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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

CÉSAR VINÍCIUS GIL BRAZ DO PRADO

Combination of stem cells from deciduous teeth and electroacupuncture in dogs with chronic spinal cord injury

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Tese apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a Obtenção do título de Doutor em Ciências

Departamento:

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Orientador:

Profa. Dra. Maria Angelica Miglino

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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

Branca entregar p/ Cesar

CERTIFICADO

Certificamos que o Projeto intitulado "Associação da eletroacupuntura e células-tronco de polpa de dente decíduo no tratamento de lesão medular crônica em cães", protocolado sob o nº 2950/2013, utilizando 20 (vinte) cães, sob a responsabilidade da Profa. Dra. Maria Angélica Miglino, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 10/4/2013.

We certify that the Research "Association of the electroacupuncture and stem cells from deciduous teeth in chronic spinal cord injury treatment in dogs", protocol number 2950/2013, utilizing 20 (twenty) dogs, under the responsibility Profa. Dra. Maria Angélica Miglino, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 4/10/2013.

São Paulo, 4 de fevereiro de 2014.

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Autor: PRADO, César Vinícius Gil Braz do

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RESUMO

PRADO, C. V. G. B. do. **Associação de células-tronco de polpa de dente decíduo e eletroacupuntura em cães com lesão medular crônica**. [Combination of stem cells from deciduous teeth and electroacupuncture in dogs with chronic spinal cord injury]. 2016. 61 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Estudos anteriores demonstraram que a associação da eletroacupuntura e células-tronco mesenquimais/estromais (CTMs) pode promover a sobrevivência e diferenciação das CTMs, assim como recuperação funcional em ratos com transecção da medula espinal. Neste estudo, foram avaliados os efeitos terapêuticos da associação de células-tronco derivadas de polpa de dente decíduo esfoliado de cães (CPDEc) e eletroacupuntura (EAP) em cães com lesão de medula espinhal crônica causada de forma natural por herniação do disco intervertebral. Os cães foram divididos aleatoriamente em quatro grupos experimentais (n=4 para cada grupo; total de 16 animais): CPDEc, EAP, CPDEc+EAP e grupo controle. Foram encontradas pequenas melhoras na pontuação do exame neurológico em um animal do grupo CPDEc (1/4; 2 pontos ganhos), um do grupo EAP (1/4; 8 pontos ganhos), três do grupo CPDEc+EAP (3/4; 16 pontos ganhos) e um do grupo controle (1/4; 2 pontos ganhos). Na avaliação funcional, pequenas melhoras também foram observadas em dois animais do grupo CPDEc (2/4; 3 pontos ganhos), dois do grupo EAP (2/3; 4 pontos ganhos), um do grupo CPDEc+EAP (1/4; 1 ponto ganho) e dois do grupo controle (2/4; 6 pontos ganhos). No entanto, não foram encontradas diferenças estatísticas entre os grupos. Os achados ressonância magnética não sugeriram melhoras comparando os exames pré e pós tratamento entre os grupos, com exceção de um animal do grupo CPDEc (1/4), e 10 animais dentre todos os grupos (10/16) apresentaram sinais de progressão na lesão da medula espinhal, que não puderam ser associados com os procedimentos do estudo, mas podem estar relacionados à progressão natural da doença. Além disso, os dentes decíduos esfoliados foram obtidos facilmente e as CPDEc foram isoladas de forma simples, ademais, não foi observada nenhuma mortalidade foi observada até 7 meses após o procedimento.

Palavras-chave: Hérnia de disco intervertebral. Células-tronco de polpa de dente humano esfoliado. Paraplegia. Terapia Celular.

ABSTRACT

PRADO, C. V. G. B. do. **Combination of stem cells from deciduous teeth and electroacupuncture in dogs with chronic spinal cord injury**. [Associação de células-tronco de polpa de dente decíduo e eletroacupuntura em cães com lesão medular crônica]. 2016. 61 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Previous studies have reported that combination of electroacupuncture (EA) and mesenchymal stem/stromal cells (MSC) promoted survival, differentiation and functional recovery in spinal cord-transected rats. In this study, it was examined the therapeutic effects of stem cells from canine exfoliated dental pulp (SCED) combined with EA treatment in dogs with chronic naturally occurred spinal cord injury due to intervertebral disc herniation (IVDH). Dogs were randomly assigned to four experimental groups (n=4 for each group; total of 16 animals): SCED, EA, SCED + EA) and control. Mild increase in the neurological scoring was found in one animal from SCED group (1/4; 2 points gained), one from EA group (1/4; 8 points gained), three from SCED+EA group (3/4; 16 points gained) and one from control group (1/4; 2 points gained). Functional outcome improvements were observed two animals from SCED group (2/4; 3 points gained), two from EA group (2/4; 4 points gained), one from SCED+EA group (1/4; 1 point gained) and two were from control group (2/4; 6 points gained). However no statistical differences were observed. Magnetic resonance imaging (MRI) findings did not suggest improvement comparing pre- and post-treatment within groups, excepted from one animal from SCED group (1/4), and 10 animals from all groups (10/16) presented signs of injury progression in the SCI in post-treatment exam, which could not be associated to the procedures from study, but could be related to the natural evolution of the disease. Limitation such as number of transplanted stem cells, delivery route, injury chronicity and intrinsic variation among naturally spinal cord injured dogs could have influence outcomes negatively. Moreover, canine deciduous exfoliated teeth were easily obtained and SCED were simply isolated, and no mortality followed up 7 month from procedure were observed.

Keywords: Intervertebral disc herniation. Stem cells from exfoliated human dental pulp. Paraplegy. Complete spinal cord injury. Cell therapy.

LISTA DE ABREVIATURAS E SIGLAS

ANXA5	Annexin A5
BDNF	Derived neurotrophic factor
BMSC	Bone marrow mesenchymal stem cells
CMRP2	Collapsing response mediator protein 2
CPDEC	Células-tronco derivadas de polpa de dente decíduo esfoliado de cães
CPDEc	Células-tronco mesenquimais/estromais derivadas de polpa dente decíduo esfoliado de cães
CSPG	Chondroitin sulphate proteoglycans
CTMSs	Células-tronco mesenquimais/estromais
DPSC	Dental pulp stem cells
DREZ	Dorsal root entry zone
EA	Electroacupuncture
EAP	Eletroacupuntura
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
hNSC	Human neural stem cells
IM	Intramuscular
IV	Intravenous
IVDD	Intervertebral disc degeneration
IVDH	Intervertebral disc herniation
KSPG	Keratin ciliary neurotrophic growth factor
mMEM/ALPHA	Modified alpha-minimum essential medium
Mod	Moderate
MSC	Mesenchymal/stromal stem cells
NGF	Nerve growth factor
NSC	Neural stem/progenitor cells
NT3	Neurotrophin-3
NT4	Neurotrophin-4

NTFs	Neurotrophic factors
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
SC	Subcutaneous
SCED	Stem cells from canine from canine exfoliated dental pulp
Sev	Severe
SHED	Stem cells from human exfoliated deciduous teeth
T2-w	T2-weighted

SUMÁRIO

1 INTRODUCTION

Spinal cord injury is a serious condition which leads to functional and sensory loss. Besides, it can lead to profound emotional, social and economic impairments (SEKHON; FEHLINGS, 2001; NSCISC, 2010). The incidence of spinal cord injury in the world lies between 10.4 and 50 people per million (TATOR, 1995; WYNDAELE; WYNDAELE, 2006). In the United States, according to the National Spinal Cord Injury Statistical Center (NSCISC), the incidence of spinal cord injury in 2016 was 54 cases per million with prevalence of approximately 282,000 persons with spinal cord injury (NSCISC, 2016). Considering traumatic spinal cord injuries, the incidence remains between 12.1 per million in Netherlands and 57.8 per million in Portugal, along with non-traumatic spinal cord injury is about 26.3 per million in Australia (VAN DEN BERG et al., 2010). The estimated economic impact can reach to US$ 1,065,980 at the first year of spinal cord injury with a lifetime cost getting up to US$4,729,788 in a high tetraplegic person injured at 25 years old (CAO; CHEN; DEVIVO, 2011).

Functional impairment following spinal cord injury is due to axon damage or disruption, neuron and glial cells loss and demyelination (CHEN et al., 2010). The first event in spinal cord injury is the direct damage on the tissue, in which the displacement or penetration of the spinal cord initiate a destructive process that expand injury site (RAMER; RAMER; STEEVES, 2005). The insult causes the breakdown of the blood-brain barrier, vascular changes along with edema, hypoxia and ischemia, migration of inflammatory cells, activation of glial cells, neuronal death and excitotoxicity caused by the production of free radicals and nitric oxide and protease release, which can amplify the initial damage (RAMER; RAMER; STEEVES, 2005; ROSSI; KEIRSTEAD, 2009). Furthermore, in contact with invading connective tissue elements, astrocytes change their morphology becoming reactive and forming the glial scar (SILVER; MILLER, 2004), a physical barrier to neurite growth that also upregulate several extracellular-matrix-associated inhibitors of regeneration, which includes chondroitin and keratin sulphate proteoglycans (CSPG; KSPG) (FILBIN, 2003; SILVER; MILLER, 2004). In addition, oligodendrocytes and degenerating myelin also restrain axonal regeneration (CARONI; SAVIO; SCHWAB, 1988; SAVIO; SCHWAB, 1990). The main inhibitory myelin associated molecule is the membrane protein Nogo and its receptor Nogo-66

(MCGEE; STRITTMATTER, 2003; STEWARD et al., 2006). The failure of regeneration following spinal cord injury can be caused, in summary, by the extremely hostile environment, lack of an adequate trophic support for maintenance and axonal growth, the glial scar and its physical and molecular related obstacles, myelin associated inhibitors and the lack of a proper regenerative response by mature neurons (STEWARD et al., 2006).

Different strategies have been studied to achieve spinal cord regeneration, attempting to overcome the inhibition of injured site (BREGMAN et al., 1995; BRÜCKNER et al., 1998; CAI et al., 1999; BRADBURY et al., 2002) and/or encouraging trophic mechanisms for regeneration (TROPEA; CALEO; MAFFEI, 2003; KAMADA et al., 2005; WANG et al., 2007).

Neurotrophic factors (NTFs) can be used to enhance intrinsic growth machinery of central nervous system (SILVER; MILLER, 2004). Application of the neurotrophic factor neurotrophin-3 (NT3) following dorsal rhizotomy in rats was shown to promote centripetal regeneration at the dorsal root entry zone (DREZ) (RAMER et al., 2001; RAMER et al., 2002) and upregulate GAP-43 within injured tissue (RAMER et al., 2001), a regeneration-associated gene (BENOWITZ; ROUTTENBERG, 1997). Moreover, NT3 can promote axonal growth and fiber extension into and beyond lesion epicenter in spinal cord dorsal column crush injury in rats (JAKEMAN et al., 1998). Exogenous Brain Derived Neurotrophic Factor (BDNF) administered intrathecally in rats with spinal cord injury could stimulate hindlimb activity as well enhanced cholinergic fibers growth (BRADBURY et al., 1999). Similar results are seen with NT3 and nerve growth factor (NGF) in transected spinal dorsal column followed by peripheral nerve transplants, which enhanced axonal growth into the graft, out the opposite end and beyond the glial scar into the host tissue (OUDEGA; HAGG, 1996; OUDEGA; HAGG, 1999). Furthermore, NT3, BDNF and ciliary neurotrophic growth factor (CNTF) were capable of stimulate regeneration of injured neurons following a 4-week chronic spinal cord hemisection in rats (YE; HOULE, 1997).

Acupuncture can also be used as an efficient spinal cord injury treatment. Electroacupuncture (EA) is a technique that involves electric stimuli at needles inserted in the acupuncture points and can promote recovery of motor neuron (LIU et al., 2014; YANG et al., 2015; MO et al., 2016) and sensory functions (LIU et al., 2014) in spinal

cord injury in rats. Moreover, EA was shown to upregulate several NTFs, such as NT3, neurotrophin-4 (NT4), BDNF (SUN et al., 2005; WANG et al., 2007; MANNI et al., 2010), NGF (WANG et al., 2007; MANNI et al., 2010), and glial cell line-derived neurotrophic factor (GDNF) mRNA expression (YANG et al., 2015). Interestingly, Liu et al. (2014) demonstrated that EA in a rat model of SCI promoted significant recovery in hindlimb locomotor and sensory functions, upregulated NT3 but also downregulated other NTFs genes expression, which contradicts the traditional view that the lack of NTFs limits spinal cord regeneration. The authors suggested, although, that downregulation of these specific genes could be related to the beneficial effects of EA, such as the reduction of glial scar formation, in the case of platelet derived growth factor (PDGF), transforming growth factor β1 and CNTF genes, and pain regulation, associated with sensory remodeling, in the case of NGF, TrkA, TrkB and TrkC genes. In addition, Liu et al. (2014) observed that EA in rats submitted to spinal cord transection upregulates annexin A5 (ANXA5) and collapsing response mediator protein 2 (CMRP2), two important proteins beneficial to neuron survival and axonal regeneration, and increased the regeneration-associated gene GAP-43, and synaptophysin, considered a marker for functional synapses (CALHOUN et al., 1996; DHILLON et al., 2016). Furthermore, Peng et al. (2007) have reported that EA can reduce glial fibrillary acidic protein (GFAP) levels in the spinal cord, an inhibitory molecule that can induce astrocyte reaction, thus, prevent glial scar forming. In a retrospective study with severe long-standing spinal cord injured dogs with natural occurred disc herniation, electroacupuncture treatment was proportionally more effective for recovery of ambulation and neurological deficits improvement than isolated decompressive surgery (JOAQUIM et al., 2010).

Cellular based therapy is another promising field, broadly studied for spinal cord injury treatment. There are several strategies of cellular transplantation such as genetically modified fibroblasts (JIN et al., 2002), Schwan cells (CHAU et al., 2004; PEARSE et al., 2004; BUNGE, 2016), olfactory ensheathing cells (CLOUTIER et al., 2016; KHANKAN et al., 2016), co-transplantation of Schwann cells and olfactory ensheathing cells (SUN et al., 2013) and stem cells (LU et al., 2003). A variety of types of stem/progenitor cells transplantation is being used for spinal cord injury treatment, such as Neural Stem Cells, Umbilical Blood Cells, MSC, Embryonic Stem Cells and induced Pluripotent Stem Cells (iPS) (FEHLINGS; VAWDA, 2011; HERNANDEZ;

TORRES-ESPIN; NAVARRO, 2011). Studies aim to their potential of forming myelin, promoting and guiding axonal growth, bridging the site of injury or differentiating to a neuronal or glial lineage, as well their intrinsic potential to secret trophic factors that promote neuroprotection and plasticity on spinal cord injury (LU et al., 2003; HERNANDEZ; TORRES-ESPIN; NAVARRO, 2011).

Mesenchymal stem/stromal cells are a heterogeneous class of multipotent cells obtained from different adult tissues (ERICES; CONGET; MINGUELL, 2000; JANKOWSKI; DEASY; HUARD, 2002; NÖTH et al., 2002). The use of MSC in SCI has been widely studied and, despite of variability and limitations, was shown to be effective for functional and sensory recovery in animal models of traumatic SCI (ANTONIC et al., 2013; OLIVERI; BELLO; BIERING-SORENSEN, 2014) and appears to be safe and effective in clinical trials in human spinal cord injured patients, particularly in chronic complete injuries (LI et al., 2015). MSC can differentiate into mesodermal tissues including bone, cartilage, muscle and blood cells (GOEL, 2016) and in addition have the potential for trans-differentiation *in vitro* and *in vivo* into different lineage, such as neural (YIM; PANG; LEONG, 2007; YAN et al., 2011; ABDULLAH, 2016) and glial cell lineage (CADDICK et al., 2006; DE ALMEIDA; 2011; YAN et al., 2011), even though differentiation into functional neuronal lineage in vivo is not fully demonstrated (WOODBURY et al., 2000; PATEL et al., 2013). In SCI studies, MSC has been shown to promote neuromodulation (CHOPP et al., 2000; HOFSTETTER et al., 2002; UCCELLI et al., 2011), immunomodulation (CHAMBERLAIN et al., 2007; NAKAJIMA et al., 2012; DASARI; VEERAVALLI; DINH, 2014), regeneration (FOROSTYAK; JENDELOVA; SYKAVO, 2013; OLIVERI; BELLO; BIERING-SORENSEN, 2014), secretion of several NTFs (CHUNG et al., 2016; HAN et al., 2016; ZHOU et al., 2016), and has been studied as a potential source for cell replacement through differentiation into neuronal and glial cells lineage (TOHILL et al., 2004; CADDICK et al., 2006; ZHANG et al., 2009).

Dental stem cells (DSC) present more beneficial features for SCI treatment compared to other sources of MSC (YAMAMOTO et al., 2014; BIANCO et al., 2016). The DSC derive embryologically from the neural crest, originated at the interface of neural plate and non-neural ectoderm where cells differentiate into multiple cells types, including neuron and glia, pigment cells, fibroblasts, adipocytes, as well odontoblasts (MAYOR; THEVENEAU, 2013). DSCs have been revealed to promote neuroplasticity and induce

endogenous axonal guidance in a host nervous system (ARTHUR et al., 2009) and capable of promoting recovery after implantation in damaged spinal cord (YAMAMOTO et al., 2014). Moreover, the neurogenic potential from DSC seems to be higher than from bone marrow mesenchymal stem cells (BMSC), a traditional tissue sources of MSCs in clinical stem cell research (OLIVERI; BELLO; BIERING-SORENSEN, 2014). It could be due to their origin in neural crest which make this cells a promising source for spinal cord injury therapy (HUANG; GRONTHOS; SHI, 2009).

Combinatorial strategies for SCI treatment appears to act synergistically leading to more effective recovery (RAMER; RAMER; STEEVES, 2005; BRADBURY; MCAHON, 2006; HERNANDEZ; TORRES-ESPIN; NAVARRO, 2011). The combination of NTFs or anti-inhibitory molecules and cellular grafting (XU et al., 1995; LU et al., 2005; KARIMI-ABDOLREZAEE et al., 2010), genetic engineered cells grafts modified to overexpress NTFs (GRILL et al., 1997; MENEI et al., 1998; MURRAY et al., 2002; LU; JONES; TUSZYNSKI, 2005) and anti-inhibitory molecules (WALTHERS; SEIDLITS, 2015) can improve growth potential through injury site. Cyclic AMP (cAMP) also appears to play an important role in the regenerative potential (RAMER; RAMER; STEEVES, 2005). Sensory and cerebellar neuron pre-treated with neurotrophic factor increased intracellular cAMP and enhanced axonal elongation through inhibitory substrates (QIU; CAI; FILBIN, 2002). Also, approaches that upturn cAMP levels improved axonal growth into the marrow mesenchymal stem cell (BMSC) graft on a cervical lesion of the dorsal columns and showed a significant axonal growth when combined with NT-3 injection (LU et al., 2004). Besides, the accumulation of cAMP in association with Schwann cells transplant enhanced axonal sparing/growth and functional recovery after moderate spinal cord contusion (PEARSE et al., 2004).

Interestingly, electroacupuncture appears to have a synergistic effect on stem cells grafts in spinal cord injury models. A group from Sun Yat-sen University, Guangzhou - China, has been studying the interaction between electroacupuncture (EA) and stem cells in spinal cord transection in rats. According to their findings, EA improves survival and migration of neural stem/progenitor cells (NSC) within injury (CHEN et al., 2008). Also, EA associated with BMSC in spinal cord transection promoted BMSC survival and differentiation into neuronal-like and oligodendrocyte-like cells, nerve fibers regeneration, NT-3 and cAMP upregulation at injury site, and partial locomotor functional recovery (DING et al., 2009; YAN et al., 2011). Another study, suggested

that the structural and functional recovery following combination of EA and BMSC in a spinal cord transection could be due to the downregulation of GFAP and CSPG (extracellular-matrix-associated inhibitors of regeneration, directly involved in glial scar formation), which prevented axonal degeneration as well as improved axonal regeneration (DING et al., 2011).

Due to limitations in translating pathophysiology of rodent SCI to human condition, companion animals have been presented as a more realistic model of SCI for stem cells therapy (HOFFMAN; DOW, 2016). Canine thoracolumbar intervertebral disc herniation (IVDH) is a spontaneous disease that bears similarities to acute SCI in human (LEVINE et al., 2011). Furthermore, intervertebral disc degeneration (IVDD) in dogs with or without disc herniation presents subclinical course or clinical features, including back pain, paresis or paralysis, along with histological and biochemical aspects resembled to human IVDD (BERGKNUT et al., 2012). Moreover, naturally occurred spinal cord contusion in dogs, like human, varies in depth, extent, and chronicity, unlike the injury experimentally generated in laboratory animal and which, in latter case, also invokes ethical concerns (LEVINE et al., 2011).

The present study investigated safety, feasibility and therapeutic effect of stem cells from canine exfoliated dental pulp (SCEDs) combined with electroacupuncture in chronic spinal cord injured dogs due to naturally occurred IVDH.

2 MATERIALS AND METHODS

2.1 PATIENT SELECTION

A total of 16 paraplegic companion dogs, from 5 to 11 years old, 9 females and 6 males, different breeds, were selected for the study, following previous formalized consent from owners. The criteria for selection of cases included the acute onset of paraplegia resulted from thoracolumbar intervertebral disc herniation (IVDH) (T10-L4), with absence of deep pain perception and/or superficial pain perception in pelvic limbs, followed up for at least 3 months at the time of initial examination. Superficial and deep pain perception was considered absent when animal did not demonstrate signs of conscious pain (e.g., crying, trying to bite or look to the limb) when interdigital webs (superficial pain) or phalanges (deep pain) in pelvic limbs were pinched using a Kelly forceps (DE LAHUNTA; GLASS, 2014). All animals were submitted to magnetic resonance imaging prior to the beginning of experiment. Injure had to be found within 10th thoracic and 4th lumbar spinal vertebrae in the thoracolumbar spinal cord region. Dogs were excluded from study if they presented at the time of initial examination signs of impairment at lumbosacral spinal cord region, such as decreased or absent patellar and/or anal reflexes. Patellar reflex was tested tapping middle patella tendon with animal in lateral recumbency and the leg loosely supported, and anal reflex was tested touching or pinching the anal or perineal area with a Kelly forceps (DE LAHUNTA; GLASS, 2014).

The animals were randomly assigned into one of the following four groups (n=4 per group). Stem cells from canine exfoliated dental pulp group (SCED): stem cells transplantation; Electroacupuncture (EA) group - injection of saline instead of stem cells and electroacupuncture treatment; SCED + Electroacupuncture group (SCED+EA) - stem cells transplantation and electroacupuncture treatment; Control group (Control): injection of saline instead of stem cells without electroacupuncture treatment. All animals were submitted to underwater treadmill training during treatment period.

The treatment lasted 13 weeks and animals were evaluated before and after treatment by neurologic examination, functional assessment and magnetic resonance imaging.

The owners signed a consent form, in which all possible procedures were explained. Each group in the study received the same consent form. At the end of the study, owners had the option of proceeding with other treatments that their pet had not received, if they wished. All animal procedures performed were in accordance with guidelines defined by the Committee of Ethic in Animal Experimentation of College of Veterinary Medicine and Zootechny, University of Sao Paulo – Brazil.

2.2 ISOLATION AND CULTURE OF STEM CELLS FROM CANINE EXFOLIATED DENTAL PULP

Following signature of a donation term from owners, exfoliated deciduous teeth from young female dogs were extracted under anesthesia, using a tooth extraction forceps, at the time of elective spaying surgery at Radial Vet Veterinary Clinic, Sao Paulo – Brazil. The teeth were put into modified alpha-minimum essential medium (mMEM/ALPHA; BR30238-01, LGC Biotecnologia) with 5% of penicillin 10.000 U/mL-streptomycin 10,000 µg/mL (BR30110-01, LGC Biotecnologia), and maintained refrigerated between 6 - 10º Celsius. The isolation procedure was previous described (DISSANAYAKA et al., 2011) and was performed within 24 hours from tooth extraction. Briefly, tooth surface was cleaned with a solution of phosphate-buffered saline (PBS) and 5% of penicillin-streptomycin 10,000 U/ml. After pulp was extracted, using a dental pulp extractor, the tissue was digested in a 3-mg/mL collagenase type I (17100017, GIBCO-Invitrogen) and 4-mg/mL dispase (17105041, GIBCO-Invitrogen) solution for 1 hour at 37°C. Then, the cells were filtered using a 70-µm strainer (352350, BD Falcon) to obtain single-cell suspensions. These cells were seeded in 8.5 cm^2 culture plates containing mMEM/ALPHA supplemented with 15% fetal bovine serum (FBS) (10-bio500, LGC Biotecnologia), 100 µM L-ascorbic acid-2-phosphate (A8960, Sigma-Aldrich) and 1% penicillin 10.000 U/mL-streptomycin 10,000 µg/mL, and cultured under

5% CO2 at 37°C. Medium was replaced every 3 days, and cells were subcultured at 80% confluence. After confluence, SCED were washed with PBS, then trypsin 0,25% (Tryple – GIBCO, Cat. A1285901) was added and plates were maintained for 5 minutes in 37°C in a humidified atmosphere of 5% CO_2 until cells detached from plates. Subsequently, enriched Alpha MEM medium was added to inactivate trypsin and the solution containing stem cells was centrifuged at 1200 RPM for 5 minutes. Afterwards, supernatant was discarded and cells were expanded in a 22 cm^2 culture plate. The process was repeated in 60,1 cm^2 culture plate until cells reach passage 4.

2.3 STEM CELLS PREPARATION

After confluence in passage 4, SCED were detached from plates with trypsin 0,25% as detailed above. After centrifugation, supernatant was discarded, cells were re-suspended in PBS and the cells were centrifuged again. The process was repeated two times more. Finally, stem cells were diluted in 150 µl of PBS, divided in 3 syringes (50 µl each syringe; 1ml syringes). The same procedure was repeated for second transplantation.

2.4 ANESTHESIA

All animals were submitted to preanesthesic evaluation with blood test (complete blood count, blood urea nitrogen, creatinine, alanine aminotransferase and alkaline phosphatase) and electrocardiography. Preanesthesic medication was done with intramuscular (IM) injection of acepromazine (0,03 mg/kg dose) and meperidine (2 mg/kg), induced into anesthetic plan with intravenous (IV) administration of propofol (5mg/kg dose) and maintained under inhalation anesthesia with isoflurane. Animals received transoperative IV ceftriaxone (30mg/kg dose), and subcutaneous (SC) dexamethasone (0,2mg/kg), tramadol hydrochloride (2mg/kg), dipyrone (25mg/kg).

2.5 SURGERY AND SCED TRANSPLANTATION

The dogs were submitted to dorsal laminectomy and spinal cord was exposed at the site injury as described in Seim III (2007). Briefly, under anesthesia, the animals were positioned in ventral decubitus, an incision over the dorsal midline was made including two spinous process cranial and caudal to the lesion, then, using a periosteal elevator or a small osteotome, epaxial muscles were subperiosteally elevated from dorsal spinous processes, laminae, articular facets, and pedicles of affected vertebrae, followed by the removal of the dorsal spinous process with large, single-action duckbill rongeurs. Then, using a pneumatic drill, the three layers of lamina (outer cortical, medullary and inner cortical) from both vertebrae were burred, and, using a dental spatula, the inner periosteum was penetrated exposing the vertebral canal. Durotomy was performed if syringomyelia was observed. The stems cells were injected intramedullary. After 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 (20mg/kg, twice daily for 7 days), prednisolone (1mg/kg, once daily for five days, then, 0,5mg/kg for five days and 0,25mg/kg for more five days), and dipyrone (25mg/kg 3 times a day for 7 days).

It was injected a total of $4x10^6$ SCED, divided in two time points. The first transplantation was performed with a dose of $2x10^6$ cells diluted in 150 µl of PBS, divided in 3 syringes using a 30-gauge (0.4mm x 13mm) needle, directly into the spinal cord parenchyma (middle of the injury, cranial and caudal margins). The second transplantation was done seven days after first procedure. Animals were put under anesthesia and received three percutaneous intraspinal injection, at cranial, midpoint and caudal margin of the injury, in a total dose of $2x10^6$ cells using a 24-gauge intravenous catheter needle (0.7mm x 19mm), guided by palpation of surgical window.

2.6 ELECTROACUPUNCTURE

Electroacupuncture treatment started after second transplantation (between days 8 and 11) and was performed three times a week for initial seven weeks, two times a week for more two weeks and then twice a week for the last three weeks. During EA treatment, the dogs remained on the exam table or on owner's lap (if it was agitated on table), without physical restraining. There were used stainless acupuncture needles with 0,25mm thickness (Dongbang). The selected acupuncture points were GV2, Bai Hui, VG3a, VG6; bilateral BL19, BL23 and BL24; unilateral KI3, ST36, LV3 and Wei Jian. Location of acupuncture point were in accordance with Xie and Preast (2007). Electrodes were connected at Bai Hui/GV6 and bilateral BL19/B24. According to Xie and Preast (2007), the points are located: at sagittal dorsal midline for points VG2 (between sacrum and first caudal vertebrae), Bai hui (7th lumbar and 1st sacral vertebrae), VG3a (4th and 5th lumbar vertebrae) and VG7 (11th and 12th thoracic vertebrae); 1,5 tsun lateral to sagittal dorsal midline (1 tsun is equivalent to a rib bone's width for acupuncture points in thoracic spine area) for points between BL19 (11th and 12th thoracic vertebrae), BL21 (13th thoracic and 1st lumbar vertebrae), BL23 (2nd and 3rd lumbar vertebrae), BL24 (4th and 5th lumbar vertebrae); in a depression approximately midpoint between medial malleolus and common calcaneal tendon insertion for KI3; at the salience of tibial cranial muscle on the craniolateral face of pelvic limb for ST36; at lateral surface of 2nd digit, distal to metatarsophalangeal joint on medial face of pelvic limb for LV3; and at the tip of the tail for *Wei Jian* point.

Electrodes were connected at Bai Hui/GV6 and bilateral BL19/B24. Electroacupuncture device (NKL EL608) was configured to alternate between 2Hz for 2 seconds and 60Hz for 1 second, for 20 minutes accordingly with the protocol of Ding et al. (2009). The intensity was adjusted to induce a slight twitch on pelvic limbs or paravertebral muscles without dog's discomfort.

2.7 UNDERWATER TREADMILL

Underwater Treadmill was performed twice a week, for the 12 weeks of treatment. The dogs were stimulated to walk on an underwater treadmill progressively from 3 to 15 minutes, respecting the physical limit of the animal. When dog could not bear its weight, or walked improperly (e.g., crossing of pelvic limbs, scuffing foot on protraction or standing on dorsum) a veterinarian corrected gait during exercise, to ensure appropriate gaiting.

2.8 NEUROLOGIC SCORING

All animals were submitted to a neurologic examination before and after treatment following guidelines from Chrisman (2003). The tests were recorded and videos were evaluated by a blind observer. It was selected five key tests to assess spinal cord injury and a score was given for each of them: urinary incontinence (Yes = 0, No = 4), fecal incontinence (Yes=0, No=4), and, for each pelvic limbs, conscious proprioception (Absent=0, Retarded=1, Present=2), hopping (Absent=0, Retarded=1, Present=2), superficial pain perception (Absent=0, Retarded=1, Present=2) and deep pain perception (Absent=0, Retarded=1, Present=2). Urinary or fecal incontinence was considered negative when dog was capable of consciously hold and control elimination of urine or feces with no incontinence. Conscious proprioception positioning was observed displacing the dog’s foot by turning it onto its dorsum while supporting the dogs weight, the animal should replace it immediately. Hopping was tested holding three legs or the contralateral tested pelvic limp off the ground and forcing 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 pelvic limb using a Kelly forceps instrument. The presence of pain was observed when animal demonstrated any sign of mental aware of the stimulus (e.g.,

crying, trying to bite of look to the limb) when pressure was applied (DE LAHUNTA; GLASS, 2009).

2.9 FUNCTIONAL ASSESSMENT

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

Chart 1 - Functional Assessment

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

Source: Olby et al. (2001) adapted by PRADO, C. V. G. B. (2016)

2.10 MAGNETIC RESONANCE IMAGING EVALUATION

All animals underwent to standard T1- and T2-weighted (T2-w) magnetic resonance imaging (MRI) scanning (0.24 Tesla VET-MR Grande - Esaote) before and after treatment. The images were analyzed by a blind examiner.

2.11 STATISTICAL ANALYSIS

For evaluation of the pre- and post-treatment scores in neurological and functional assessment, Paired Wilcoxon Test was used. Posteriorly, difference of the post- and the pre-treatment neurologic and 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 analysis were made with Graphpad Prism 5.0 Software (GRAPHPAD, 2007).

3 RESULTS

No mortality was observed in dogs followed up 7 months after SCED transplantation. Minor post-operative pain was observed in animals 1, 9 and 15, and was extinguished within 2-7 days. Detailed information is described in Chart 2.

3.1 NEUROLOGICAL SCORING

Using the scoring method adopted for neurologic examination, in SCED group, animal 1 showed an improvement in score from 0 to 2 points, with absent conscious proprioception before and retarded conscious proprioception after treatment. The animals 2, 3 and 4 did not score before or after treatment. In EA group, animal 8 showed an improvement in score from 2 to 10 points, with positive deep pain perception on left pelvic limb before and normal conscious proprioception on left pelvic limb, positive deep pain perception and superficial pain perception in both left and right pelvic limbs after treatment. Animals 5, 6 and 7 did not score before or after treatment. In SCED+EA group, animal 9 showed an improvement in score from 0 to 6 points, with conscious proprioception and hopping test negative for pelvic limbs before and positive conscious proprioception on left and right pelvic limbs and positive hopping in left pelvic limb after. Animal 10 showed also showed an improvement in score from 0 to 8 points, with negative conscious proprioception and urinary incontinence before and positive conscious proprioception on left and right pelvic limbs and with no urinary incontinence after treatment. Furthermore, animal 12 showed an improvement in score from 0 to 2 points, with conscious proprioception negative before and retarded after treatment. Animal 11 did not score before or after treatment. Finally, in control group, animal 16 showed an improvement in score from 0 to 6 points, with conscious proprioception and hopping test negative for right pelvic limb and retarded after treatment. Animals 13, 14 and 15 did not score before or after treatment (Chart 2).

Chart 2 - Detailed data of the cases

Animal, breed, gender and age (years)	Injury chronicity	Moment	T2-w MRI Hyperintensity / Spinal Cord Atrophy	MRI Compression (spinal vertebrae)	SCI length (cm)	Associated Syringomyelia	Neurologic scoring	Funcional assessment
SCED								
Animal 1, Mongrel, F, 5	6 months	Pre-treatment	T8-L1; sev spinal cord atrophy	T10-12 (mild ventral disc)	9.3	Yes	0	0 - No PL movement
		Post-treatment	No changes	No changes	9.3	Yes	2	0 - No PL movement
Animal 2, Daschshund, F, 4	3 months	Pre-treatment	T11-L2 (sev)	T12-13 (mild ventral disc)	6.9	Yes	0	0 - No PL movement
		Post-treatment	T11-L1 (sev)	No changes	5.8	Yes	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
Animal 3, Dachshund, M, 6	6 months	Pre-treatment	T12-13	No compression	0.7	No	0	0 - No PL movement
		Post-treatment	Progression along caudal thoracic and cranial lumbar spine (sev); spinal cord atrophy (sev)	No changes	7.7	Yes	0	0 - No PL movement
Animal 4, Mongrel, F, 11	4 months	Pre-treatment	L1-2	T13-L1 (mild ventral)	0,4	No	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	Did not underwent to post-treatment RMI	Did not underwent to post-treatment RMI	-	-	0	3 - Non-WB protraction PL with > 1 joint involved > 50% time
Eletroacupuncture								
Animal 5, Dachshund, F, 6	13 months	Pre-treatment	T9-13	No compression	5.8	No	0	0 - No PL movement
		Post-treatment	progressive spinal cord atrophy	No changes	3.7	Yes	0	2 - Non-WB protraction PL with > 1 joint involved < 50% time
Animal 6, Dachshund, F, 7	14 months	Pre-treatment	No hyperintensity	L1-2 (sev right ventral)	1.4	Yes	0	3 - Non-WB protraction PL with > 1 joint involved > 50% time
		Post-treatment	L1-3, spinal cord atrophy	No changes	4.3	Yes	0	3 - Non-WB protraction PL with > 1 joint involved > 50% time
Animal 7, Lhasa Apso, F, 4	6 months	Pre-treatment	L4	No compression	1.8	Yes	0	0 - No PL movement
		Post-treatment	No changes	No changes	2.3	Yes	0	0 - No PL movement
Animal 8, Dachshund, M, 7	8 months	Pre-treatment	No hyperintensity	T13-L1 (mild ventral; focal central canal dilatation)	0		2	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	No changes	T13-L1 (mild ventral; progressive dilatation of central canal)	1,3		10	3 - Non-WB protraction PL with > 1 joint involved > 50% time
SCED + Electroacupuncture								
Animal 9, Mongrel, M, 11	5 months	Pre-treatment	No hyperintensity	T12-13 (mild right ventral disc)	1.9	Yes	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	No changes	No changes	2.8	Yes	6	1 - Minimal non-WB protraction PL (movement of 1 joint)
Animal 10, Dachshung, F, 4	17 months	Pre-treatment	L1-T13	T11-12 (mild ventral)	2.1	Yes	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	No changes	No changes	2.7	Yes	8	2 - Non-WB protraction PL with > 1 joint involved < 50% time
Animal 11, Lhasa Apso, M, 7	16 months	Pre-treatment	No hyperintensity	T10-11 (mild right ventral); right-sided hemilaminectomy T13-L1	4.6	Yes	0	0 - No PL movement
		Post-treatment	No changes	No changes	4.7	Yes	0	0 - No PL movement
Animal 12, Dachshund, M, 2	7 months	Pre-treatment	T10-11	L1-2 (right vental; right-sided hemilaminectomy)	1.6	Yes	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	No changes	No changes	1.6	Yes	2	1 - Minimal non-WB protraction PL (movement of 1 joint)
Control								
Animal 13, Yorkshire, M, 7	14 months	Pre-treatment	No hyperintensity	T13-L1 (righ ventral)	3.7	Yes	0	1 - Minimal non-WB protraction PL (movement of 1 joint)
		Post-treatment	spinal cord atrophy	No changes	3.9	Yes	0	5 - WB protraction of PL 10 to 50% time
Animal 14, Dachshund, F, 8	13 months	Pre-treatment	T13-L1	T11-12 (mod ventral)	3.6	Yes	0	0 - No PL movement
		Post-treatment	No changes	No changes	4.4	Yes	0	0 - No PL movement
Animal 15, Pug, M, 7	6 months	Pre-treatment	T10-11	T10-11 (ventral)	1	No	0	0 - No PL movement
		Post-treatment	No changes	No changes	0.9	No	0	2 - Non-WB protraction PL with > 1 joint involved < 50% time
Animal 16, Dachshund, F, 4	18 months	Pre-treatment	No hyperintensity; spinal cord atrophy	T11-12 (mild right ventral; right-sided hemilaminectomy)	2	No	0	3 - Non-WB protraction PL with > 1 joint involved > 50% time
		Post-treatment	No changes	No changes	1.9	No	2	3 - Non-WB protraction PL with > 1 joint involved > 50% time

Source: Prado, C. V. G. B. Spinal vertebrae was used to localize injury localization, considering 13 thoracic (T) and 7 lumbar (L) vertebrae. Spinal cord compression was classified in mild, moderate (mod) and severe (sev). Each intervertebral space within vertebrae was considered. Pelvic limb (PL), weight-bearing (WB) was observed to determine score in functional assessment.

3.2 FUNCTIONAL ASSESSMENT

Using the adapted functional recovery score from Olby et al. (2001), in SCED group, animal 2 has improved from score 0 (no pelvic limb movement) to 1 (minimal non-weight-bearing protraction of the pelvic limb with movement of 1 joint) and animal 4 has improved from score 1 to 3 (non-weight-bearing protraction of the pelvic limb with > 1 joint involved > 50% of the time). The animals 1 and 3 did not score. In EA group, animal 5 has improved from score 0 to 2 (non-weight-bearing protraction of the pelvic limb with > 1 joint involved < 50% of the time), animal 6 remained at the initial score of 3, and animal 8 has improved from score 1 to 3. Animal 7 did not score. In SCED+EA group, animals 9 and 12 remained at the same score of 1 and animal 10 has improved from score 1 to 2. Animal 11 did not score. In control group, animal 13 has improved from score 1 to 5 (weight-bearing protraction of pelvic limb, 10 to 50% of the time), animal 15 has improved from 0 to 2, animal 16 remained at initial score of 3 and animal 14 did not score (Chart 2).

3.3 MAGNETIC RESONANCE IMAGING

At pre-treatment time point, all dogs presented injury at 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 to 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 from animals 6, 8, 9, 11, 13 and 16, had presented different grades and extent of hyperintense signal in T2-w MRI in the spinal cord. All animals had signs of injury within thoracolumbar spinal cord region. Detailed information is described in chart 2. After treatment, data generated by MRI revealed differences in injury extent, hyperintense signal and spinal cord atrophy. In SCED, animal 2 had a reduction in spinal cord hypersignal from vertebrae T11-L2 to T11-L1 and injury extent from 6.9 to

5.8 cm; and animal 3 presented a progression of severe hypersignal along cranial thoracic and caudal lumbar spine, with an increase of the injury extent from 0.7 to 7.7 cm. Animal 1 presented no changes in MRI and the owner of animal 4 did not bring it for second MRI. In EA group, animal 5 presented a progressive spinal atrophy, with reduction in injury extent from 5.8 to 3.7cm; animal 6 presented a hyperintense signal in spinal cord within L1-L3, not seen in pre-treatment MRI, and had an increase in injury extent from 1.4 to 4.3 cm; and animal 7 presented an increase in injury extent from 1.8 to 2.3 cm. Animal 8 presented no changes in MRI. In SCED + EA, animal 9 presented an increase in injury extent from 1.9 to 2.8 cm; and animal 10 presented and increase in injury extent from 2.1 to 2.7. Animals 11 and 12 did not present any significant change in MRI. In Control group, animal 13 presented atrophy within spinal cord injury and had a mild increase in injury extent from 3.7 to 3.9 cm; and animal 14 presented an increase in injury extent from 3.6 to 4.4 cm. Animals 15 and 16 presented no changes in MRI.

Data generated by MRI revealed no changes in spinal cord parenchyma between pre- and post-treatment in all groups.

4 DISCUSSION

Stem cell-based therapies for spinal cord injury aim three basic strategies: neuroprotection to prevent or modulate damage caused by secondary injury after first insult, regenerative potential to permit axonal regeneration through the inhibitory microenvironment and replacement of dead cells, particularly oligodendrocytes, to facilitate remyelinating of spared axons (HERNANDEZ; TORRES-ESPIN; NAVARRO, 2011; TASHIRO et al., 2016). Some advantages in using MSC in SCI is the feasibility to be harvested, expanded and stored, the possibility of obtaining them directly from patient for autologous transplantation (NAKAJIMA et al., 2012), and its minimal or no immunoreactivity and graft-versus-host reaction in allogeneic transplantation (LE BLANC; RINGDÉN, 2006; YI; SONG, 2012).

Dental pulp stem cells (DPSC) and stem cells from human exfoliated deciduous teeth (SHED) are sources of MSC found in perivascular niche of the dental pulp (GRONTHOS et al., 2002) that simultaneously express early mesenchymal, neuroectodermal stem/progenitor cells markers and certain embryonic stem cells markers (GRONTHOS et al., 2000; MIURA et al., 2003; KERKIS et al., 2007; SAKAI et al., 2012). These cells express several NTFs following SCI, such as GDNF, BDNF, CNTF and NGF (SAKAI et al., 2012; BIANCO et al., 2016). Furthermore, SHEDs exhibit a higher number of population doublings in *vitro* compared with BMSC and its proliferation rate is 1.5 times higher than the observed in DPSCs (MIURA et al., 2003). In addition, both DPSCs and SHEDs expressed GDNF, BDNF and CNTF at more than 3 to 5 times more the levels expressed by BMSC, and promoted neuroprotection and regeneration of transected axons by directly inhibiting multiple axon growth inhibitors, including chondroitin sulfate proteoglycan and myelin-associated glycoprotein, via paracrine mechanisms (SAKAI et al., 2012). Moreover, it has been shown the capacity of SHEDs to differentiate into glial and neuronal cells *in vitro* (MIURA et al., 2003; MORSCZECK et al., 2010) and express glial markers *in vivo* (DE ALMEIDA et al., 2011) and differentiate into mature oligodendrocyte when transplanted into a spinal cord injury (SAKAI et al., 2012). Taghipour et al. (2011) transplanted undifferentiated SHEDs and neural induced SHEDs (iSHEDs) into acute contused spinal cord in rats and reported neuronal and glial differentiation, as well as a significant functional recovery in both SHEDs and iSHEDs groups. Interestingly, Sakai et al. (2012)

demonstrated that the functional improvement after spinal cord injury in rats in was higher when transplanted with SHEDs than DPSC which in turn was higher than BMSC. Thus, the regenerative potential of SHEDs and the safety and feasibility in obtaining this source of stem cells without any harmful or painful procedure for donor, make the stem cells from canine exfoliated dental pulp (SCED) a great candidate for SCI treatment in dogs. Furthermore, translational findings using SCEDs could represent a potential source for stem cell-based therapy for human SCI.

The abnormal findings in the neurologic examination of dogs with severe thoracolumbar IVDH are related to the injury at the level of thoracolumbar spinal cord (injured upper motor neurons) and the lack of spinal reflexes inhibition of lower motor neuron at lumbosacral spinal cord (spared lower motor neurons) caudal to (bellow) the SCI. The first can cause urinary and fecal incontinence, and no conscious movement, negative conscious proprioception and hopping tests, absent of superficial pain perception with or with no deep pain perception in pelvic limbs. The later can cause spasticity and increased/normal spinal reflexes in pelvic limbs (e.g., increased patellar and sciatic reflex) (DE LAHUNTA; GLASS, 2009). Hence, in this study, we aimed to evaluate neurological signs which could be impaired directly by the thoracolumbar injury. Considering the ascending and/or descending spinal tracts involved in a normal response in these neurological signs it is possible to determine indirectly the structures within injury recovered after treatment. A positive conscious proprioception test requires the integrity of the fasciculus gracilis (afferent pathway which brings the impulses from mechanoreceptors, stretch, or touch receptors in pelvic limb to parietal cortex), and the descending lateral corticospinal, rubrospinal, medullary and pontino reticulospinal tracts (major motor response pathways) (BAGLEY, 2005). The hopping test requires, in addition to the later pathways, the integrity of dorsal and ventral spinocerebellar tracts (part of the afferent pathway which transmit unconscious proprioceptive information from the muscle spindles and Golgi tendon organs to cerebellum) (BAGLEY, 2005; JAGGY; PLATT, 2010). Moreover, for a normal urinary function and defecation, spinothalamic tract and fasciculus gracilis (which ascend sensory information), tectospinal and reticulospinal pahtways (which descend information) integrity within thoracolumbar spinal cord are required (BAGLEY, 2005). Finally, superficial pain perception goes through myelinated fibers in the afferent spinothalamic tract, while deep pain perception ascends in unmyelinated axonal fibers

though the spinoreticular tract and are required to be present in neurological examination (PRADA, 2014).

Mild increase in the neurological scoring was found in six dogs (6/16; 28 points gained), within one was from SCED group (1/4; 2 points gained), one from EA group (1/4; 8 points gained), three from SCED+EA group (3/4; 16 points gained) and one from control group (1/4; 2 points gained) (Chart 2). Moreover, functional outcome improvements were observed in seven dogs (7/16; 14 points gained), within two were from SCED group (2/4; 3 points gained), two from EA group (2/4; 4 points gained), one from SCED+EA group (1/4; 1 point gained) and two were from control group (2/4; 6 points gained) (Chart 2). Considering the therapeutic potential of stem cells and/or electroacupuncture in SCI (WANG et al., 2009; DING et al., 2011; RUFF; WILCOX; FEHLINGS, 2012), mild improvements observed could be related to the isolated and combined treatments. However, no statistical difference was found between groups in neurologic examination and functional assessment, thus, improvements could not be associated to any isolated or combined treatments. Additionally, the influence of the decompressive surgery and underwater treadmill training, in which all animals were submitted, could not be disregarded, due to their potential to promote regeneration within injury site (OLBY et al., 2003; ENGESSER-CESAR et al., 2005; THOMAS; GORASSINI, 2005; JEFFERY et al., 2016) and neuroplasticity of spared spinal circuitry spinal cord caudal to (bellow) the injury at lumbar spinal cord (EDGERTON et al., 2014). Besides, the possibility of spontaneous recovery cannot be excluded (BALLERMANN; FOUAD, 2006).

Curiously, from the seven dogs that had improved scores in functional assessment, only two dogs (animal 8 from EA and animal 10 from SCED+EA) had improved scores in neurological examination as well. These findings could suggest, even though no statistical association could be made, that the partial recovery in within injury site (indirect assessed by neurological scoring) could not be directly associated with improvement in stepping and coordination of pelvic limbs (observed in functional assessment), which, on the other hand, are related to enhanced plasticity in lumbar spinal cord spared circuitry (WOLPAW; TENNISSEN, 2001; ICHIYAMA et al., 2005; ROUSSE et al., 2016). Moreover, the two dogs which had improved in both neurological and functional scoring obtained the higher improvement in neurologic scoring (animals 8 and 10 with 8 points gained each), but low functional improvement.

Therefore, it could indicate that regeneration of injured spinal cord and neuroplasticity of spared lower motor neurons at lumbar spinal cord caudal to the SCI point could occur, somewhat, independently. Although, a higher recovery at injury site in spinal cord could have some influence in functional improvement. However further studies would be necessary to test this hypothesis.

For the MRI analysis in the present study, hyperintensity observed in T2-w MRI, considering the clinical history of acute onset of paraplegy, chronicity and actual clinical signs, were related to gliosis/glial scar. MRI findings did not suggest improvement comparing pre- and post-treatment within groups, excepted from animal 2 from SCED group, in which a reduction in T2-w MRI hyperintensity and SCI extents. Instead, 10 animals among all groups (10/16) showed signs of progression of the SCI in different grades observed in increased T2-w MRI hyperintensity and SCI extents (Chart 2), which is compatible with the natural progression of the SCI (BRAMLETT; DIETRICH, 2007).

In the present study, mild improvements observed in neurologic and functional scoring could not be associated to any isolated or combined treatments. However, limitations for a better assessment can be summarized in four major causes: number of transplanted stem cells, delivery route, injury chronicity and intrinsic variation among naturally spinal cord injured dogs.

The number of cells transplanted into injury site appear to have an important role in spinal cord regeneration. Higher doses of transplanted stem cells are associated with better regenerative outcome in pre-clinical studies (ANTONIC et al., 2013). Oliveri, Bello and Biering-Sørensen (2014) observed that the total dose of stem cells in rodent models of spinal cord injury used in intraparenchymal injection is between 3×10^3 and 5×10^6 cells (5×10^5 – median dose). Li et al. (2015) showed that in clinical trials in human patients with complete and chronic SCI, higher doses of transplanted stem cells (between $n \times 10^8$ - 10^9) seemed to be more beneficial. In canine models of spinal cord injury, studies have used a total dose of stem cells from $1 \text{ X } 10^6$ (RYU et al., 2009; SARMENTO et al., 2014) to 1×10^7 (LIM et al., 2007; JUNG et al., 2009; PARK et al., 2012; KIM et al., 2016). In present study, we used a total of 4×10^6 SCED, which is in accordance with usual range in number of cells used in previous studies, however due to the severity, extent and late chronic condition of spinal cord injury in the dogs, an

increased number of SCED could have been necessary to improve neurological scoring and functional outcomes.

The route for cell delivery may also have a critical impact in SCI. Intraparenchymal injection is the most frequent route for cell delivery in pre-clinical studies (BELLO; BIERING-SORENSEN, 2014), however, intraspinal injection have the risk of secondary damage in the already injured spinal cord (JUNG et al., 2009) which could be caused by needle trauma, the relatively large volume injected, high cell concentration and high delivery rate (OLIVERI; BELLO; BIERING-SORENSEN, 2014). Therefore, different approaches have been used for SCI cell-based treatment such as, intra-arterial (SYKOVÁ et al., 2006), intra-venous (SYKOVÁ et al., 2006; MORITA et al., 2016), subdural (CHENG et al., 2015) and into cerebrospinal fluid via intrathecal (JUNG et al., 2009; CHENG et al., 2015), brain's 4th ventricle (OHTA et al., 2004) and lumbar puncture (BAKSHI et al., 2006). In a few comparative studies, intravenous injections showed similar (KHALATBARY; TIRAIHI, 2009; KANG; HA; KIM, 2012; KIM et al., 2013) or even better results than intraspinal injections (ANTONIC et al., 2013). Furthermore, intrathecal delivery route allows larger volume of cell transplantation compared to intraparenchymal delivery (JUNG et al., 2009). Likewise, avoiding inject cells into injury site in chronic injured spinal cord, thus, avoiding glial scar and growth-inhibiting microenvironment (KUMAMARU et al., 2013), may result in an advantage for spinal cord regeneration (ANTONIC et al., 2013; CHENG et al., 2015). In the present study, we injected SCED directly into the injury in the spinal cord parenchyma, so, even though it could not be confirmed, the lack of effective neurological recovery could be influenced, among other factors, to the limited action of the stem cells within the late chronic injury microenvironment.

Studies using acute or subacute models of SCI for stem cells engraftment have been used extensively and have reported their efficacy (CUSIMANO et al., 2012; ANTONIC et al., 2013; OLIVERI; BELLO; BIERING-SORENSEN, 2014). Nevertheless, some studies reported that stem cells in chronically injured spinal cord were not capable to promote significant functional locomotor recovery in rodent models, despite of their capacity to improve neurobehavior (CUSIMANO et al., 2012; NISHIMURA et al., 2013; JIN et al., 2016). Kumamaru et al. (2013) demonstrated that neural stem/progenitor cells injected in chronic injured spinal cord has provided a more neurogenic environment and increased significantly the transcriptional activity of

regenerative/neurotrophic molecules, but did not promote functional recovery. Nishimura et al. (2013) reported NSC in subacute or chronic SCI have the same survival rate of grafted cells, but in later case, cells were restricted and enclosure around the epicenter of the chronic injury by the prominent glial scar formed. Accordingly, cell transplantation in an early stage of chronic injury may have higher potential for regeneration (SALAZAR et al., 2010; TASHIRO et al., 2016). Salazar et al. (2010) transplanted human neural stem cells (hNSC) 30 days after injured spinal cord mice, in an early chronic microenvironment and reported that local hNSC transplantation were capable of survive, differentiate, integrate to host tissues and enhance locomotor function. De Almeida et al. (2011), transplanted DPSCs into a subacute and early chronic (7 and 28 days after injury) model of contusive SCI in mouse and reported a functional locomotor improvement in both stages, although better results were observed in subacute transplantation. Comparing with acute treatment, a more delayed transplantation into a chronic injury could be more beneficial (COUMANS et al., 2001; KARIMI-ABDOLREAZAEE et al., 2010). The reason could be the alteration of inflammatory response that provide better conditions for both endogenous and transplanted populations (MORENO-MANZANO et al., 2009; ROLLS; SHECHET; SCHWARTZ, 2009; DAVID; LOPEZ-VALES; WEE, 2011). However, with time, glial scar stabilizes and up-regulation of NTFs attenuates in such way that regenerative potential is reduced substantially (CREGG et al., 2014; SATAKE et al., 2000). Although, a few studies achieved better outcomes in functional recovery of late chronic SCI treatment in which stem cells transplantation was combined with treadmill training (TASHIRO et al., 2016) or immunosuppressive treatment (MORITA et al., 2016).

The challenge of cell-based therapy is upon the refractory state of chronically injured spinal cord, that can result in failure in functional recovery, despite of the therapeutic activity of stem cells (KUMAMARU et al., 2013). Thus, modification in microenvironment focusing on glial scar formation and inflammatory phenotype should be considered (NISHIMURA et al., 2013) besides of synergistic strategies to enhance stem cells regenerative capacities (KARIMI-ABDOLREAZAEE et al., 2006; KUMAMARU et al., 2013; MORITA et al., 2016; TASHIRO et al., 2016). The optimal therapeutic window in early chronic spinal cord injury is suggest to be within 2-4 weeks, when acute phase of secondary injury has diminished and severed axon and

interneuron are still capable of responding to the host of axon guidance molecules and neurotrophins upregulated after SCI (HAYASHI et al., 2000; BAREYRE et al., 2004; HOLLIS II, 2016). Therefore, the late chronic SCI (from 3 to 18 months) in studied dogs could have influenced negatively the regenerative potential of the proposed treatment.

Studies using canine model of SCI have shown that MSC were capable of promote functional improvement when transplanted into a subacute condition (7 days post injury), even thought, no significant difference could be observed in spinal cord parenchyma using MRI (LIM et al., 2007; RYU et al., 2009). Kim et al. (2016) reported that transplantation of ADSC following decompressive surgery (DSX), in subacute SCI (7 days post injury) caused by acute thoracolumbar disc disease in dogs with no deep pain perception, promoted a success rate of 77.8% (7/9 dogs), in which 55.6% (5/9) obtained full recovery and 22.2% (2/9) partial recovery. On the other hand, studies aiming MSC treatment in naturally occurred chronic spinal cord injury in dogs cause by severe compressive herniation suggested a lesser functional improvement than subacute SCI. Penha et al. (2014) delivered a total of 5×10^6 autologous BMSC in four dogs with surgically refractory, chronic IVDD (> 60 days). It was observed partial gain in different points at neurological evaluation but no functional recovery and no changes in MRI. Similar results were reported in chronic naturally spinal cord injured dogs (>30 days after decompressive surgery) transplanted with allogeneic fetal canine BMSC (1×10^6 cells) that showed mild degree of neurologic-locomotor recovery with no effective functional recovery and no changes in MRI (SARMENTO et al., 2014). Moreover, Besalti et al. (2015) examined the therapeutic potential of autologous BMSC differentiated into neurospheres in seven dogs with chronic SCI (>42 days) caused by IVDD. The animals received 2 percutaneous intraspinal injections of 5×10^6 cells 2 weeks apart and followed up to 8 months after transplantation. At 8 months, there was a 1-2 points improvement in gait, proprioception and nociception in three of four dogs which remained in the study. Despite these minor changes, no conclusive beneficial effect could be associated with the stem cells therapy (BESALTI et al., 2015). In the present study, the lack of a uniform injury and individual differences within naturally spinal cord injured dogs, along with late chronicity, could have prevented a better assessment of animals, however, mild neurological and locomotor improvements observed were similar to previous studies using MSC in chronic SCI of dogs naturally affected by IVDH.

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. Despite mild changes, no robust improvements were found in neurological scoring and functional assessment. The failure of the proposed treatments should be considered. Additionally, the long period between SCI and the beginning of treatment (up to 18 months of chronicity) could have had a negative influence in spinal cord regeneration (CREGG et al., 2014; SATAKE et al., 2000), possibly due to the established destructive cascade of secondary damage following the first injury (STEWARD et al., 2006; NISHIMURA et al., 2013). Hence, an earlier chronic SCI model (within 2 and 4 weeks) could be more adequate model for comparing treatments (COUMANS et al., 2001; KARIMI-ABDOLREAZAEE et al., 2010; SALAZAR et al., 2010; DE ALMEIDA et al., 2011). In addition, a higher dose of transplanted stem cells and different delivery routes (e.g., intra-venous, intra-thecal and lumbar puncture) cold promote better outcomes by improving regenerative potential of stem cells (LI et al., 2015) and avoiding hostile microenvironment (BAKSHI et al., 2006; JUNG et al., 2009; ANTONIC et al., 2013; CHENG et al., 2015,) in SCI focus, and would maintain the same feasibility and safety as observed in this experiment.

5 CONCLUSION

SCED isolation was relatively simple and feasible, and transplantation was shown to be safe, with no mortality followed up 7 month from procedure. However, although mild improvements were seen, no beneficial could be associated with EA, SCEAD or combined treatment. Variation in injury extent and severity could have prevented a uniform assessment. Thus, a higher number of animals and more homogeneous SCI would be necessary to make a more concrete evaluation. In addition, the late chronic spinal cord injury, along with the relatively low number of cells transplantation and intraspinal delivery route could have had a negative influence for spinal cord regeneration. Further investigations considering the limitations reported in the present study should be made to minimize limiting variables and assess the therapeutic effect of SCED combined with electroacupuncture in chronic spinal cord injured dogs.

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APPENDIX A – CONSENT TERM

COLLEGE OF VETERINARY MEDICINE AND ZOOTECNY FROM UNIVERISITY OF SAO PAULO

CONSENT TERM

OWNER'S IDENTIFICATION

NAME: _____

IDENTITY DOCUMENT NUMBER: _____ GENDER: M ☐ F ☐

BIRTHDATE: ____/____/_____

DDRESS: _____

NEIGHBORHOOD: _____CITY: _____

STATE: _____

ZIPCODE: _____ TELEPHONE: _____

ANIMAL'S IDENTIFICATION

ANIMAL'S NAME: _____

SPECIE: _____BREED: _____

SEX: _____ BIRTHDATE: _____

INFORMATION ABOUT THE STUDY

PROJECT TITLE: Combination of stem cells from deciduous teeth and electroacupuncture in dogs with chronic spinal cord injury.

RESEARCHER: César Vinícius Gil Braz do Prado.

Post/Function: Veterinarian.

REGIONAL VETERINARY COUNCILL REGISTRATION: CRMV-SP 28.466

Department: Surgery department, Anatomy of Domestic and Wild Animals section– FMVZ – USP

RISK ASSESSMENT:

MINIMUM RISK	☐	MEDIUM RISK	☐
LOW RISK	x	HIGH RISK	☐

STUDY DURATION: 13 weeks.

COLLEGE OF VETERINARY MEDICINE AND ZOOTECNY FROM UNIVERISITY OF SAO PAULO

The study aims to evaluate the combination of electroacupuncture and stem cells from canine exfoliated dental pulp in spinal cord injury of dogs. The objective of this study is to examine if combination of these two therapies would be capable of improve neurological and locomotor improvement in paraplegic dogs due to natural intervertebral disc herniation followed up to 2 months after injury.

The selected dogs will be assigned randomly into one of the four different experimental groups, 1) Stem cells transplantation, 2) Electroacupuncture treatment, 3) Stem cells + Electroacupuncture, and 4) Control group.

The procedures following selection is summarized in the steps bellow:

- Blood tests and electrocardiography;

- Magnetic resonance imaging before and after study (animal will be anesthetized) ;

- Decompressive surgery and injection of stem cells or saline solution directly into the spinal cord at injury site (animal will be anesthetized);

- After 7 days from surgery, another injection of stem cells or saline solution, percutaneously into the spinal cord at injury site (animal will be anesthetized);

- Electroacupuncture treatment (only for animal assigned to group that received electroacupuncture) 3 times a week for 7 weeks, then twice a week for 2 week, finally once a week for 3 weeks. Electroacupuncture will begin up to 7 days after second injection;

- Underwater treadmill training 2 times a week for 12 weeks. It will begin up to 7 days after second injection;

- Neurologic examination and functional assessment, that will be recorded for posterior evaluation, before and after the study.

All dogs will be medicated during the procedure and at post-surgery medication will be prescribed in order to prevent pain related to the surgery. In addition, discomfort related to electroacupuncture is minimum and is due to the needle insertion and electric stimuli, although majority of dogs do not express any sign of pain and relax during this therapy. These procedures, along with the possible pain or discomfort for blood collection are all moments that dogs would experience any discomfort, pain or suffering within study.

Considering the treatments as part of an experimental study, results can vary. Even though similar studies had reported functional recovery in another animal models, the dog selected for the study could no present any improvement or could show a great functional recovery. Hence, we will only know about any beneficial effect.

The animals from all groups will be submitted to decompressive surgery and underwater treadmill exercise. However, some dogs will not receive stem cells transplantation and/or electroacupuncture treatment. For this reason, at the end of the study, owner will have the option of proceeding with other treatments that their pet had not received, if they wish.

There will be no charges for any procedure, however the owner would need to be responsible for bringing the dog to the treatments and examinations. Moreover, the owner will have access to the professionals responsible of the research in any time in order to clarify eventual questions. The main researcher is the veterinarian César Vinícius Gil Braz do Prado, that can be found at 87, Professor Doutor Orlando Marques de Paiva, Cidade Universitária, College of Veterinary Medicine and Zootechny, Surgery department – University of Sao Paulo. Phone number: (11) 98536-3246. Furthermore, it is ensured to the owner to withdraw consent at any time point of study and stop participation of his dog, without prejudicing animal's treatment.

The data collect in this study will be analyzed together with other patients. No personal identification will be divulged. The main researcher compromises to use collected data and material for only this study and after Committee of Ethic in Animal Experimentation approval.

I believe to have been sufficiently informed about the confidentiality and permanent possibility of contact, as well the procedures, potential benefits, discomforts and risks related to the study “Combination of stem cells from deciduous teeth and electroacupuncture in dogs with chronic spinal cord injury”.

I have discussed with Mr. César Vinícius Gil Braz do Prado about the decision in including my animal in this study. I voluntarily agree my animal participate in this study and I could withdraw my consent in any moment, without any prejudice or loss of benefit that my animal could have acquire.

Owner’s signature Date ___/___/___

_____

Attestant’s signature* Date ___/___/___

_____

*In case of illiterate, semi-illiterate or visually impaired person.

(Only for responsible for project)

I declare that obtained in a appropriated and voluntary manner the consent term from this owner to participation of his dog in this study

_____

Study responsible signature Date ___/___/___

APPENDIX B – NEUROLOGIC EXAMINATION SCORING RESULTS

		Hopping - hindlimbs (A/R/P)		Conscious Proprioception - hindlimbs (A/R/P)		Superficial pain perception - hindlimbs (A/R/P)		Deep pain perception - hindlimbs (A/R/P)		Urinary incontinence (Y/N)	Fecal Incontinence (Y/N)	Total score
		Left	Right	Left	Right	Left	Right	Left	Right			
Stem Cells												
Animal 1	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	1	1	0	0	0	0	0	0	**2**
Animal 2	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 3	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 4	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Total	Pre-treatment											**0**
	Post-treatment											**2**
Electroacupuncture												
Animal 5	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 6	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 7	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 8	Pre-treatment	0	0	0	0	0	0	2	0	0	0	**2**
	Post-treatment	0	0	2	0	2	2	2	2	0	0	**10**
Total	Pre-treatment											**2**
	Post-treatment											**10**
Stem Cells + Electroacupuncture												
Animal 9	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	2	0	2	2	0	0	0	0	0	0	**6**
Animal 10	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	2	2	0	0	0	0	4	0	**8**
Animal 11	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 12	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	1	1	0	0	0	0	0	0	**2**
Total	Pre-treatment											**0**
	Post-treatment											**16**
Control												
Animal 13	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 14	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 15	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	0	0	0	0	0	0	0	0	0	**0**
Animal 16	Pre-treatment	0	0	0	0	0	0	0	0	0	0	**0**
	Post-treatment	0	1	0	1	0	0	0	0	0	0	**2**
Total	Pre-treatment											**0**
	Post-treatment											**2**

Y= Yes (0), N= No (4), A= Absent (0), R= Retarded (0), P= Present (2)

APPENDIX C – FUNCTIONAL ASSESSMENT RESULTS

Animal	Moment	Score	
Stem Cells			
Animal 1	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**0**	No pelvic limb movement
Animal 2	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
Animal 3	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**0**	No pelvic limb movement
Animal 4	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
Total	Pre-treatment	**1**	
	Post-treatment	**4**	
Electroacupuncture			
Animal 5	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**2**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved < 50% time
Animal 6	Pre-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
	Post-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
Animal 7	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**0**	No pelvic limb movement
Animal 8	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
Total	Pre-treatment	**4**	
	Post-treatment	**8**	
Stem Cells + Electroacupuncture			
Animal 9	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
Animal 10	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**2**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved < 50% time
Animal 11	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**0**	No pelvic limb movement
Animal 12	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
Total	Pre-treatment	**3**	
	Post-treatment	**4**	
Control			
Animal 13	Pre-treatment	**1**	Minimal non-weigh-bearing protraction pelvic limb (movement of 1 joint)
	Post-treatment	**5**	weigh-bearing protraction of pelvic limb 10 to 50% time
Animal 14	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**0**	No pelvic limb movement
Animal 15	Pre-treatment	**0**	No pelvic limb movement
	Post-treatment	**2**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved < 50% time
Animal 16	Pre-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
	Post-treatment	**3**	Non-weigh-bearing protraction pelvic limb with > 1 joint involved > 50% time
Total	Pre-treatment	**4**	
	Post-treatment	**10**	