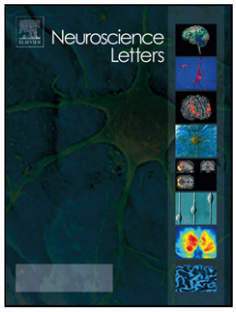

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Authors: E. Krueger, L.M. S. Magri, A.S. Botelho, F.S. Bach, C.L.K. Rebellato, L. Fracaro, F.Y.I. Fragoso, J.A. Villanova JR, P.R.S. Brofman, L. Popović-maneski

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Effects of Low-intensity electrical stimulation and adipose derived stem cells transplantation on the time-domain analysis-based electromyographic signals in dogs with SCI

E. KRUEGER[1,2], L. M. S. MAGRI[2], A. S. BOTELHO[3], F. S. BACH[4], C. L. K. REBELLATO[4], L. FRACARO[4], F. Y. I. FRAGOSO[4], J. A. VILLANOVA JR[4], P. R. S. BROFMAN[4], L. POPOVIĆ-MANESKI[5]

[1]Neural Engineering and Rehabilitation Laboratory, Master and Doctoral Program in Rehabilitation Sciences UEL-UNOPAR, Anatomy department, State University of Londrina, Londrina, Brazil.
[2]Graduate Program in Biomedical Engineering, Technological Federal University - Paraná, Curitiba, Brazil.
[3]Federal University of Pará, Belém, Brazil
[4]Pontificial Catholical University of Paraná, Curitiba, Brazil
[5]Institute of Technical Sciences of the Serbian Academy of Sciences and Arts, Belgrade, Serbia.

E-mail: kruegereddy@gmail.com; botelhoagatha@outlook.com

Highlights

- **Adipose derived mesenchymal stem cells transplantation contributed positively to the clinical improvement**
- **The percutaneous electromyography response during loading on hind limb showed voluntary contraction improvements 30 and 60 days after the experiments**
- **The low-intensity electrical stimulation, stem cell therapy, and both therapies together, showed positive effects in the patients, but without statistical differences between them**
- **The therapies also showed positive results in chronic patients**

Introduction. The application of low-intensity electrical stimulation (LIES) to neural tissue increases neurochemical factors responsible for regeneration as nerve growth factor. Stem cell (SC) therapy for patients with Spinal cord injury (SCI) promote some increase functional improvement. ***Objective.*** Investigate the electromyographic response in paraplegic dogs

undergoing LIES and SC transplantation. ***Methods.*** 27 dogs paraplegics with SCI were divided into three groups with different types of therapy. G_{ADSC}: two SC transplants (n=9); G_{LIES}: LIES (n=8); G_{COMB}: two SC transplants and LIES (n=10). Adipose derived mesenchymal stem cells (ADSCs) were transplanted by lumbar puncture in the amount of $1.2x10^6$ cells/50µL. Acupuncture needles positioned in the interspinous space were used for stimulation. The electrical stimulation was applied with a mean voltage ~30 mV and four consecutive modulated frequencies (5Hz, 10Hz, 15Hz and 20Hz) within 5 minutes each. The patients motor performance was evaluated before (Pre) the procedure and after 30 ($Post_{30}$) and 60 ($Post_{60}$) days, from electromyography root mean square (EMG_{RMS}) registered with subcutaneous electrodes in the vastus lateralis muscle, while the animals were in quadrupedal position. ***Results.*** All three groups showed a significant intra-group increase of EMG_{RMS} (Pre vs. $Post_{30}$ or Pre vs. $Post_{60}$). However, there were no statistically significant differences between $Post_{30}$ and $Post_{60}$. The inter-group test (G_{ADSC} X G_{LIES} X G_{COMB}) did not present significance when compared the instants Pre ($p = 0.34$), $Post_{30}$ ($p = 0.78$) and $Post_{60}$ ($p = 0.64$). ***Conclusion.*** Some dogs recovered motor activity, expressed by the $EMG_{RMS,}$ in all groups, in pre vs. post (30 or 60 days) comparisons.

Key words: Electrotherapy, Neural Rehabilitation, Spinal Cord Injury, Adipose derived Mesenchymal Stem Cells.

Introduction

Spinal cord injury (SCI) is classified as a neurological disorder that affects motor and sensory functions below the injury level, causes various degrees of deterioration and functional loss to the axons of the spine and their downstream targets [20]. There are several methodologies that can be used as assistive and/or therapeutic approaches after spinal cord injuries, such as functional electrical stimulation, robot-assisted training, epidural stimulation and intraspinal microstimulation [10]. A recent study of Donati et al. [16] showed that intensive training leads to partial recovery of functions below the lesion level and improvements from level A to C on ASIA scale; however, it is more likely that initial classification of patients is inaccurate in most cases when assessed immediately after injury, and after a period of initial care, because intensive training allows patients to reach maximal potential of the remaining functions. Recently, Capogrosso et al. [8] tested a brain–spine interface in monkeys, as a bypass on a lesion, with promising but incipient results.

One interesting finding of regenerative medicine is the applicability of stem cells (SC) in different human and animal tissues. Stem cells are defined as cells that have the capacity of

self-renewal as well the differentiation capacity in several lineages of cells [19, 34]. Mesenchymal stem cells (MSCs) are adult stem cells and have the advantage of easy isolation, expansion and low antigenicity, which allow the use of allogeneic MSCs [7]. The mechanisms through which this type of cell therapy achieves neurological recovery have not yet been fully understood. After MSC transplantation, various repair processes take place, including the release of neurotrophic factors by the cells [40], reduction of neuroinflammation [23], or the activation of endogenous mechanisms of the spinal cord, able to partially restore neurological functions previously abolished [46].

Low-intensity electrical stimulation (LIES) uses current intensities around 0.5-1mA and voltage amplitudes around 3-5V, that do not activate motor nerves. Since 1982 the LIES has been tested as an adjuvant to peripheral nerve regeneration [1] and could effectively improve the speed and accuracy of nerve regeneration after peripheral nerve injury [52]. Controversially, electrical stimulation with high voltage pulses can retard the effective nerve growth [53]. Studies like those from Franz et al. [21] have demonstrated that electrical stimulation applied for 1 hour to transected peripheral nerves accelerates the reinnervation of distal target tissues like muscles.

Despite the positive results obtained in experimental models using a variety of animal species and significant recent advances in cell transplantation, the development of effective strategies to treat chronic spinal cord injuries remains a major clinical challenge [28]. In order to solve this challenge, the aim of this study was to evaluate the effects of treatment with low-intensity electrical stimulation and/or ADSCs (Adipose-derived stem cells) transplantation in paraplegic dogs. Our hypothesis is that both types of therapies would lead to some motor recovery, but combined treatment with low-intensity electrical stimulation and ADSCs transplantations would result in highest recovery rates.

Methods and materials

Participants

This work was approved by Animal Ethics Committee of Technological Federal University - Paraná according to the protocol number 2015 – 016. The animal care and all operative procedures were performed according to animal care and safety guidelines. The dog proprietors signed the informed consent. The study included 33 dogs with spinal cord injury (Table 1). Inclusive criterion was adult paraplegic dogs. Exclusive criteria were tetraplegia, the presence of infections, absence of quadricipital reflex, the presence of voluntary contraction or treatment not finalized. After the experimental procedures, both hind limbs (right and left) were included in the evaluation analysis. Four dogs were excluded due to the presence of voluntary contractions (N=4), and two dogs did not conclude the experiments (N=2). We included twenty-seven adult dogs paraplegics aged between two and seven years with a history of chronic thoracolumbar spinal cord injury (between six months and two years), without concomitant disease. They were subjected to clinical evaluations to determine the severity of the lesion as described in [43].The included participants (N=27) were randomly divided into three groups: ADSC two stem cells transplants, with an interval of one week (N=9), LIES - low-intensity electrical stimulation (N=8) and COMB – two stem cells transplants, with an interval of one week, plus low-intensity electrical stimulation (N=10).

Research design

Figure 1 illustrates the research design. The sequence of experiments was:

- First day: the first electromyography assessments (Pre);

- One week after first evaluation: the experimental treatment was applied (ADSC, LIES or COMB) twice with one week of interval between them;
- One month after injury: the second electromyography assessments ($Post_{30}$);
- Two months after injury: the third electromyography assessments ($Post_{60}$).

Figure 1

Electromyography acquisition

The equipment used for the signal acquisition was purchased and calibrated by EMG System do Brasil Ltda®, model 430C. The amplification was 2000, and sampling frequency was 1 kHz. The pair of bipolar EMG electrodes (disposable acupuncture sterilized needle type, stainless steel, 0.25 x 30mm - DongBang®), connected with alligator clamps, were inserted subcutaneously (not intramuscular) in the *vastus lateralis* muscle. On the lateral aspect of the thigh in the craniocaudal direction are the muscles: sartorius (fine and long); tensor of the fascia lata (triangular shape); vastus lateralis (covered by fascia lata) and femoral biceps. The vastus lateralis and femoral biceps muscles are the most voluminous and it is possible to identify a mild depression in non-obese animals. Another way to differentiate them is that only the vastus lateralis muscle is inserted in the greater trochanter of the femur, an easily palpable bone eminence, even in obese animals.

The reference electrode was positioned subcutaneously in the region of the spinous process of the fourth lumbar vertebrae. The acquisition was initiated while the animals were supported by a technician in quadrupedal position. After that, the animal was raised by the animal's front limbs to get loading (in total ~10s) on the hind limbs.

Signal processing

The signal processing was performed by a customized routine in MatLab® (MathWorks, Inc, version 2013). The signals during the assisted bipedal position, 2s (in the middle of 10s) were used for EMG analysis. The EMG signals were filtered using a 3rd order Butterworth band-pass filter (20-450 Hz) with notch filters on power line harmonics (60, 120, 180, 240, 300, 360 and 420 Hz). The root mean square (EMG_{RMS}) was calculated.

Anesthetic and surgical procedure

The surgery procedure was accomplished in PUCPR Veterinary Hospital, Curitiba - Brazil. As premedication, first, the fentanyl (2 µg/kg) and ketamine (1 mg/kg), and after that propofol bolus (5 mg/kg), were administered intravenously. The anesthesia was maintained with isoflurane inhalation and fentanyl (0.1 µg/kg/min). Wide trichotomy was performed in the dorsal region, followed by antisepsis with polyvinylpyrrolidone. With the patient positioned on the ventral side, the ADSCs transplantation was performed in epidural space according to the technique described by Dewey [15].

Postoperative care

The patient remained under observation for 72 hours in the semi-intensive care unit of the PUCPR veterinary hospital. During five days, the patient received analgesia with tramadol (2mg/kg/bid) and Dipyrone (25 mg/kg/bid) subcutaneously. Antibiotic prophylaxis was performed by subcutaneous injection of enrofloxacin (5 mg/kg/bid) during seven days. The dogs' owners received instructions for the rest of the period, as well as for the monitoring of urinary function and mild physical activities.

Adipose derived stem cells and cell transplantation

Adipose derived stem cells (n=3) were isolated using enzymatic dissociation [56]. Briefly, after consent of the dog owners, part of the omentum was removed, to serve as a source of adipose tissue, the material was collected during elective ovariohysterectomy surgery. The omentum, was cut into small pieces and washed with sterile phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA). The dissociation step was performed with 1 mg/mL collagenase type I (Gibco, Grand Island, NY, USA), for 30 minutes, at 37°C, under permanent shaking, followed by filtration through a 100 μm mesh filter (BD Biosciences Discovery Labware, Bedford, MA, USA). Cell suspension was centrifuged at 800 g for 10 minutes, and contaminating erythrocytes were removed with cell lysis buffer. Cells were then washed, counted and $1x10^5$ cells/cm^2 were seeded in 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium (DMEM-F12) medium (Gibco, Grand Island, NY, USA), supplemented with 10% of fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (100 units/mL, Sigma-Aldrich, Saint Louis, MO, USA) and streptomycin (100 μg/mL, Sigma-Aldrich, Saint Louis, MO, USA). Cells were kept at 37°C in a 5% CO_2 atmosphere and the medium was changed after two days. Culture medium was then replaced twice-a-week. ADSC were cultivated until cells reached 80%-90% confluence; then cells were detached with 0.25% trypsin/EDTA (Gibco, Grand Island, NY, USA) and were seeded as passage-1 cells. The cells were expanded until the desired cell numbers were obtained for transplantation.

The characterization of cells was established by the potential of cell differentiation and the expression of cell-surface antigen profile. ADSC were assessed for their potency by inducing their differentiation into adipocytes, osteoblasts and chondrocytes. ADSC were seeded on glass coverslips in twenty-four wells plates (Sarstedt, Newton, USA) to evaluate adipogenic

and osteogenic differentiation. Commercial differentiation media (GibcoTM Invitrogen, NY, USA) was used during 21 days. ADSC were stained with Oil Red O to analyze adipogenic induction and with Alizarin Red S for osteogenic differentiation. In chondrogenic differentiation assays, the cells were cultivated in three-dimensional conformation and stained with Toluidine Blue [30].

In order to determine cell-surface antigen profile, ADSCs were labelled with antibodies against several dog proteins to analyse cell-surface expression of typical marker proteins: CD29 PE (canidae reactivity – Abcam - Cambridge, United Kingdom), CD44 Alexa fluor 488 (dog reactivity – AbD Serotec - Bio-Rad, Hercules, CA, USA), CD90 PE (human reactivity – BD Pharmingen - San Jose, CA, USA), CD45 FITC (canidae reactivity – eBioscience - Thermo Fisher, San Diego, CA, USA), CD34 PE (canidae reactivity – eBioscience - Thermo Fisher, San Diego, CA, USA) and CD14 APC (human reactivity – BD Pharmingen - San Jose, CA, USA). For cell viability and apoptosis cells were labeled with 7-aminoactinomycin D dye (7-AAD) and annexin V, respectively. Cells were washed with PBS, and incubated in the dark for 30 min at room temperature with the respective antibody. Cells were then washed with PBS and resuspended in 500 µL of 1% formaldehyde solution. Mouse isotype IgG1 antibodies were employed as controls (BD Pharmingen - San Jose, CA, USA). Approximately 100,000 labeled cells were passed through a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and were analyzed by FlowJo software (Flowjo, Ashland, Oregon, USA).

The cells were transplanted into spinal cord by lumbar puncture in the space (L7–S1) in the amount of 1 x 10^7 [31, 35, 41] cells/50µL, with a volume rate of 10µL/min. The needle was maintained for five minutes to avoid any reflux [39].

Electrical stimulation therapy

The low-intensity electrical stimulation was applied on sub sensorial (~500 μA) level with rectangular waves. The acupuncture needle type electrodes (disposable acupuncture sterilized needle type, stainless steel, 0.25 x 10 mm - DongBang®), connected with alligator clamps were used subcutaneously to lower the tissue impedance to less than 1 kΩ [5] with high current density. They were positioned in the interspinal space, above (anode) and below (cathode) the lesion level. The mean voltage applied was ~30 mV, and the burst frequency was modulated from 5 to 20 Hz. The carrier frequency was ~1.3 kHz with ~200 μs pulse width. Each modulated frequency was applied during 5 minutes in the following order: 5 Hz, 10 Hz, 15 Hz and 20 Hz (20 min total application period) with approximately 15% duty cycle, where first 14% consisted of standard pulse polarity followed by 1% of reverse polarity to prevent charge build-up and possible biochemical changes of the tissue during treatment [38].

Statistical analysis

For statistical analysis, a custom routine was developed the MatLab® software (MathWorks R2012b), applying: (1) Shapiro-Wilk test, to confirm that the data do not have a Gaussian distribution; (2) Friedman test for intra-group comparison (Pre vs. $Post_{30}$, Pre vs. $Post_{60}$ and $Post_{30}$ vs. $Post_{60}$) and consequent posthoc Bonferroni (multcompare in MatLab®); (3) Kruskal-Wallis test inter-group (SC vs. ES vs. SCES) at the Pre, $Post_{30}$ and $Post_{60}$ instants; (4) Effect size (*d*) was calculated for the results in the groups that showed statistical difference.

Results

Cell viability and apoptosis average using 7-AAD and annexin V dual staining were 98.44% and 1.58% respectively, demonstrating that cells were viable before transplantation (Figure 2).

Figure 2

Differentiation into adipocyte lineage was demonstrated by staining with Oil Red O and it was observed large, rounded cells with cytoplasmic lipid-rich vacuoles. Mineralization of the extracellular matrix in osteogenic differentiation was observed by Alizarin Red S staining. In chondrogenic differentiation assays, high-density micromass ADSC cultures generated cellular nodules, which produced large amounts of cartilage-related extra-cellular matrix molecules like collagen. Paraffin sections of the aggregates stained with toluidine blue showed a cartilaginous extracellular matrix stained in purple (metachromasia) while undifferentiated or fibrous tissue stained in blue. Untreated control cultures, which were growing in regular medium without adipogenic, osteogenic or chondrogenic differentiation stimuli, did not exhibit spontaneous adipocyte, osteoblast or chondrocyte formation after 21 days of cultivation (Figure 3).

Figure 3

The expression of the surface antigens in the ADSCs was evaluated by flow cytometry and the following results were obtained: CD29 (99.8%), CD44 (68.8%), CD90 (3.81%), CD45 (6.9%), CD34 (4.84%), CD14 (0.079%) (Figure 4).

Figure 4

The statistical results calculated from EMG signals is demonstrated in Figure 5. The Shapiro-Wilk test showed that none of the data groups followed the Gaussian distribution. The Friedman test showed $p < 0.01$ for all groups (G_{ADSC}, G_{LIES}, G_{COMB}). All three groups had a

significant increase of EMG_{RMS} after the application of the therapies (Pre vs. $Post_{30}$ or Pre vs. $Post_{60}$). However, there were no statistically significant differences between $Post_{30}$ and $Post_{60}$. The inter-group test of Kruskal-Wallis (G_{ADSC} X G_{LIES} X G_{COMB}) did not show statistical significance in any of the instants Pre ($p = 0.34$), $Post_{30}$ ($p = 0.78$) and $Post_{60}$ ($p = 0.64$).

Figure 5

Discussion

The aim of this study was to evaluate the effects of treatment with low-intensity electrical stimulation and/or stem cells in paraplegic dogs. We evaluated the motor response of *vastus lateralis* muscle by EMG recordings during loading on the hind limbs, and dogs from three test groups exhibited motor improvements confirmed by EMG recordings ("I" and "N" motor responses showed in Table 1); however, in contrary to our expectations, the combined therapy group, SCES, was not superior to the ES or ST groups.

Adipose derived stem cell group

Our hypothesis was that ADSC promotes the axon growth in the injured spinal cord contributing to the restoration of the function [50]. Stem cell transplantation may lead to axon remyelination and reconnection of the neural pathway [45]. In our study, the ADSC were administered by lumbar puncture, in contrary to Bakshi et al. [3] who showed that most experiments in animals had been performed by injecting cells directly into the injured parenchyma. This invasive technique compromises the injured spinal cord, although it delivers

cells into the friendly environment of the acutely injured cord. Also, lumbar puncture more efficiently distributes the stem cells in the injured spinal cord compared to the intravenous route.

As mesenchymal stem cells transplantation should increase the nerve fiber count and accelerate the functional recovery in the peripheral nervous system [13], it may have been responsible for the increase in EMG_{RMS} after the therapy, consequently improving the motor response. The mesenchymal stem cell transplantation is more effective in acute stages and sub-acute tissue injury, and in later stages often does not show functional benefits [49]; however, in our study, positive results emerged in a chronic patient, Pt3 (table 1) with 3 years of lesion, but with cause of injure not conclusive, that can beneficiated this improvement.

In this study, with the use of allogeneic stem cell, there was a slight improvement in motor function. This is in accordance with results observed by Azari et al. [18], Pal et al. [39], Rodrigues [44], Carvalho [9] and Kaminski [32] with autologous cells.

We presume that ADSC transplantation contributed positively to the clinical improvement. This effect is possible due to the paracrine effect that stem cells can perform on the site of injury, the release of bioactive molecules that mediate proliferation and the homing of endogenous stem cells at the injury site [14, 25, 39]. In this study, we showed improvement in the motor recovery in early stage (30 days) after transplantation. This result corroborates the previous studies of Cho et al. [12], showing that paracrine action has the main contribution in this therapeutic modality.

Low-intensity electrical stimulation group

The application of LIES accelerates the axonal regeneration and improves the specificity of sensory reinnervation [6, 21, 47]. The use of low-intensity electrical stimulation accelerates the tissue regeneration mainly due to the increasing concentration of adenosine triphosphate

molecules (~500%) [37]. The current intensity was less than 1mA, although there is no standard for these parameters [37]. Only 2 hours of LIES with a current of 10 µA applied to neural proteins in culture, provides organelles' movement and a discreet stretching of structures [33]. Related to nervous systems, it is known that LIES induces neuron growing and elongation in the direction of the cathode (negative pole) [4, 42].

Borgens and Bohnert [4] evaluated the regeneration for a month with the electrical stimulator and electrodes implanted on the spinal cord of adult pigs. The cathode was positioned above the level of the injury, with an amplitude of 0.4 mV/mm (extremely low-intensity) and continuous current with intensity of 45 µA. At the end of two months, there was no significant improvement in the experimental group. Maybe the negative results of Borgens and Bohnert [4] were due to the continuous application and extremely low-intensity. In our study, we applied just 40 min of LIES (divided in two days) because brief trans-vertebral electrical stimulation accelerates the reinnervation of damaged femoral nerve [21] better than longer stimulation periods.

Han et al. [27] stated that one month after a lesion in sciatic nerve of a rat the LIES fails due to distal fibrosis. However, Elzinga et al. [17] found significant improvements using the same frequency as Han *et al.* [27] (20Hz) in femoral nerves of rats 3 months after lesion. In our study, the time from injury for different participants varied from 2 months to 8 years, although, subjects with the shorter time from injury, as Pt 17 (4 months) did not show improvements, and subjects as Pt3 (3 years) showed improvements. Moreover, our participants did not undergo any surgical procedure to remove the astrocytic scar because modern theories say that the astrocytic scar is important to promote the axonal regrowth [51].

Our protocol applied the LIES subcutaneously with needles to reach the central nerve systems (spinal cord) on the dorsal side. The presence of several layers of tissue between needle electrodes and spinal cord can reduce the effects of already very low-intensity stimulation.

However, if the needles are positioned near the dorsal rami of spinal nerves [48], they can lead electrical stimuli to spinal cord because of much greater electrical conductivity (1.2 S/m) than other neighboring tissues, e.g. muscles (0.175 S/m) [22].

The electroacupuncture is usually applied via needle electrodes at meridian points. Zhang et al. [55] evaluated the locomotor recovery of SCI rats who had been treated with electroacupuncture. The acupoints were proximal (posterior region of the neck) and distal (leg) to the lesion level (T10). They found that LIES improved the locomotor recovery by inhibiting the activation of astrocytes and microglia, and apoptosis. With a similar electroacupuncture protocol on SCI rats, Chen and Wu [11] applied (during 30 min) a constant ES with 2 Hz frequency and 0.2 mA intensity, 2 and 8 hours after the surgical injury. The authors found a significant decrease on neural apoptosis through inhibition of P38 mitogen-activated protein kinases. These results are corroborated with our data, indicating that neuroprotective effects may allow the patient recovery (through neuroplasticity) and can be registered by techniques as electromyography.

Combined therapies group

Huang et al. [29] suggest that stem cell therapy combined with stimulation techniques, such as deep brain stimulation, functional electrical stimulation, transcranial magnetic stimulation or electroacupuncture, may be a promising non-pharmaceutic approach for recovery of the central nervous system. In this sense, Yamada et al. [53] proved that embryonic stem cells, when electrically stimulated, initiate mechanisms of differentiation.

In our study, the COMB group that used combined therapy did not achieve better recovery than other groups, although it showed positive results. Moreover, our results may be associated with the importance of LIES for ADSC differentiation [24]. Liu [36] showed similar

results in patients with multiple sclerosis. They combined ES with transplantation of bone marrow mesenchymal stem cells (BM-MSC), and the results showed increased not only BM-MSC differentiation but also the promoted myelin repair. Gu et al. [26] proved that low-frequency (~20 Hz) ES promoted proliferation and contributed to the differentiation of blood stem cells in Schwann cells.

Ashour et al. [2] reported very promising results evaluation both techniques, LIES and stem cells transplantation, on repair of sciatic nerve crush injury in rats. They showed that both, LIES or stem cells, accelerate and promote the functional nerve regeneration in a period of 8 weeks. They analyzed the nerve conduction velocity (mm/s), showing that stem cells transplantation group (34.87 ± 1.3 mm/s) and LIES group (34.96 ± 1.29 mm/s) had a significantly higher nerve conduction velocity than SCI control group (26.71 ± 1.3 mm/s) [2]. As in our results, Ashour et al. [2] proved that stem cells and LIES groups showed EMG improvements, without demonstrating a superiority of one technique over another.

Hybrid therapies with stem cell showed positive results. Yan et al. [54] investigated the effect of electroacupuncture on the differentiation of mesenchymal stem cells of nerve fibers in mice (N=20). Mice were separated into four groups (N= 5 each), (1) control, (2) electroacupuncture, (3) stem cells and (4) electroacupuncture with stem cells. The authors showed that experimental groups 2, 3 and 4 had an increase in neurotrophin-3 (NT-3), in other words, the electroacupuncture was efficient in nerve regeneration; furthermore, the combined application of electroacupuncture with stem cells made this regeneration even more pronounced.

Conclusion

We found that application of low-intensity electrical stimulation or adipose derived stem cells transplantation can lead to a similar motor recovery in paraplegic dogs 30 ($Post_{30}$) and 60

($Post_{60}$) days after the procedure. A combined therapy of both, LIES and ADSC led to improvements but there was not sufficient statistical significance for inter-group results at any instant (Pre, $Post_{30}$, and $Post_{60}$). The mechanisms involved in potential underachievement of combined therapy are unclear. The heterogeneous characteristics of dogs participants indicate that our results were not biased by intrinsic factors like breed, age, weight, time and cause of lesion. All participants were domestic dogs, although this research is an experimental treatment, a control group was not included to provide therapy to all participants. Further studies should be conducted to test the optimal number of sessions and period of application to promote neural plasticity on the spinal cord.

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Figure Legend

Figure 1. Research design. EMG: electromyography; ADSC: adipose derived stem cells group; LIES: low-intensity electrical stimulation group; COMB: adipose derived stem cells + low-intensity electrical stimulation group.

Figure 2. Cell viability by flow cytometry. Annexin V it was used to apoptosis detection and 7-AAD for cell viability. Representative histograms are displayed. Isotype control is shown as red line histogram.

Figure 3. Differentiation of MSC. Differentiation into adipocyte lineage was demonstrated by staining with Oil Red O (D), Alizarin Red S staining shows mineralization of the extracellular matrix in osteogenic differentiation (E) and toluidine blue shows the deposition of proteoglycans and lacunaes in chondrogenic differentiation (F). Untreated control cultures without adipogenic, osteogenic or chondrogenic differentiation stimuli are shown (A, B, C). Bar scale: 50 µm.

Figure 4. Immune phenotype by flow cytometry. ADSCs were labelled with antibodies against the indicated antigens and quantitative analyses were performed using a FlowJo software. Isotype control is shown as red line histogram.

Figure 5. Box-plot of electromyography response. (A) ADSC; (B) LIES; (C) COMB; *d*: effect size; *: $p < 0.05$. Whiskers indicate the minimum and maximum value respectively.

Figures

Figure 1.

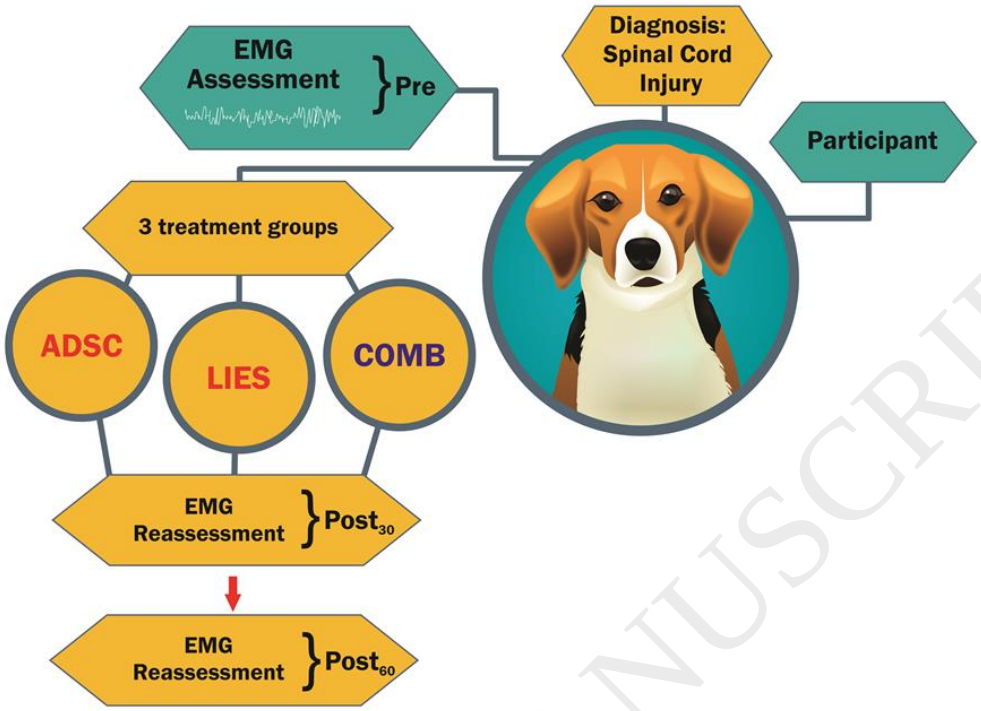

Figure 2.

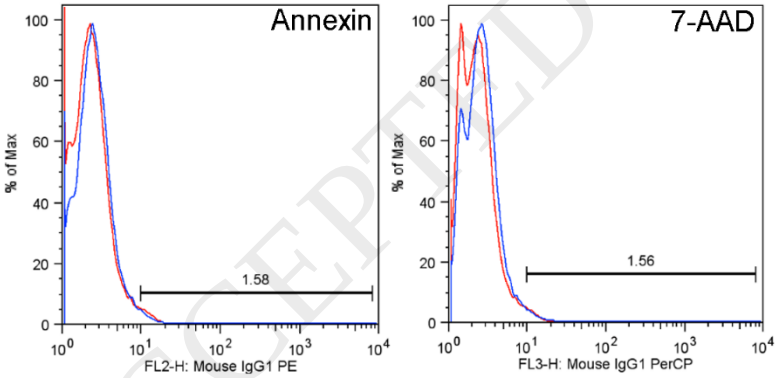

Figure 3.

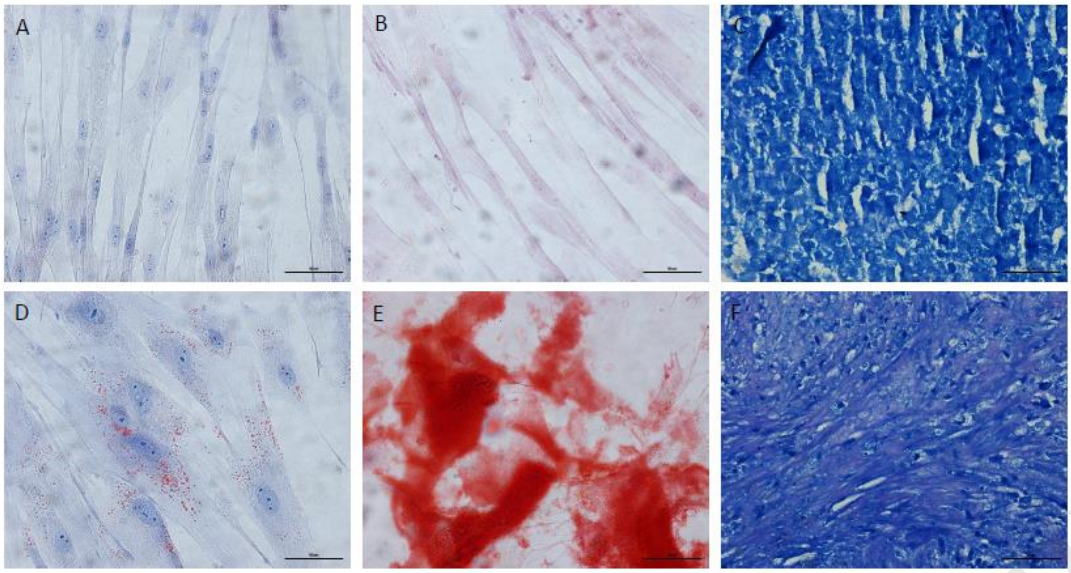

Figure 4.

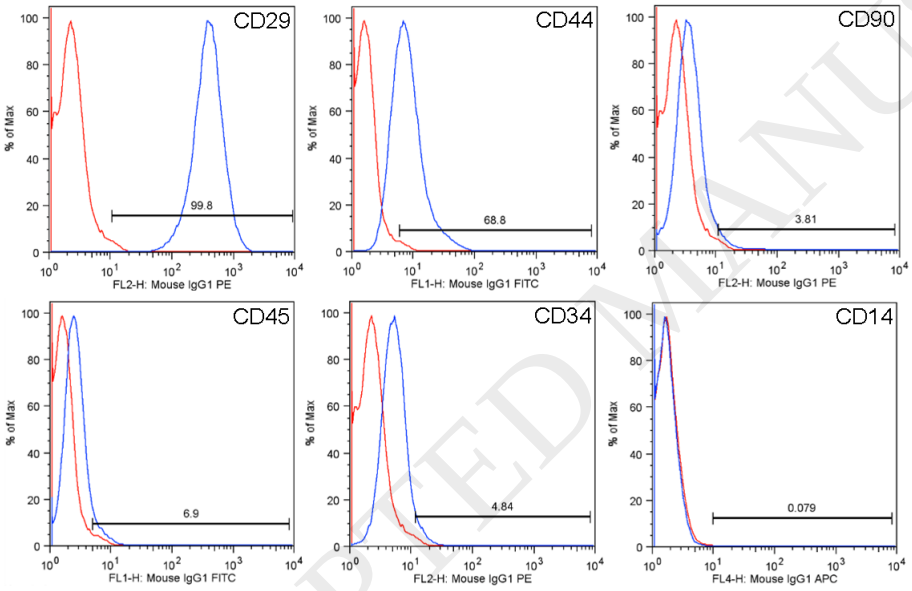

Figure 5.

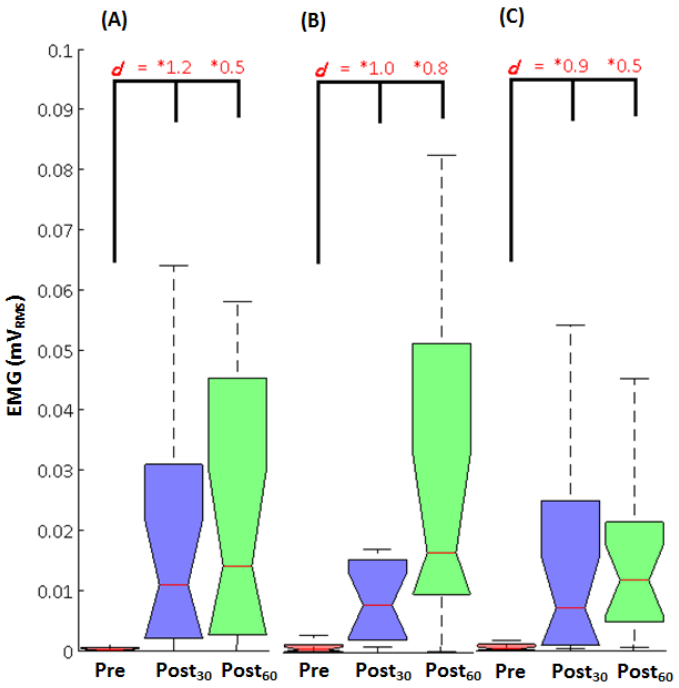

Table 1. Participants demography

Pt – Participant; F – Female; M – Male; ADSC – Adipose derived stem cells; LIES – Electrical Stimulation, COMB –

Pt	Group	Sex	Age (years)	Weight (kg)	Breed	Injury Time	Cause of Injury	Motor responses
1	ADSC	F	4	5.2	DA	7m	DH	I
2		M	2	9.2	MBD	3m	CA	I
3		F	7	4.5	PI	3y	FA/DH	I
4		F	1	15.0	MBD	3m	AT	L
5		F	6	17.6	LA	7m	DH	L
6		F	3	5.6	LA	7m	DH	L
7		M	3	13.8	MBD	2y	CA	L
8		M	8	3.7	LA	1y	DH	N
9		F	10	6.1	DA	8y	TR	N
10	LIES	F	5	3.9	PO	1y	DH	I
11		M	7	9.1	FB	1y	DH	I
12		M	4	27.5	MBD	3m	CA	I
13		M	5	9.0	FB	2m	DH	I
14		F	9	7.6	DA	1y	DH	L
15		M	2	10.2	MBD	5m	CA	L
16		M	4	10.4	PO	1y	DH	N
17		M	4	12.5	MBD	4 m	DH	N
18	COMB	M	6	9.8	LA	1y 5m	DH	I
19		M	2	2.6	YO	1y 5m	FA	I
20		F	4	10.0	FB	5m	DH	L
21		F	9	8.4	DA	3m	DH	L
22		F	1	19.3	MBD	8 m	UN	L
23		F	6	45.0	SB	6y	UN	L
24		M	8	4.7	YO	6y	TR	N
25		M	3	15.0	MBD	1y 5m	CA	N
26		F	5	6.2	DA	3m	DH	N
27		F	4	8.0	LA	5m	FA	N

Adipose derived stem cells and Electrical Stimulation; DA - Dachshund, MBD – Mixed Breed Dog; PI – Pinscher; LA - Lhasa Apso; PO – Poodle; FB - French Bulldog; YO – Yorkshire; SB - St Bernard; y – Year; m – Month; DH - Disk Herniation; CA - Car Accident; FA – Fall; TR – Trauma; UN – Unknown; I – motor improvement (keeping the quadrupedal position for 10 s or more); L – Low motor improvement (keeping the quadrupedal position for less than 10 s) ; N – None motor improvement.