Canine Adipose-Derived Mesenchymal Stromal Cells Enhance Neuroregeneration in a Rat Model of Sciatic Nerve Crush Injury

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Abstract

Crush injuries in peripheral nerves are frequent and induce long-term disability with motor and sensory deficits. Due to axonal and myelin sheath disruptions, strategies for optimized axonal regeneration are needed. Multipotent mesenchymal stromal cells (MSC) are promising because of their anti-inflammatory properties and secretion of neurotrophins. The present study investigated the effect of canine adipose tissue MSC (Ad-MSC) transplantation in an experimental sciatic nerve crush injury. Wistar rats were divided into three groups: sham ($n = 8$); Crush+PBS ($n = 8$); Crush+MSC ($n = 8$). Measurements of sciatic nerve functional index (SFI), muscle mass, and electromyography (EMG) were performed. Canine Ad-MSC showed meso-dermal characteristics (CD34-, CD45-, CD44+, CD90+ and CD105+) and multipotentiality due to chondrogenic, adipogenic, and osteogenic differentiation. SFI during weeks 3 and 4 was significantly higher in the Crush+MSC group ($p < 0.001$). During week 4, the EMG latency in the Crush+MSC groups had better near normality ($p < 0.05$). The EMG amplitude showed results close to normality during week 4 in the Crush+MSC group ($p < 0.04$). There were no statistical differences in muscle weight between the groups ($p > 0.05$), but there was a tendency toward weight gain in the Crush+MSC groups. Better motor functional recovery after crush and perineural canine Ad-MSC transplantation was observed during week 2. This was maintained till week 4. In conclusion, the canine Ad-MSC transplantation showed early pro-regenerative effects between 2–4 weeks in the rat model of sciatic nerve crush injury.

Keywords

cell-based therapy, mesenchymal stem cells, nerve regeneration, sciatic nerve injury, crush injury, myelin sheath

Introduction

Multipotent mesenchymal stromal cells derived from adipose tissue (Ad-MSC) have potential for use in translational medicine because they can be easily harvested with low morbidity and have high self-renovation capacity[1–3]. Several *in vivo* and *in vitro* studies have demonstrated the ability of Ad-MSC to repair the nervous system through substitution and fusion with myelinating cells, resulting in re-myelination; production of neurotrophins, which provides axonal protection; and production of anti-inflammatory cytokines, which decreases inflammatory reaction and supports axonal growth[1,4,5]. Other mechanisms of action include immuno-modulation, homing capacity, limitation of apoptosis, and angiogenesis promotion[4–9]. The therapeutic effectiveness of allogeneic Ad-MSC transplantation has generated

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fundamental interest[10]. Research on MSC in several animal models is crucial and may contribute to clarifying the effect, relocation, permanency, and functionality of cells in a non-self-environment[11,12].

Traumatic lesions of peripheral nerves lead to long-term disability, with motor and sensory deficits of the affected region, neuropathic pain, and reduction in quality of life[13,14]. Sciatic nerve and brachial plexus injuries are common in dogs[14]. Compression, stretching or traction, laceration, crushing and local injections of drugs are the most common etiologies of nerve injuries in dogs[14,15]. Kouyoumdjian[16] reviewed 456 patients with peripheral nerve injury. Vehicle accidents affecting the brachial plexus or radial, sciatic, facial, and peroneal nerves were the most common. Neurotmesis occurred in 41% of cases, while axonotmesis occurred in 45% of cases. Vehicle accidents resulted in injuries to the upper limb nerves in 75.3% of cases[16].

The peripheral nervous system (PNS) shows intrinsic regeneration post-trauma. However, functional recovery fails in severe injuries, leading to axon degeneration and subsequently Wallerian degeneration and loss of muscle innervation[17–19]. Schwann cells in the distal stump maintain their regenerative phenotype. However, diffuse axonal impairment and myelin sheath disruptions, lesion of the proximal nerve, chronic insult, and motor neuron damage impede recovery[20,21]. There are ethical restrictions regarding the induction of injuries in companion animals; thus, animal models are crucial for peripheral nerve research. The sciatic nerve crush in rats (axonotmesis) causes myelin and axon disruption, and is widely used to investigate functional achievement and organ re-innervation[2,22].

Our hypothesis is that canine Ad-MSC transplantation in rat sciatic nerve injuries stimulates neuroregeneration. In this context, the aim of this study was to evaluate the regenerative effects of Ad-MSC local transplantation in experimental sciatic nerve injury in rats.

Material and Methods

Experimental Design

Ninety-day-old Wistar rats ($n = 24$) (from central bioterium of the São Paulo State University, Brazil) weighing between 200 and 300 g were subjected to axonotmesis. They were maintained in controlled conditions (12/12 light: dark cycle, at a room temperature between 20 and 22°C) with ad libitum access to food and water. This study was approved by the committee for experimental ethics in animal use (CEUA/FMB/UNESP protocol PE-2/2015).

The rats were randomly assigned to three groups. The Sham group ($n = 8$) was subjected only to approach of the sciatic nerve. The Crush+PBS group ($n = 8$) was subjected to crush lesion followed by perineural application of phosphate-buffered saline (PBS). The Crush+MSC group ($n = 8$) was subjected to crush lesion followed by perineural transplantation of canine Ad-MSC.

Evaluations were performed by electromyography (EMG) at pre-treatment (week 0) and 4 weeks post-treatment. The sciatic nerve functional index (SFI) was measured pre- (week 0) and post-treatment during weeks 1, 2, 3, and 4. During week 4, muscle mass measurement was performed on the tibial cranial and gastrocnemius muscles.

Isolation, Culture, and Characterization of Canine Ad-MSC

Inguinal subcutaneous fat segments were harvested from the inguinal area from each dog under inhalation anesthesia. These segments were finely cut with scissors, digested with collagenase type 1 (0.04%) (Sigma-Aldrich, St. Louis, MO, USA), and shaken for 60 min at 37°C. The cells were seeded in T-75 cell culture flasks with 15 ml of culture medium containing 90% Dulbecco's Modified Eagle Medium (DMEM) high glucose, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (10,000 U/mL), and 1.2% amphotericin B (3 mg/mL) (all from Invitrogen, São Paulo, Brazil) and 0.005% amikacin (250 mg/mL) (Novafarma, Anapolis, Brazil). The culture flasks were incubated at 37°C in a humidified chamber containing 5% CO_2 and 95% oxygen. The medium was changed twice weekly. When cells reached 80% confluence within 2–3 weeks, they were harvested using trypsin (trypsin, 0.25%, Invitrogen, São Paulo, Brazil) and cryopreserved in liquid nitrogen. Cryopreserved cell stock was used for characterization *in vitro* and transplantation.

The canine Ad-MSC was characterized by plastic adherence, colony formation, and fibroblast morphology 14–21 days after start of culture. Furthermore, the Ad-MSC showed tri-lineage differentiation potential (the ability to differentiate into chondrocytes, adipocytes, and osteocytes) and expression of surface markers including CD34-, CD45-, CD44+, CD90+, and CD105+ by flow cytometry. These features support the mesodermal fate of these cells.

Cell viability was evaluated using the trypan blue (0.4%) exclusion method. Viable cells were determined according to the formula: Viability (%) = cells unstained (viable) × 100/total cells (stained+unstained). The viability of the transplanted canine MSC was between 70% and 80%.

MSC Transplantation

Rats were anesthetized using isoflurane (Isoforine®, Cristália, Brazil). The surgical posterior approach to the left sciatic nerve through the gluteal muscle was performed using microsurgery instruments. The axonotmesis injury (crush) was performed using a hemostatic forceps for 60 s in the Crush+PBS and Crush+MSC groups as previously described[23,24]. The observation of translucent nerve bands confirmed the crush lesion. In the Crush+MSC group, perineural transplantation of 10^6 cells in suspension was performed using the Hamilton microsyringe. In the Crush+PBS group 10 µl of PBS buffer solution was applied by perineural injection.

Sciatic Nerve Functional Index

This test was performed in weeks 1, 2, 3 and 4 in the Sham, Crush+PBS, and Crush+MSC groups. Footprints of the hind limbs were taken for walking track analysis. Several measurements were obtained from the footprints to obtain the SFI. They include (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). These parameters were measured for both normal (N) and experimental (E) feet. The SFI were analyzed using the following equation: -38.3 ([EPL-NPL]/NPL) +109.5 ([ETS-NTS]/NTS) +13.3 ([EIT-NIT]/NIT) - 8.8. An SFI value of -100 indicates total crush or transection of the sciatic nerve, while an SFI value of 0 means intact sciatic nerve[25].

Electrophysiological Test

The latency (m/s) and amplitude (mV) were measured using EMG in the Sham, Crush+PBS, and Crush+MSC groups before experimental injury of the sciatic nerve and 4 weeks after the injury. A bipolar stimulation electrode was positioned in the proximal stump of the sciatic nerve. The active surface electrode (black) and a reference electrode (red) were placed in the belly of the tibial cranial muscle and near its insertion point, respectively. A dispersive electrode (green) was placed in the ventral abdominal region. A Sapphire II 4ME instrument (Teca Medelec, USA) was used; the intensity ranges for stimulation and the filter were 10–20 mA and 20–2,000 Hz, respectively.

Muscle Atrophy

The tibial cranial and gastrocnemius muscles of the left (atrophied) and right (normal) hind limbs were collected 4 weeks later to compare muscle weight among Sham, Crush+PBS, and Crush+MSC groups. To do this, the animals were humanely euthanized at the end of the experiment.

Statistical Analysis

The variables (amplitude, latency, SFI, and muscle mass) were evaluated for their normality with statistical tests (Shapiro–Wilk) and graphical analyses. An analysis of variance test followed by Tukey's test was performed to verify the difference in means of the variables between each group and moment of the experiment. All analyses were performed using the Statistical Analysis Software – SAS version 9.3 (SAS Institute, 2011). Non-repeated and unpaired measures were analyzed with the Mann–Whitney test (GraphPad Prism version 5.01 for Windows, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.
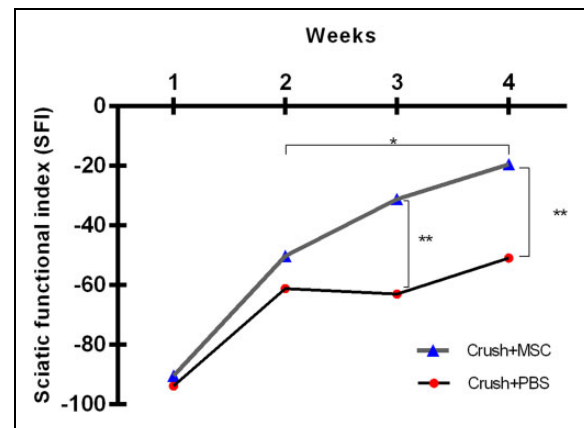

Fig 1. Sciatic nerve functional index (SFI). The test was performed during 4 weeks in the Crush+PBS and Crush+MSC groups, after Ad-MSC transplantation. *Difference between groups ($p < 0.05$).

Results

Sciatic Nerve Functional Index

Significant differences were observed after 3 and 4 weeks between the Crush+MSC (SFI; –31.15 during week 3 and –19.42 during week 4) and Crush+PBS groups (SFI; –62.91 during week 3 and –50.85 during week 4) ($p < 0.001$), as shown in Fig 1. The Crush+MSC group showed mean values close to normal.

A significant difference was observed within the Crush+MSC group after 2 and 4 weeks, with values of –50.19 after 2 weeks and –19.42 after 4 weeks ($p < 0.01$), as shown in Fig 1. No significant difference was observed within the Crush+PBS group after 2 and 4 weeks, with values of 61.12 after 2 weeks and –50.85 after 4 weeks ($p > 0.05$).

Electromyography

Latency. The mean latency of the Crush+MSC group did not show any significant difference between week 0 (pre-treatment; 1.54 m/s) and week 4 (post-treatment; 2.23 m/s) ($p > 0.05$). The mean latency values of the Crush+PBS group were not significantly different between weeks 0 (1.74 m/s) and 4 (2.16 m/s) ($p > 0.05$), as shown in Fig 2. These results show that the latency of both groups was higher than the normal values.

No significant differences in latency were observed among the Sham, Crush+MSC, and Crush+PBS groups ($p > 0.05$) after week 4 with values of 1.59, 2.23, and 2.16 m/s, respectively, as shown in Fig 2.

Amplitude. Significant differences were observed in the mean amplitude during week 4 between the Sham (23.75 mV) and Crush+PBS (10.04 mV) groups ($p < 0.05$). However, there were no significant differences between the Sham (23.75 mV) and Crush+MSC (17.26 mV) groups, as shown in Fig 3. These findings show better amplitude results close to normal in the Crush+MSC group.
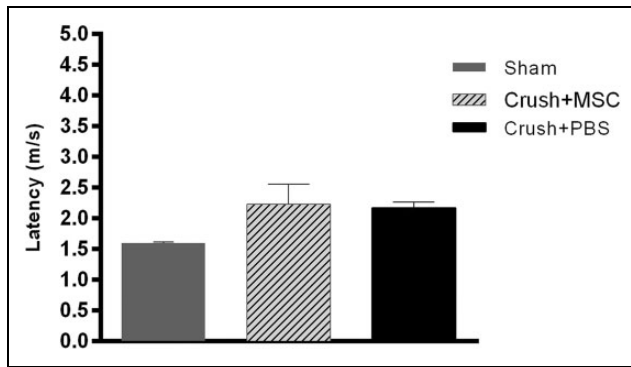
Fig 2. Electromyographic latency. The test was performed in the Sham, Crush+PBS, and Crush+MSC groups before experimental injury of the sciatic nerve and 4 weeks after the injury. *Difference between groups ($p < 0.05$).
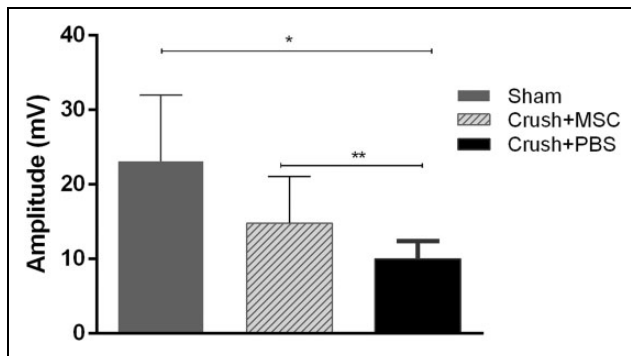

Fig 3. Electromyographic amplitude. The test was performed in the Sham, Crush+PBS, and Crush+MSC groups before experimental injury of the sciatic nerve and 4 weeks after the injury. *Difference between groups ($p < 0.05$).

Significant positive differences in amplitude were observed among the Crush+MSC and Crush+PBS groups ($p < 0.001$) during week 4 with values 17.26 and 10.04 mV, respectively, as shown in Fig 3. This suggests the presence of more myelinated fibers innervating the muscle in the Crush+MSC group.

Weight of the tibial cranial and gastrocnemius muscles. The weight of both tibial cranial and gastrocnemius muscles in the right limb during week 4 was significantly different among all experimental groups ($p < 0.001$), as shown in Table 1. There were no differences in weights of both gastrocnemius ($p = 0.139$) and tibial cranial ($p = 0.075$) muscles among the groups. However, there was a tendency toward an increase in muscle mass in the Crush+MSC group.

Discussion

Promoting a permissive microenvironment for neuroregeneration after peripheral nerve injury is crucial[2,20]. The alignment and organization of nerve fibers and neurotrophins play

an important role in nerve regeneration[8,26]. However, cellular components are indispensable for optimized nerve regeneration[27,28]. Cell transplantation has therefore become a challenging field of interest. Local implantation after nerve repair with guiding tubes for neural stem cells or bone marrow and adipose tissue mesenchymal stem cells has been shown to improve peripheral nerve regeneration in neurotmesis injury[12,28–30]. Transplantation of Schwann cells has shown beneficial effects. Despite the results, there are limitations owing to limited availability and expensive culture[21,31].

To overcome this difficulty, MSC transplantation has been an area of active research owing to high self-renewal, growth capacity, and ease of acquisition and culture[1,32]. Evidence suggests that MSC confer beneficial effects on peripheral nerve regeneration in several animal models[12,24,33–38]. Neuroprotective effects were demonstrated using human MSC in rats[27,39]. However, to date, only few studies have evaluated the clinical and electrophysiological effects of canine Ad-MSC on experimental sciatic nerve injury in rats.

The SFI gait analysis is a well-established method for assessing motor recovery and performance in hind limbs in rats after experimental sciatic nerve injury[25,40,41]. In our study, there was better gait recovery in the Crush+MSC group treated with canine Ad-MSC after axonotmesis lesion compared with the non-treated group (Crush+PBS). It was observed that the perineural transplantation of canine Ad-MSC 2 weeks after axonotmesis accelerated motor functional recovery. The SFI analysis indicated that Ad-MSC contributed to significant early improvement 2 weeks after perineural transplantation and this was maintained until the end of the experiment (week 4). These observations were consistent with the positive results obtained in the EMG latency and amplitude.

Electrophysiological analysis was used to determine the motor functional recovery of myelinated axons. The group treated with canine Ad-MSC (Crush+MSC) showed better electrophysiological results in latency and amplitude compared with the non-treated group (Crush+PBS). The results indicate greater axonal sprouting and presence of myelinated fibers, which are responsible for the electrophysiological response and neuromuscular transmission. Our study demonstrates that the latency and amplitude in Crush+MSC groups were close to normal values. However, the recovery was incomplete. These findings are reinforced by the positive results obtained in the SFI analysis. Our results are similar to those of other studies[36,38,42,43].

Although there was a tendency toward recovery of muscle mass in the Crush+MSC group, a significant difference was not observed. Muscular homeostasis and the regenerative capacity of muscles depend on electrophysiological and biochemical communication between nerves and muscles[2,20,41]. In clinical settings, denervation at the proximal segment of the nerve leads to rapid muscle mass loss[13–15]. Accelerated restoration of the nerve function to prevent muscle atrophy is essential[19,21]. The absence of complete muscle recovery

Table 1. Weight of the Tibial Cranial and Gastrocnemius Muscles at Week 4.

Groups	Right Gastrocnemius (g) Mean/SD	Left Gastrocnemius (g) Mean/SD	Right Tibial Cranial (g) Mean / SD	Left Tibial Cranial (g) Mean / SD
[†]Crush+PBS	1.71 ± 0.41	0.54 ± 0.10*	0.54 ± 0.10	0.39 ± 0.11*
[‡]Crush+MSC	1.70 ± 0.41	0.68 ± 0.15*	0.68 ± 0.15	0.50 ± 0.11*

Abbreviations: [†]Crush+PBS: crush lesion followed by perineural application of PBS; [‡]Crush+MSC: crush lesion followed by perineural transplantation of canine Ad-MSC; SD: standard deviation; g: grams.
*Compared with contralateral side (right). $p < 0.01$.

might be justified by insufficient time necessary for the regeneration of the muscle, long distance from the site of nerve injury to the muscle, and possible decreases in the receptivity of regenerated axons in the muscle[2,19,21,41].

From the results of our study, the positive effects of local perineural transplantation of canine Ad-MSC may be related to the high concentration of pro-regenerative molecules in the microenvironment of the lesion[44]. Importantly, intracytoplasmic vesicles in the MSC seem to have paracrine regenerative effects because they contain neurotrophic and proangiogenic factors[9,45]. The most important factors include the nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3/4 (NT-3/4), vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor, and glial-derived neurotrophic factor[9,44–46]. Neurotrophins can stimulate axon growth, preventing apoptosis and stimulating cell proliferation and regeneration by several pathways of axon growth promoters[47,48]. Studies have shown high expression of BDNF after MSC transplantation in the first 4–8 weeks after experimental lesion of the sciatic nerve. The effects were related to the production of neurotrophins[38,45,49]. In addition, MSC transplantation can promote cell replacement, angiogenic capacity, immunomodulatory and anti-inflammatory effects, migration, and survival in damaged tissues[1,32,35,50,51].

The distal environment of the damaged nerve and Schwann cells play an important role in axonal sprouting, formation of Büngner bands, and guidance of regenerating axons. The conduction of action potentials depends on the proliferation of Schwann cells and myelin production[19,20]. In our study, we observed significant motor functional improvement in nerve conduction owing to a high number of myelinated axons, determined by the EMG latency and amplitude. Possible differentiation of MSC into Schwann-like cells or the increase in intrinsic Schwann cell proliferation mediated by neurotrophins can explain these observations[5,8,52–55]. Greater expression of the S-100 Schwann cell marker was observed in rats treated with allogeneic and xenogeneic Ad-MSC after peripheral nerve injury compared with the control group[30,42,56]. Other *in vivo* studies have demonstrated the co-expression of S-100, the receptor specific for the BDNF, and NT-3,4,5 neurotrophins following experimental axonotmesis and neurotmesis in the sciatic nerve in rats after MSC transplantation[42,49].

Canine MSC perineural transplantation promotes motor functional and electrophysiological recovery in axonotmesis injury. Canine MSC perineural transplantation is easy and results in a higher concentration of cells at the lesion site. However, the fact that the perineural cells are preserved in this model can positively influence the regeneration process maintaining the guiding structure for the growth of axons and Schwann cells[57,58]. Spontaneous functional recovery in experimental murine axonotmesis was observed between 7 and 9 weeks[59]. Our results demonstrated motor functional and electrophysiological recovery in a short period (3 weeks) after canine Ad-MSC perineural transplantation, reinforcing the cell-mediated effect at the lesion site after experimental axonotmesis. Our study suggests that the canine cells were effective and presented adaptive capacity in the inflammatory environment facilitating regeneration.

Lastly, canine Ad-MSC presents a relevant therapeutic potential in the acute axonal injury model, representing a valuable tool for the treatment of PNS traumatic, inflammatory, or degenerative diseases in animals. A limitation of the study is the short time for evaluating recovery (4 weeks) necessary to reach maximum neuroregeneration. We believe that functional evaluation is important in nerve regeneration studies, since morphological recovery does not often reach maximum values between 3 and 6 months[60].

Conclusion

Canine Ad-MSC promoted electrophysiological and motor recovery in the rat sciatic nerve 3 weeks after crush injury. Future studies are needed to evaluate the therapeutic potential of canine MSC in the regeneration of peripheral nerves through clinical trials in dogs.

Authors' Note

Diego N.R. Sánchez, Luis A.L. Resende, and Rogério M. Amorim made contributions to the definition of intellectual content. Diego N.R. Sánchez, Giovana B.A. Pinto, Ana L.C. Bovolato, and Elenice Deffune performed the experimental study and data acquisition. Diego N.R. Sánchez, Fábio S. Possebon, and Rogério M. Amorim analyzed the data. All authors wrote, reviewed, and approved the manuscript.

Acknowledgments

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