



## Transplantation of human immature dental pulp stem cell in dogs with chronic spinal cord injury<sup>1</sup>

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### Abstract

**Purpose:** To investigate the therapeutic potential of human immature dental pulp stem cells in the treatment of chronic spinal cord injury in dogs.

**Methods:** Three dogs of different breeds with chronic SCI were presented as animal clinical cases. Human immature dental pulp stem cells were injected at three points into the spinal cord, and the animals were evaluated by limb function and magnetic resonance imaging (MRI) pre and post-operative.

**Results:** There was significant improvement from the limb function evaluated by Olby Scale, though it was not supported by the imaging data provided by MRI and clinical sign and evaluation.

**Conclusion:** Human dental pulp stem cell therapy presents promising clinical results in dogs with chronic spinal cord injuries, if used in association with physical therapy.

**Key words:** Cell- and Tissue-Based Therapy. Spinal Cord Injuries. Dental Pulp. Dogs.

## ■ Introduction

Spinal cord injury (SCI), with loss of neurological function, represents a major challenge, not only in terms of adjusting patients, owners, and their environment to daily life with a dog with SCI, but also regarding the therapeutic options that improve the outcome in patients<sup>1</sup>.

Ramón Y Cajal<sup>2</sup> was the first to describe the limited regeneration capacity of the central nervous system. Only after five decades were these information questioned by Richardson *et al.*<sup>3</sup>. These authors performed an assay on rats by removing axons from the spinal cord and inserting them in the peripheral nervous system. They noticed that the regeneration difficulties of the CNS neural cells were more related to their cellular niche than to their regenerative or reposition capability.

Animal models are important tools for the study of the underline physiopathology, and possibilities of new therapies<sup>4</sup>. Stem cell therapy may become more effective than other therapeutic alternatives, such as transplantations and drugs, as long as some limiting factors are taken into account, among which the characteristics of the glial scar would be the most important aspect in this context.

A great number of studies have been performed with stem cells from different sources using animal models of spinal cord injury, and even therapeutic attempts on humans, presenting promising results<sup>5-7</sup>. However, there are some difficulties to perform a direct comparison of such results, due to the large variability of characterization grade of the transplanted cells, different animal models, and the time fluctuation between injury event and its treatment<sup>8</sup>.

Commonly, dogs present clinical injuries of the thoracic and lumbar spinal medulla mainly due to herniation or protrusion of the intervertebral discs<sup>9</sup>. Animals that exhibit

limb paralysis, but with preserved deep pain reflexes are suitable for decompressive spinal surgery. If the dog has lost the deep pain reflex for more than 48 hours, they usually are no longer suitable for surgical treatment because previous studies demonstrated poor prognosis on the return of the locomotive function in these cases<sup>10</sup>.

On the other hand, some studies performed in rats reported coherent evidences of functional recovery in cases of spinal cord injury treated with mesenchymal stem cells (MSC)<sup>11,12</sup>. These “non-neural” cellular types display different mechanisms for recovery in spinal cord injury, in which there is no reposition of neurons or glial cells. It is highly probable that these types of cells provide trophic support, modulate inflammatory response or provide substratum for axon growth, guiding the spinal cord regeneration and therefore promoting functional recovery<sup>13,14</sup>.

The ideal stem cells for transplantations should be immunologically inert, developed from easily accessible sources, possess fast *in vitro* expansibility, present competence for both long term survival and integration to the host site of interesting, besides being suitable for transfection and expression of exogenous genes<sup>15</sup>.

Human dental pulp was uncovered as a source of MSC derivative from the neural crest, with promising therapeutic possibilities. Many authors have studied the capacity of self-regeneration, multi-lineage differentiation and clonogenic efficiency of dental pulp stem cells. They reported their capacity of differentiation into neural cells and adipocytes<sup>16-17</sup>.

Kerkis *et al.*<sup>18</sup> reported the isolation and characterization of human immature dental pulp stem cells (h-IDPSC) using various embryonic stem cell markers. The authors differentiated these cells in both skeletal and smooth muscle, bones, cartilage and neurons in pre-defined culture conditions, suggesting

the use of such cells in pre-clinical assays.

The aim of the study was to transplant human dental pulp stem cells in chronic spinal cord injury in dogs with intervertebral disc herniation in order to evaluate the condition of the canine patients using magnetic resonance imaging and behavioral clinical tests before and after they underwent the experimental surgical stem cell application associated with a physical therapy protocol.

## Methods

The experiment was carried out and approved according to the ethical principles of the Ethics Committee on Animal Use of The Veterinarian Medicine and Animal Science College, Universidade de São Paulo (USP), protocol number 2081/2010.

Three dogs of different breeds selected from local private veterinary practices within the state of São Paulo, Brazil, in the period between December of 2008 and January of 2011. The criteria of inclusion used were: paraplegia, absence of conscious proprioception, presence of sciatic reflexes, exaggerated patellar and cranial tibial reflexes, absence of deep pain reflexes, and the diagnosis of thoracolumbar intervertebral disc disease on the MRI. Animal number one was a six-year-old male Lhasa Apso dog with chronic spinal cord injury for over two years, that has been treated with the use of ozone therapy and physical therapy for over a year, although unsuccessfully so far. Animal number two was a nine-year-old male Dachshund dog with hindlimb paralysis for at least one and a half year. Subject number two had never undergone surgical treatment, and has been in ozone therapy for a year. Animal number three was a nine-year-old male Yorkshire Terrier with chronic paralysis for two years, under physical therapy for a year. This animal had undergone previous surgical spinal decompression.

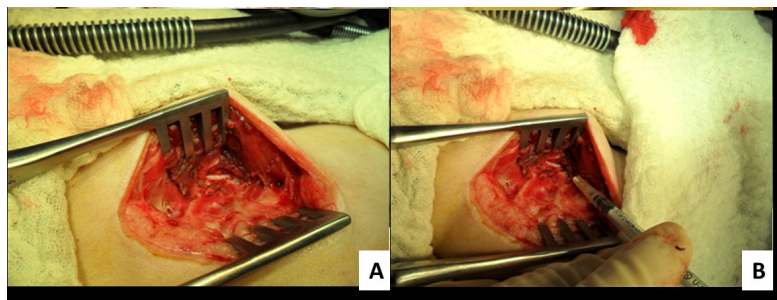
## Human IDPSC isolation and culture

Human immature dental pulp stem cells (hiDPSC, 2n=46, XY) were isolated and previously characterized and cultured as described by Kerkis *et al.*<sup>23</sup>. These cells were cryopreserved and available at the Stem Cell Laboratory (SCL), USP. *In vitro* expansion and differentiation followed the protocols already developed by this group: the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1, Invitrogen, Carlsbad, Calif., USA), containing 15% Fetal Bovine Serum (FBS, HyClone, Logan, Utah, USA), 100U/mL Penicillin + 100µg/ml Streptomycin, 2mM L-Glutamine and 2mM Non-Essential Amino Acids.

## Surgical procedure

Intervertebral disc disease was diagnosed by magnetic resonance imaging (MRI). The animals were pre-medicated with deep intramuscular application of 0.05 mg/Kg acepromazine maleate in association with 2 mg/Kg meperidine. Anesthesia was induced by propofol (5 mg/Kg) and maintained with isoflurane. The animals were monitored through electrocardiogram and pulse oximetry. The same anesthetic protocol was used in the performance of the surgical procedure.

Spinal decompressive surgery was performed a few weeks after the MRI. Hemilaminectomy procedure was performed on the ipsilateral side to the spinal cord compression, as determined by the MRI results. The herniated disc was removed and  $1 \times 10^6$  hiDPSC were injected in three different sites: directly at the site of the spinal cord injury, 2mm cranially and 2mm caudally to the lesion (Figure 1). Post-operative management included the administration of antimicrobials and analgesics, as well as urinary incontinence management, and nursing care to prevent scar pressure ulcers.



**Figure 1** - Procedure for human immature dental pulp stem cell (h-IDPSC) intramedullary injection in dog with chronic spinal cord injury. **A.** Hemilaminectomy surgical approach for spinal cord. **B.** Intramedullary injection of h-IDPSC in site of disc compression.

All animals received a veterinary physical therapy protocol including hydrotherapy, in three times per week sessions during two months post-surgery. The animals were placed on underwater treadmill to eliminate the animal's weight and facilitating ambulation for strengthening the pelvic muscles. No

procedures were used to interfere with the inflammatory process or to avoid interference with stem cell chemotaxis by inflammation. The sessions were recorded at date times 0, 15, 30, 60 (in days), when scores were assigned for each sessions by two veterinarians, according to the Olby Scale<sup>19</sup> (Table 1).

**Table 1** - Assessment of pelvic limb function by Olby Scale<sup>19</sup>.

Stage	Point	Neurological Status
	0	No pelvic limb movement and no deep pain sensation.
1	1	No pelvic limb movement with deep pain sensation.
	2	No pelvic limb movement but voluntary tail movement
	3	Minimal non-weight-bearing protection of pelvic limb (movement of one joint)
2	4	Non-weight-bearing protection of pelvic limb with no more than one jt. Involved less than 50% of the time
	5	Non-weight-bearing protection of pelvic limb with no more than one jt. Involved more than 50% of the time
3	6	Weight-bearing protection of pelvic limb less than 10% of the time
	7	Weight-bearing protection of pelvic limb 10-50% of the time.
	8	Weight-bearing protection of pelvic limb more than 50% of the time.
4	9	Weight-bearing protection 100% of time with reduced strength of pelvic limb. Mistake > 90% of the time.
	10	Weight-bearing protection 100% of time with reduced strength of pelvic limb. Mistake 50-90% of the time.
	11	Weight-bearing protection 100% of time with reduced strength of pelvic limb. Mistake < 50% of the time.
5	12	Ataxic pelvic limb gait with normal strength, but mistakes made > 50% of time.
	13	Ataxic pelvic limb gait with normal strength, but mistakes made < 50% of time.
	14	Normal pelvic limb gait.

The protocol for MRI exams was sagittal and transversal T1 and T2 weighted images, dorsal STIR (short-tau inversion recovery), and transversal 3DHyce (a type of Balanced Steady State Free Precession). Recheck MRI evaluation was performed approximately 60 days after the transplantation of the hIDPSCs, in order to check the spinal cord status.

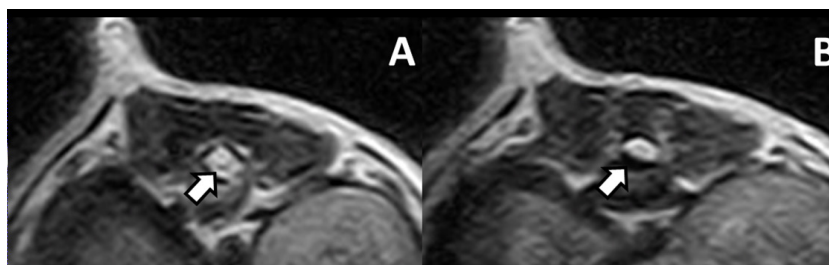
## ■ Results

### Case 1

On animal 1, the pre-surgical MRI showed a right ventrolateral extradural myelopathy with adjacent syringohydromyelia

on T12-13. Neurofunctional evaluation by the Olby Scale was then performed. The animal showed irregular movements of the hind limbs and did not support its own weight. Based on these data, this animal was scored 3, according to the Olby Scale.

In surgery, the disc material was removed and  $1 \times 10^6$  cells diluted in 300 $\mu$ l PBS were applied at three points (cranial, caudal and in the center of the spinal cord injury). Post-operative neurofunctional evaluation by the Olby Scale was then performed, and this animal showed weight-bearing protraction of pelvic limb about 25% of the time, which scored a final 7 points for this animal. No improvements were observed in final MRI (Figure 2).

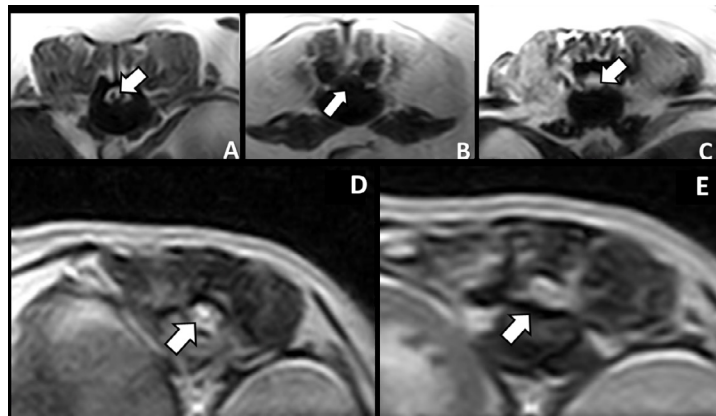


**Figure 2** – Case 1 pre-operative MRI. **A.** White arrow points to central channel dilation. **B.** White arrow points to ventrolateral extradural compression.

### Case 2

On the MRI study of the animal 2, was observed presence of moderate to severe ventromedial extradural compressive myelopathy in L4-5 and areas of gliosis or focal hemorrhages at T13 and L1, with probable syringohydromyelia from T11 to L2. Pre-operative analysis of this animal gives him 2 points in Olby Scale, with no pelvic limb movement, but presence of voluntary movement of the tail.

Surgical decompression was performed and human dental pulp stem cells were then applied. This application was made three times with  $1 \times 10^6$  hIDPSC diluted in 300  $\mu$ l PBS, from which 100  $\mu$ l were applied each in the T11-12 and T12-13 intervertebral spaces, and the remaining 100  $\mu$ l were applied in the L4-5 space. After that, the animal showed an improvement in Olby Scale, getting a 4 in final score with no improvement in final MRI (Figure 3).

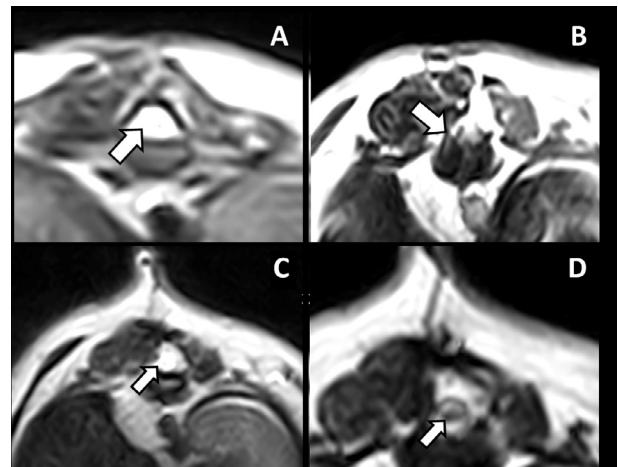


**Figure 3** – Case 2 pre and post-operative MRI. **A.** Central channel dilation. **B.** Severe ventromedian extradural compressive myelopathy in L4-5. **C.** Gliosis or focal hemorrhages at T13-L1. **D.** White arrow points to permanent central channel. **E.** Right ventrolateral compression.

### Case 3

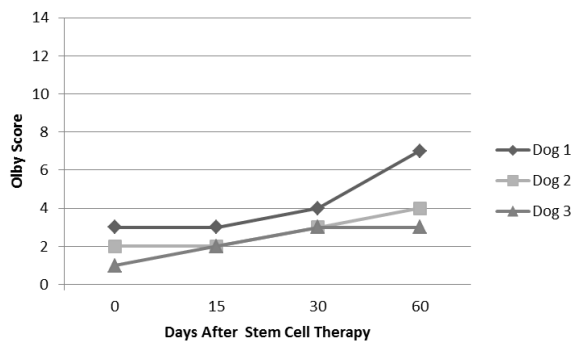
Dog number 3 also underwent pre-surgical MRI that demonstrated spinal cord atrophy in the thoracic-lumbar region (mainly from T10 to L2) and syringohydromyelia at the level of L1. Previous surgical intervention, probably left hemilaminectomy was noted on T11-12, and associated adjacent muscular paravertebral atrophy was also observed. This animal presents no pelvic limb movement and deep pain sensation, getting score 1 in Olby Scale.

The surgical procedure was performed in this animal, differently from the others. The hemilaminectomy was not necessary, since no disc material on the vertebral canal was present. Three applications of immature human dental pulp stem cells, diluted in 300  $\mu$ l PBS were made, 100  $\mu$ l each on the T10-11, T11-12 and T12-13 intervertebral spaces. After surgery, the animal was evaluated by Olby Scale and presents movement of one joint, with minimal non-weight-bearing protection, getting a 3 in final score. MRI evaluation showed no improvement (Figure 4).



**Figure 4** – Case 3 pre and post-operative MRI evaluation. **A.** Medullary hypersignal. **B.** Previous hemilaminectomy surgery. **C.** Permanent medullary hypersignal. **D.** Medullary central channel dilation.

In all cases, using the Olby Scale<sup>19</sup> it was observed clinical improvement of the scores through time, despite it was not supported by the post-surgical MRI data, performed approximately 60 days after the surgical procedure (Figure 5).



**Figure 5** – Olby Scale assessment of three dogs with chronic spinal cord injury undergoing human immature dental pulp stem cell transplantation.

## ■ Discussion

There are few studies that use intramedullary stem cell transplantation, of which the most notorious is the one performed by Mackay-Sim *et al.*<sup>20</sup>. Intramedullary injections of stem cells were performed in dogs with spinal cord injury in this study, favoring the delivery of cells in a form that is more efficient than the epidural or intravenous access.

Furthermore, it is probable that the cellular niche is the provider of nourishment and subsides for the differentiation of the injected cells in nervous origin cell. Such statements were confirmed in previous studies by Paul *et al.*<sup>21</sup> in an assay using rats. In this study, a number of  $1 \times 10^6$  human IDPSC were injected in 3 pre-defined points in the spinal cord injury, in the same manner as in the current study. This same method was proposed by Ryu *et al.*<sup>22</sup>, in experimentations on dogs with induced acute spinal cord injury and treatment with stem cells derived from the adipose tissue. Another study was performed by Lee *et al.*<sup>12</sup> with dogs whose spinal cord was injured through compression with an angiography balloon and treated with umbilical cord stem cells. They showed significant improvement in

the behavioral scores in the Olby Scale without apparent improvement in MRI, the same result being found like in the present study. The injury caused in both studies, however, was of acute character, that probably developed a spontaneous resolution.

The cases selected for the present study had chronic spinal cord injury with no response to either usual or alternative therapies that are currently used in veterinarian medicine. Despite the behavioral clinical improvement shown by the animals in this experimentation, there is no evidence of spinal cord regeneration after post-surgical MRI. Ryu *et al.*<sup>22</sup> reported that even though there was improvement in behavior, there were no changes in the histopathological evaluation, and no improvement in MRI, despite the affirmed presence of the injected adipose stem cells, that were confirmed by the Green Fluorescent Protein – GFP - gene reporter. There are consistent reports of function recovery in rats with spinal cord injury that were treated with mesenchymal stem cells (MSC)<sup>12,23</sup>. This fact was observed in the present study by using human dental pulp stem cells, which are an example of MSC. Nosrat *et al.*<sup>24</sup> evaluated the neurogenic potential of these stem cells *in vitro*, and in rats that underwent spinal cord hemisection. The injection of such cells in those rats increased the survival rate of motor neurons, showing functional bioactivity.

The results obtained in the present study, in association with the results obtained by the referred authors, lead us to the belief that the transplanted cells promoted a functional response in the animals. The mesenchymal stem cells present different mechanics for spinal cord injury recovery, with no reposition of neurons or glial cells<sup>8</sup>.

The h-IDPSC that was used in this experimentation with dogs consists of a source of “non-neural” cells with great capacity for neurogenic regeneration. It is highly probable that this kind of cells provides nourishing

support, modulates inflammatory response or provides substratum for axon growth, acts a guide for spinal cord regeneration, promoting functional recovery, as previously reported by Hofstetter *et al.*<sup>11</sup>. Such mechanism is credited for the clinical improvement shown in the Olby behavioral evaluation. Human dental pulp stem cells have already been differentiated into neuronal tissue in cell culture conditions, influenced by supplemented culture medium<sup>18</sup>.

Authors report that these cells have strong tendency to differentiate into neuronal tissue, even without the addition of specific culture media, and this was the main reason for their choice as a cell source for the therapy of the dogs in the present study. The applied cells probably differentiated into neural origin cells, having as influences the chemical substances generated by the cells of the niche to where they were transplanted. Studies using clinical tests with therapy and stem cells in dogs with spontaneous spinal cord injury are rare in the literature<sup>25</sup>, but with increase of data reports during last years.

In the present study, we chose to use animals with spontaneous spinal cord injury, because this presentation is very common in dogs and also due to the possibility of evaluating the results of stem cell therapy in a real life situation.

Even though significant improvement was achieved in the dogs that underwent treatment, our results confirmed the findings by Sykova *et al.*<sup>26</sup>, who affirmed that in complete and extended injuries, the isolated application of stem cells is not enough for tissue repairing, and so, a search for other alternatives that can complement the action of the cells and fill the gap caused by tissue death is required. Up-to-date, studies aim to the resolution of the glial scar associated to the stem cell therapy, as a treatment for chronic spinal cord injury<sup>14,27</sup>. Immunization with recombinant vaccines that act on the site of the axonal growth inhibitors

(Nogo A, AMG e OMP) in the axonal cells<sup>28</sup>, and even treatment with anti-Nogo A antibodies<sup>29</sup> show promising results among the therapies for spinal cord injury. Other alternative therapies are related with benefic effects for the spinal cord injury<sup>30</sup>, and in association with stem cells, might be a reasonable hope in the search for the cure of this disease.

## ■ Conclusions

Human immature dental pulp stem cell therapy presents promising clinical results in dogs with chronic spinal cord injuries, if used in association with physical therapy. The imaging data gathered, however, does not support the clinical results presented.

The postoperative evaluation of only 60 days is not enough to evidence the results presented, and it is also worth mentioning that a single application was made, despite the high concentration of cells used.

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