Research in Veterinary Science 123 (2019) 247–251

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Combination of stem cells from deciduous teeth and electroacupuncture for therapy in dogs with chronic spinal cord injury: A pilot study

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ARTICLE INFO

Keywords:
Intervertebral disc herniation
Intervertebral disc disease
Stem cells from exfoliated human dental pulp
Cell therapy
Electroacupuncture

ABSTRACT

Spinal cord injury (SCI) is a serious condition that causes profound economic and emotional impact in human patients and companion animal owners. It has been shown that the neurogenic effects of the stem cells are enhanced when combined with electroacupuncture (EA) in rodent models of SCI. To determine the safety and feasibility of combining transplantation of allogenic stem cells derived from canine exfoliated deciduous teeth (SCED) and EA in dogs with chronic spinal cord injury a canine pilot clinical study was conducted. A total of 16 individuals ranging from 5 to 11 years at 3 to 18 months of injury were investigated and randomly assigned to 4 experimental groups (SCED, EA, SCED + EA, control). Mild neurological and functional improvements were seen in all 4 groups. There was no clinical progression or mortality of the cases occurred in a follow up of 7 months after procedure. The study shows that SCED transplantation and electroacupuncture were feasible, safe and potentially beneficial. However Long-term patient monitoring is necessary to rule out any delayed side effects and assess any further improvements.

1. Introduction

Spinal cord injury is a serious condition that leads to motor functional and sensory. It can cause profound emotional, social and economic impairments (Sekhon and Fehlings, 2001). Different strategies have been studied to achieve spinal cord regeneration, by attempting to overcome the inhibition of the injured site (Bradbury et al., 2002) and/or encouraging trophic mechanisms of regeneration (Tropea et al., 2003).

Mesenchymal stem/stromal cells (MSC) transplantation has been widely studied in SCI and, despite variability and limitations, was shown to be effective for functional and sensory recovery in animal models of traumatic SCI (Antonic et al., 2013; Oliveri et al., 2014). Stem cells from human exfoliated deciduous teeth (SHED) are a source of MSC found in perivascular niche of the dental pulp that simultaneously express early mesenchymal, neuroectodermal stem/progenitor cells markers and certain embryonic stem cells markers (Sakai et al., 2012). Also, the neurogenic potential from SHED seems to be higher than bone marrow mesenchymal stem cells (BMSC) (Sakai et al., 2012), a traditional tissue sources of MSCs in clinical stem cell research (Oliveri et al., 2014). Moreover, it was shown that neurogenic induction in both canine and human dental pulp stem cells causes morphological changes and the expression of the neurogenic marker ßIII-tubulin (Dissanayaka et al., 2011).

Combined strategies using electroacupuncture (EA) and neural stem/progenitor cells (NSC) or BMSC in rats with spinal cord transection have improved regeneration, survival and migration of the transplanted stem cells towards injury (Ding et al., 2009; Yan et al., 2011); increased differentiation of stem cells into neuronal-like and oligodendrocyte-like cells, and upregulated neurotrophin-3 (Ding et al., 2009; Yan et al., 2011); and downregulated extracellular-matrix-associated inhibitors of regeneration, directly involved in glial scar

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https://doi.org/10.1016/j.rvsc.2019.01.011
Received 26 October 2018; Received in revised form 10 December 2018; Accepted 10 January 2019

formation (Ding et al., 2011).

Due to limitations in transpose pathophysiology of rodent SCI to the human condition, companion animals have been proposed as a more realistic model of SCI for stem cells therapy. Canine thoracolumbar intervertebral disc herniation (IVDH) is a spontaneous disease that bears similarities to acute SCI in humans (Bergknut et al., 2012; Hoffman and Dow, 2016). The present study investigated the safety, feasibility and therapeutic effect of stem cells from canine exfoliated deciduous teeth (SCED) transplantation combined with electroacupuncture in chronic spinal cord injured dogs due to naturally occurring IVDH.

2. Materials and methods

2.1. Patient selection

A total of 16 paraplegic dogs, with history of acute onset of paralysis (< 24 h) and chronicity of at least 3 months, caused by thoracolumbar intervertebral disc herniation (IVDH) (T10-L4), with absence of deep pain perception and increased spinal reflexes in pelvic limbs (PLs) were selected for the study. Deep pain perception was considered absent when animal did not demonstrate signs of conscious pain (de Lahunta et al., 2009). The animals were randomly assigned to one of the following four groups ($n = 4$ per group). Stem cells from canine exfoliated deciduous teeth (SCED); Electroacupuncture (EA); SCED + Electroacupuncture group (SCED + EA) and control. The experiment lasted 13 weeks and animals were evaluated before and after treatment by neurological examination, functional assessment and magnetic resonance imaging.

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and in accordance by the Ethics Committee in Animal Experimentation of College of Veterinary Medicine and Animal Sciences, University of Sao Paulo – Brazil (permit number 2950/2013).

2.2. Isolation, culture and preparation of stem cells from canine exfoliated deciduous teeth

Exfoliated deciduous teeth from young female dogs were extracted (Dissanayaka et al., 2011). Briefly, the tooth surface was cleaned with a solution of phosphate-buffered saline (PBS) and 5% penicillin-streptomycin (LGC Biotecnologia). After the extraction, the tissue was digested in a 3-mg/mL collagenase type I and 4-mg/mL dispase (GIBCO-Invitrogen) solution for 1 h at 37 °C. Then, the cells were filtered using a 70-μm strainer (352,350, BD Falcon) to obtain single-cell suspensions. These cells were seeded into culture plates containing minimum essential medium (MEM/ALPHA; BR30238–01, LGC Biotecnologia), supplemented with 15% fetal bovine serum (FBS) (10-bio500, LGC Biotecnologia), 100 μM L-ascorbic acid-2-phosphate (A8960, Sigma-Aldrich) and 1% penicillin-streptomycin 10,000 μg/mL, and cultured under 5% CO2 at 37 °C. Medium was replaced every 3 days. After confluence, SCED were washed with PBS and detached from plate using 0,25% trypsin (Tryple – GIBCO, Cat. A1285901) and maintaining the plate for 5 min in incubator, followed by addition of enriched Alpha MEM medium to inactivate the trypsin and centrifugation at 1200 RPM for 5 min. Afterwards, supernatant was discarded, and the cells were expanded into a 22cm^2 culture plate. The process was repeated into a 60 cm^2 culture plate until the cells reached passage 4.

2.3. Stem cells from canine exfoliated teeth (SCED) phenotyping

For characterization, protein expression quantification by flow citometry analysis was performed. The cells were washed with PBS and incubated with 1 μg of the antibodies CD105, CD90 and CD45 (Santa Cruz Biotechnology Inc.; California, USA). The analysis was conducted on a FACS Caliber flow cytometer (Becton Dickinson, San Jose, California, USA) and analyzed on the WinMDI 2.9 software. The expression of markers was determined by comparison with an isotype control labeled with FITC non-specific fluorochrome (Alexa Fluor 488).

2.4. Surgery and SCED transplantation

Animals received intramuscular injection of morphine hydrochloride (1 mg/kg) and diazepam (0,3 mg/kg) as preanesthesic drugs and was put under general anesthesia with intravenous propofol (5 mg/kg) for induction and maintained with isoflurane. The dogs were submitted to dorsal laminectomy and the spinal cord was exposed at the site injury as previous described (HB, 2007). A dose of 2×10^6 cells diluted in 150 μL of PBS, divided into 3 syringes were then injected intramedullary using a 30-gauge (0.4 mm × 13 mm) needle, directly into the spinal cord parenchyma (middle of the injury, cranial and caudal margins). After the procedure, a piece of subcutaneous fat was harvested and placed over the laminectomy site, then the fascia, epaxial muscles, subcutaneous and skin were closed. Post-surgery prescription included orally cephalexin (20 mg/kg, twice daily for 7 days), prednisolone (1 mg/kg, once daily for five days, then, 0,5 mg/kg for five days and 0,25 mg/kg for more five days), and dipyrone (25 mg/kg 3 times a day for 7 days). The second transplantation was done seven days after the first procedure. Animals were put under anesthesia and received three percutaneous intraspinal injections, at cranial, midpoint and caudal margin of the injury, for a total dose of 2×10^6 cells using a 24-gauge intravenous catheter needle (0.7 mm × 19 mm), guided by palpation of surgical window. Animals from EA and control groups received injections of 150 μL of PBS in both procedures. Considering the first and second injection, a total of 4×10^6 SCED was injected.

2.5. Electroacupuncture

Electroacupuncture treatment started after the second transplantation (between days 8 and 11) and it was performed three times a week for the initial seven weeks, two times a week for more five weeks. Stainless acupuncture needles were used with 0,25 mm thickness (Dongbang). The acupuncture points used were GV2, Bai Hui, GV3a, GV6; bilateral BL19, BL23 and BL24; unilateral KI3, ST36, LV3 and Wei Jian (Fig. 1). The chose and location of the acupuncture points was in accordance with (Xie and Preast, 2008) for thoracolumbar spinal cord injury. Electrodes were connected at Bai Hui/GV6 and bilateral BL19/B24. Electroacupuncture device (NKL EL608) was configured to alternate between 2 Hz for 2 s and 60 Hz for 1 s, for 20 min accordingly with the protocol of (Ding et al., 2009). The intensity was adjusted to induce

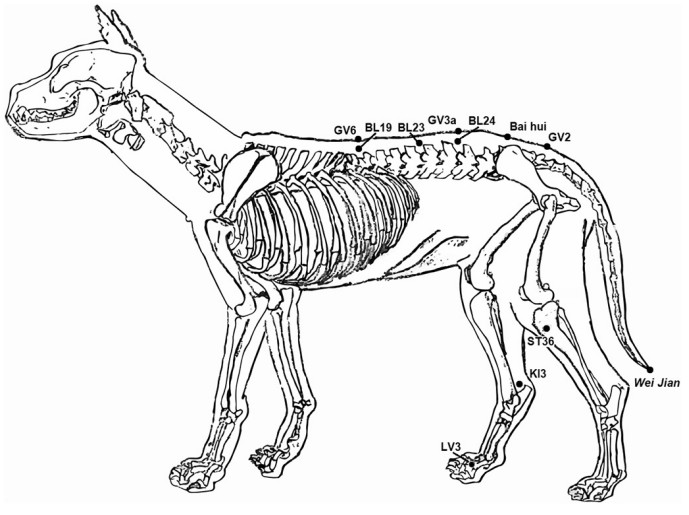

Fig. 1. Acupuncture points used. Locations were in accordance with Xie and Preast (2008).

a slight twitch on PLs or paravertebral muscles without discomfort to the dog.

2.6. Underwater treadmill

Underwater Treadmill was performed twice a week in all animals, for the 12 weeks of treatment. The dogs were stimulated to walk on an underwater treadmill progressively from 3 to 15 min, according to the physical limits of the animal.

2.7. Neurological examination

All animals were submitted to a complete neurological examination before and after treatment following guidelines from (Chrisman et al., 2003). For this study, six key neurological tests were selected to assess spinal cord injury. They were: urinary incontinence, fecal incontinence, conscious proprioception (CP), hopping, superficial pain perception (SPP) and deep pain perception (DPP) for each PL. Conscious proprioception positioning was observed by displacing the dog's foot by turning it onto its dorsum while supporting the dog's weight; the animal should replace it immediately. Hopping was tested holding three legs or the contralateral tested pelvic limp off the ground and forcing the animal to hop or move the leg while being pushed laterally on the fourth limb. Superficial and deep pain perception was observed by pinching the interdigital webs and phalanges of each digit of PL using a Kelly forceps. The presence of pain was observed when an animal demonstrated any sign of mental awareness of the stimulus (e.g., crying, trying to bite of look at the limb) when pressure was applied (de Lahunta et al., 2009).

2.8. Functional assessment

The dogs were evaluated using a functional score for thoracolumbar spinal cord injury in dogs adapted from Olby et al. (Olby et al., 2001) (Table 1). Briefly, each dog was recorded from both sides and behind when walking in a nonslippery surface for at least 10 steps at each observation angle. Dogs that could not bear weight on their PLs were sustained by supporting the base of the tail in order to allow non-weight bearing movements to be seen. The score ranged from 0 (no PL movement) to 12 (normal PL gait) and the evaluation depended on weight bearing ability, number of joints involved on gait and the percentage of time of PL protraction. Deep pain sensation and voluntary tail movement was excluded from the original score.

2.9. Magnetic resonance imaging evaluation

All animals underwent standard sagittal and transverse T1- and T2-weighted as well as dorsal STIR images, using a low-field magnetic resonance imaging (MRI) scanner (0.24 Tesla VET-MR Grande - Esaote) before the surgery in the study and after 12 weeks of treatment.

2.10. Statistical analysis

For evaluation of the pre- and post-treatment scores in functional assessment, the Paired Wilcoxon Test was used. Posteriorly, the difference of the post- and the pre-treatment functional scores was assessed (post - pre) for each animal in the four experimental groups. The medians of the difference in each treatment group were compared with Kruskal-Wallis test. Significance level adopted was 0.05, and all analyses were made with Graphpad Instant Sofware V5.0 (Graphpad Software, USA).

3. Results

The study sample of dogs ranged from 5 to 11 years old, 9 spayed females and 6 neutered males of different breeds, including 9 dachshunds (9/16), 3 mongrels (3/16), 2 Lhasa Apsos (2/16), 1 Yorkshire Terrier (1/16) and 1 Pug (1/16). The chronicity of the SCI before ranged from 3 to 18 months. No mortality was observed in dogs followed up to 7 months after SCED transplantation. Minor post-operative pain was observed in animals 1, 9 and 15, and was absent within 2–7 days.

3.1. Stem cells from canine exfoliated teeth (SCED) characterization

Flow cytometry analysis showed positive marker expression for CD90 (72,1%) and CD105 (49,8%). CD45 was not expressed by stem cells from exfoliated teeth (Fig. 2).

3.2. Neurological examination

All animals were presented with negative proprioception and hopping test, absence of superficial and of deep pain perception and increased spinal reflexes in both PLs, along with urinary and fecal incontinence at the beginning of the study. There were observed neurological improvements in animals from SCED group (1/4), EA group (1/4), SCED + EA group (3/4) and Control group (1/4), when comparing pre and post treatment. In SCED group, animal 1: CP was absent on both PLs before and retarded after treatment. In EA group, animal 8: SPP and DPP on right PL were absent before and present after

Table 1
Functional assessment score.

Grade	Gait
0	No Pelvic limb movement.
1	Minimal non-weight-bearing protraction of the pelvic limb (movement of 1 joint).
2	Non-weight-bearing protraction of the pelvic limb with > 1 joint involved < 50% of the time.
3	Non-weight-bearing protraction of the pelvic limb with > 1 joint involved > 50% of the time.
4	Weight-bearing protraction of pelvic limb < 10% of the time.
5	Weight-bearing protraction of pelvic limb 10 to 50% of the time.
6	Weight-bearing protraction of pelvic limb > 50% of the time.
7	Weight-bearing protraction 100% of the time with reduced strength of pelvic limb. Mistakes > 90% of the time (e.g., crossing of pelvic limbs, scuffing foot on protraction, standing on dorsum of foot, falling).
8	Weight-bearing protraction 100% of the time with reduced strength of pelvic limb. Mistakes 50 to 90% of the time
9	Weight-bearing protraction of pelvic limb 100% of the time with reduced strength. Mistakes < 50% of the time.
10	Ataxic pelvic limb gait with normal strength, but mistakes > 50% of the time (e.g., lack of coordination with thoracic limb, crossing of pelvic limbs, skipping steps, bunny-hopping, scuffing foot on protraction).
11	Ataxic pelvic limb gait with normal strength, but mistakes < 50% of the time.
12	Normal pelvic limb gait

Scoring used in functional assessment, adapted from Olby et al., (2001).

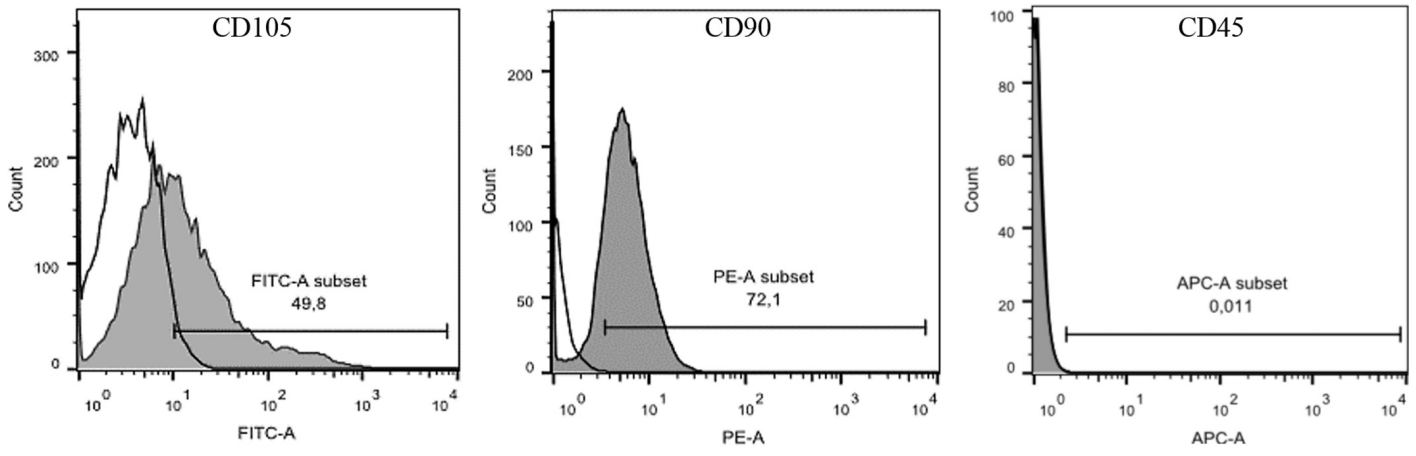

Fig. 2. The flow cytometry results for mesenchymal stem cell markers CD105, CD95 and CD45.

treatment, and conscious proprioception on left PL was absent before and present after treatment. In SCED + EA group, animal 9: CP was absent on both PLs before and present after treatment, and hopping test on left PLs was absent before and present after treatment; animal 10; CP was absent on both PLs before and present after treatment, and urinary incontinence was positive before and no urinary incontinence after treatment; animal 12: CP was absent on both PLs before and retarded after treatment. Finally, in control group, animal 16: CP was absent on right PL before and retarded after treatment, and hopping test on right PL was absent before and retarded after treatment (S1 Table).

3.3. Functional assessment

The functional assessment score showed improvements in 2 animals in SCED group, 2 animals in EA group, 1 animal in group SCED + EA and 2 animals in Control group. In SCED group, animal 2 improved from score 0 to 1 and animal 4 improved from score 1 to 3. In EA group, animal 5 improved from score 0 to 2 and animal 8 improved from score 1 to 3. In SCED + EA group, animal 10 improved from score 1 to 2. In the control group, animal 13 improved from score 1 to 5 and animal 15 improved from 0 to 2 (S2 Table). No statistical significance was observed between groups in functional assessment (Fig. 3).

3.4. Magnetic resonance imaging

At the pre-treatment time point, all dogs presented injury in the thoracolumbar spinal cord region, with extent from 0.4 to 9.3 cm, and different grades of injury compatible with or without spinal cord compression IVDH (Henke et al., 2013). In addition, in animals 11, 12 and 16 that had previously undergone decompressive surgery before it was observed compression that could be caused by the scar in the soft tissue developed in laminectomy site. Animals 1 and 16 presented spinal cord atrophy at injury site. All animals, except 6, 8, 9, 11, 13 and 16, had presented different grades and extent of hyperintense signal in T2-w sequences within the spinal cord. All animals had signs of injury within thoracolumbar spinal cord region. Detailed information is described in S3 Table.

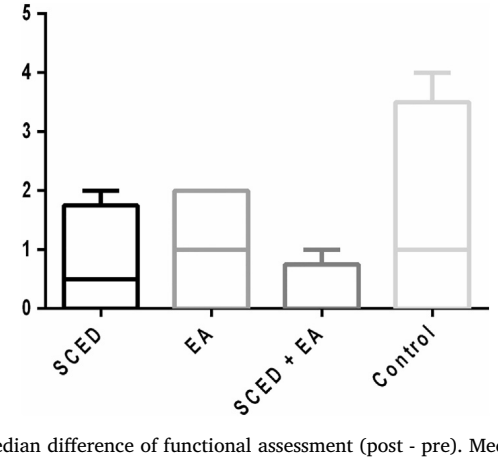

Fig. 3. Median difference of functional assessment (post - pre). Medians of the difference in each treatment group compared with Kruskal-Wallis test.

After treatment, data generated by MRI revealed differences in injury extent (IE), hyperintense signal (HS) and spinal cord atrophy (SCA). In SCED, animal 1 presented no changes in MRI; animal 2 had a reduction in spinal cord HS from vertebrae T11-L2 to T11-L1 and IE from 6.9 to 5.8 cm; animal 3 presented a progression of severe HS along cranial thoracic and caudal lumbar spine, with an increase of the IE from 0.7 to 7.7 cm; and the owner of animal 4 did not bring it for second MRI. In the EA group, animal 5 presented a progressive SCA, with reduction in IE from 5.8 to 3.7 cm, animal 6 presented a HS in spinal cord within L1-L3, not seen in pre-treatment MRI, and had an increase in IE from 1.4 to 4.3 cm, and animal 7 presented an increase in IE from 1.8 to 2.3 cm; animal 8 presented no changes in MRI. In SCED + EA, animal 9 presented an increase in IE from 1.9 to 2.8 cm, and animal 10 presented an increase in IE from 2.1 to 2.7 cm; animals 11 and 12 did not present any significant change in MRI. In the control group, animal 13 presented SCA within the injury and had a mild increase in IE from 3.7 to 3.9 cm, and animal 14 presented an increase in IE from 3.6 to 4.4 cm, animals 15 and 16 presented no changes in MRI (S2 Table).

4. Discussion

Despite of mild neurological and functional improvement, no beneficial effect could be associated with SCED, EA or combined treatment. The lack of statistical difference between groups in neurologic scoring and functional assessment can be regarded to the small number of dogs in the experiment. Also, the pronounced variation of injury severity and extent with thoracolumbar spinal cord could implicated in heterogenous results. The failure of the proposed treatments should be considered. Regardless, the results observed were similar to previous studies using MSC transplantation in naturally occurring chronic SCI in dogs, in which mild neurological improvements were observed (Besalti et al., 2015; Penha et al., 2014; Sarmento et al., 2014). According to Li et al. (2018), human dental pulp stem cells has a potential for neurogenic differentiation, which makes this cell a promising source for neural regeneration.

The allogenic transplantation of MSCs was shown to be effective to rehabilitate locomotor and nociceptive function in SCI dogs (Jung et al., 2009). Although immunosuppressive agent administration reduces

secondary damage and may improve outcome following SCI in rats, it could not be associated with a locomotor recovery in studies using allogenic and xenogeneic grafts, and that can be due to the hypo-immunogenic properties of MSCs and the lack of long-term engraftment (Oliveri et al., 2014). In the present study, the allogenic transplantation of the SCED was done without immunosuppresive therapy, simmilarly with previous studies using allogenic MSC in SCI dogs (Kim et al., 2016; Jung et al., 2009; Ryu et al., 2009; Lim et al., 2007).

Compared to acute treatment, a more delayed transplantation into a chronic injury could be more beneficial (Karimi-Abdolrezaee, 2006)] due to the alteration of inflammatory response that provides better conditions for both endogenous and transplanted populations (David et al., 2012). However, the long period between SCI and the beginning of treatment in this study (up to 18 months of chronicity) could have had a negative influence in spinal cord regeneration. Also, the total dose of SCED transplanted in this study (4×10^6) was in accordance with usual range in number of cells used in previous studies, which in canine models of spinal cord injury, varies from 1×10^6 (Ryu et al., 2009; Sarmento et al., 2014) to 10×10^6 (Jung et al., 2009; Kim et al., 2016; Lim et al., 2007). Nevertheless, higher doses of transplanted stem cells in pre-clinical studies are associated with better regenerative outcome (Antonic et al., 2013). Additionally, MRI findings did not suggest improvement comparing pre- and post-treatment within groups, except for animal 2 from the SCED group. Instead, signs of progression of the SCI, compatible with the natural progression of the SCI (Bramlett and Dietrich, 2007), were observed.

5. Conclusion

SCED isolation was relatively simple and feasible, and transplantation was shown to be safe, with no mortality during a follow up of 7 months. Despite mild neurological and functional improvements, no conclusive beneficial effect could be associated with SCED, EA or combined treatment. Thus, further investigations with a higher number of animals and more homogeneous SCI would be necessary to make a more concrete evaluation and assess the therapeutic effect of SCED combined with electroacupuncture in chronic spinal cord injured dogs.

Acknowledgments

This study was partially supported by the National Institute of Science and Stem Cells Technology (INCTC-Brazil, n° 2014/50947-7). The authors would like to acknowledge the team of Cães e Gatos Animal Hospital (Osasco, Brazil), Nato Veterinany Clinic (Sao Paulo, Brazil), StellaFisiovet for the support in performing the magnetic resonance, surgery of the animals and underwater treadmill, and the colleagues Lara Carolina Mario and Jessica Borghesi for collaboration in laboratory procedures of this study.

Conflict of interests

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2019.01.011.

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