle innervation which is well documented [22,73]. Procedures to mitigate the development of foot flexion-contractions in limb transplantation and crushed/transsected sciatic nerve injury models have resulted in modest success. However, in the present study, flexion contractures were significantly reduced and motor function recovery improved (based on foot print analyses) in animals that received MSC and G-CSF or Dihexa injections into their gastrocnemius muscle, which perhaps was due to improved muscle innervation.

We demonstrated improved functional recovery with the administration of MSCs, G-CSF and/or Dihexa in to target muscle gastrocnemius. Increased neurotrophic factor expression at the site of nerve injury via

stem cell therapy or exogenous administration of growth factors results in an increase in axon sprouting, improved nerve and muscle regeneration of the target organ and consequent accelerated motor function recovery [79]. It is documented that neurons express G-CSF receptors, and exogenous G-CSF promotes peripheral nerve regeneration and function significantly [56]. We believe G-CSF and Dihexa when combined with MSCs have high potential to enrich the tissue-injured microenvironment and enhance peripheral nerve damage repair and functional recovery.

In this study, we administered MSCs topically and systemically. Topical administration is advantageous as MSCs arrive at the site of the lesion. When administered intravenously, MSCs migrate, and extravasate at the SNR lesion site in response to chemoattractants such as stromal cell derived factor 1 (SDF-1); this is due to the interaction of CXCR4 receptor expressed on MSCs and SDF-1 [33,65] which is referred to systemic homing [52]. However, there is increasing evidence that MSCs administered intravenously are easily trapped in several tissues (lung, liver or spleen) due to their larger size and adhesive nature (expression integrin CD49f or CD49d) as a result about 2% of cells delivered reach the target lesion site [16,54]. To enhance MSC/G-CSF/Dihexa survival, localization and sustained release at the site of injury, we delivered them via hydrogel.

## 5. Limitations

There were a few limitations in this study and we believe addressing them will improve future studies. We evaluated the sensory function by manually pinching the nerve boundary with forceps and recorded response, which is a standard and acceptable methodology. However, electrostimulation is preferred to precisely stimulate nerve boundaries at particular stimulus strength consistently in all animals and measure the response. Manual physiotherapy to rats included gentle flexing and extending the limb and housing animals in a wire-mesh floor cage for about 8 h a day. Though these physiotherapy methods are generally acceptable, using an animal treadmill would provide consistent and effective physiotherapy.

## 6. Conclusions

The strategy of utilizing novel agents (Dihexa; G-CSF) in conjunction with MSCs is attractive, feasible, and promising in the improvement of functional recovery in peripheral nerve injury. Limb functional recovery following SNR was superior when we delivered MSCs and G-CSF or Dihexa directly to the target muscle in addition to local and systemic administrations. We believe the insights gathered from this study regarding the role of novel agents and delivery systems tested in peripheral nerve damage repair would make a significant impact in peripheral nerve injury repair and limb transplantation outcomes. The findings warrant further investigation to understand the cellular/molecular mechanisms involved in the improved functional outcome.

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## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2021.102917>.

## Disclosures/disclaimers

The views expressed are those of the author(s) and do not reflect the official policy of the Department of the Army, the Department of Defense or the U.S. Government.

## Ethical Approval

Animals were used, cared, and maintained according to the 'Guide for the Care and Use of Laboratory Animals' published by the National Research Council/Institute of Laboratory Animal Research (ILAR). Animal housing, husbandry, and experiments were conducted as per the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) following approval by the Institutional Animal Care and Use Committee (IACUC) of the Madigan Army Medical Center, as per protocol and institutional guidelines.

Yes. Approved by the Madigan Army Medical Institutional Animal Care and Use Committee (IACUC). Approved Protocol No 215064.

## Provenance and peer review

Not commissioned, externally peer reviewed.

## Author contribution

Shashikumar K. Salgar: Conceptualization, study design, performed experiments, data analysis & interpretation, project administration, execution & supervision, Data curation, Formal Analysis, Funding support, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing Original Manuscript draft, Critical review, editing and approval of the final version of the manuscript.

Jessica B. Weiss: performed surgery & experiments, data collection, analysis & interpretation, critical review. editing & approval of final version of the manuscript.

Data curation, Formal analysis, Investigation, Visualization, Writing, review and editing.

Cody J. Phillips: performed surgery & experiments, data collection, analysis & interpretation, critical review, editing & approval of final version of the manuscript.

Data curation, Formal analysis, and Investigation, Writing, review and editing.

Edward W. Malin: Microsurgery advise, Critical review, editing &

approval of final version of the manuscript.

Vijay S. Gorantla: Scientific and microsurgery advise from clinical perspective, and critical review, editing & approval of final version of the manuscript.

Joseph W. Harding: Resources (Dihexa reagent preparation and supply), scientific/protocol advice, critical review, editing & approval of final version of the manuscript.

CONTRIBUTORS in other ways:

Ms. Mary DeHart, Ms. Jennifer Damcis and Ms. Elisabeth Dornisch provided technical/laboratory support.

Mr. Juan Tercero, Mr. John Schaphorst, Ms. Joanna Dandeneau and Mr. Branden Hubbard provided animal care/testing support.

## Guarantor

Shashikumar K. Salgar, PhD.

## Consent

Animal studies, Not Human studies.

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