

Retrospective study of mesenchymal stem cell therapy in dogs with neurological complications resulting from infection by canine distemper virus

Hilana dos Santos Sena Brunel¹, Carla Lujan Villaroel^{1,2}, Bruno Stéfano Lima Dallago³, Robert Pogue² & Patrícia Furtado Malard¹

¹ Bio Cell Cell Therapy, Brasilia, Brazil

² Department of Genomics and Biotechnology, Catholic University of Brasilia, Brasilia, Brazil

³ Department of Veterinary Medicine, University of Brasilia, Brasilia, Brazil

Correspondence: Hilana dos Santos Sena Brunel, Bio Cell Cell Therapy, Brasilia, Brazil. E-mail: lanasena@gmail.com

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Abstract

Canine distemper virus causes death in a large proportion of infected dogs. For the survivors, various physiological systems can be damaged, including the nervous system, resulting in neurological signs such as ataxia, paresis or plegias, myoclonus, tremors and epileptic crises. Mesenchymal stem cells are undifferentiated cells with the capacity to release trophic factors with neuroprotective, anti-inflammatory and immunomodulatory properties, and as such may represent an alternative to treat or mitigate the clinical symptoms in dogs with such neurological sequelae. In the current retrospective study, we evaluated clinical data and films from 14 dogs that presented myoclonus, epileptic episodes, and/or ambulatory difficulties after distemper virus infection, and that were treated with allogeneic mesenchymal stem cells from a cell bank. The animals that had presented epileptic crises and myoclonus presented a reduction in the frequency of these episodes, and of the 14 animals that presented with ambulatory difficulties, ten regained the ability to walk without aid after the therapy. No animal presented with any adverse reaction to the cell transplant. These results suggest that mesenchymal stem cell therapy may be an alternative for treatment of neurological sequelae, however, further controlled studies should be carried out in order to obtain further data regarding the number of cells to be transplanted, the time interval between transplants, and even about the ideal time for initiation of such therapy.

Keywords: ambulatory difficulties, epileptic crises, myoclonus, remyelination, safety.

Resumo

O vírus da cinomose canina causa a morte em uma grande proporção de cães infectados. Para os sobreviventes, vários sistemas fisiológicos podem ser danificados, incluindo o sistema nervoso, resultando em sinais neurológicos como ataxia, paresia ou plegia, mioclonia, tremores e crises epiléticas. As células-tronco mesenquimais são células indiferenciadas com capacidade de liberar fatores tróficos com propriedades neuroprotetoras, anti-inflamatórias e imunomoduladoras e, como tal, podem representar uma alternativa para tratar ou atenuar os sintomas clínicos em cães com essas sequelas neurológicas. No atual estudo retrospectivo, avaliamos dados clínicos e filmes de 14 cães que apresentaram mioclonia, episódios epiléticos e/ou dificuldades deambulatorias após infecção pelo vírus da cinomose, e que foram tratados com células-tronco mesenquimais alogênicas de um banco de células. Os animais que apresentavam crises epiléticas e mioclonias apresentaram redução na frequência desses episódios, e dos 14 animais que apresentavam dificuldade de deambulação, dez recuperaram a capacidade de deambular sem auxílio após a terapia. Nenhum animal apresentou qualquer reação adversa ao transplante celular. Esses resultados sugerem que a terapia com células-tronco mesenquimais pode ser uma alternativa para o tratamento de sequelas neurológicas, porém, mais estudos controlados devem ser realizados a fim de obter maiores dados sobre o número de células a serem transplantadas, o intervalo de tempo entre os transplantes e até mesmo sobre o momento ideal para o início dessa terapia.

Palavras-chave: dificuldades ambulatorias, crises epiléticas, mioclonias, remielinização, segurança.

Resumen

El virus del moquillo canino causa la muerte en una gran proporción de perros infectados. Para los sobrevivientes, se pueden dañar varios sistemas fisiológicos, incluido el sistema nervioso, lo que resulta en signos neurológicos como ataxia, paresia o plejía, mioclonías, temblores y ataques epilépticos. Las células madre mesenquimales son células indiferenciadas capaces de liberar factores tróficos con propiedades neuroprotectoras, antiinflamatorias e inmunomoduladoras y, como tales, pueden representar una alternativa para tratar o aliviar los síntomas clínicos en perros con estas secuelas neurológicas. En el estudio retrospectivo actual, evaluamos datos clínicos y películas de 14 perros que presentaron mioclonías, episodios epilépticos y/o dificultades para caminar después de la infección por el virus del moquillo, y que fueron tratados con células madre mesenquimales alogénicas de un banco de células. Los animales que tenían ataques epilépticos y mioclonías mostraron una reducción en la frecuencia de estos episodios, y de los 14 animales que tenían dificultad para caminar, diez recuperaron la capacidad de caminar sin ayuda después de la terapia. Ningún animal mostró ninguna reacción adversa al trasplante de células. Estos resultados sugieren que la terapia con células madre mesenquimales puede ser una alternativa para el tratamiento de secuelas neurológicas, sin embargo, se deben realizar estudios más controlados para obtener más datos sobre el número de células a trasplantar, el intervalo de tiempo entre trasplantes y incluso sobre el momento ideal para iniciar esta terapia.

Palabras clave: dificultades ambulatorias, crisis epilépticas, mioclonias, remielinização, segurança.

1. Introduction

Canine distemper virus (CDV) belongs to the Paramyxoviridae family, genus Morbillivirus, and is the etiological agent of canine distemper (Murphy et al., 2012). The host range of CDV mainly includes species from the order Carnivora which belongs to the families Mustelidae (weasel, ferret, fishers, mink, skunk, badger, marten, otter), a wide range of members of the family Felidae (lions, leopards, cheetahs, tigers) and the principal for veterinary clinics, Canidae, which includes the dogs. Also, a minor extension other important family belonging to different orders such as Artiodactyla, Primates, Rodentia, and Proboscidea (Martinez-Gutierrez., 2016).

The virus infects the host through the nasal or oral passages, in aerosols, and begins its replication using macrophages or dendritic cells of the respiratory system. This virus is associated with multiple cell tropism (epithelial, lymphoid and neurological), which leads to a systemic infection including respiratory, digestive, urinary, lymphatic, cutaneous, skeletal, and central nervous system (CNS) diseases (Lempp et al., 2014), and is considered as a highly contagious and an acutely febrile disease in dogs that has been known since 1760 (MacLachlan et al., 2011). Respiratory and gastrointestinal clinical pathologies are the most common signs by the end of 6 to 10 post-infection days along with rashes (a typical symptom of CDV) in the form of erythematous patches whose diameter ranges between 3 and 8 mm. The neck and the face are the first body parts that are affected. An increasing number of patches appear around the mouth while the infection progresses (Pfeffermann et al., 2018; Delpeut et al., 2017).

After invasion, the virus reaches the choroid plexus, and then disperses throughout the CNS, especially the region of the cortex, optic tract, encephalic trunk, cerebellum, and medulla (Greene & Appel, 2006), resulting in a variety of neurological signs, such as ataxia, pareses or plegias, muscular atrophy, hyperesthesia, myoclonus, tremors and epileptic crises (Moro et al., 2003). Animals that manage to survive the viral infection can suffer permanent neurological sequelae, and there are no specific treatments of cures for these clinical signs, though they can be attenuated with physiotherapy and acupuncture (Santos, 2013).

Considering the difficulties of treating demyelinating diseases, several research groups have investigated the therapeutic use of mesenchymal stem cells (MSCs) (Dulamea, 2015; Cruz-Martínez et al., 2017). Some results have suggested that MSCs could promote endogenous repair and exert positive immunomodulatory effects to reduce demyelination, increase neuroprotection, modulate inflammation, and promote the differentiation of neural MSCs into oligodendrocytes (myelin-producing cells in the central nervous system) (Jadasz et al., 2013). In addition, some clinical trials have shown promising results in the use of MSCs in multiple sclerosis (Dulamea, 2015; Llufríu et al., 2014). As mesenchymal stem cells possess paracrine anti-inflammatory and immunomodulatory effects, it makes these cells a viable alternative for treating or improving the standard of life of animals affected by neurological sequelae following infection by canine distemper virus (Oliveira-Sales et al., 2013). In this context, this study aimed to evaluate the use of MSC therapy in dogs with neurological signs caused by infection by this virus.

2. Materials and Methods

A multicenter retrospective study was carried out using data from medical charts for dogs with clinical and/or serological diagnosis of infection with the canine distemper virus, at veterinary clinics and hospitals in Brazil, and at the laboratory that supplied the allogeneic mesenchymal stem cells, during the period from April 2014 to January 2018. All dogs had passed through the viremic phase and presented with neurological sequelae (epileptic crises, myoclonus and/or walking difficulties) due to infection by the virus for at least six months. The dogs were evaluated clinically to ensure that they did not present respiratory or digestive clinical signs consistent with the viremic phase and were subsequently submitted to therapy with allogeneic mesenchymal stem cells from a cell bank.

The MSC were isolated from adipose tissue donated by healthy dogs, according to previously described protocols (Lindroos et al., 2011; Romagnoli e Brandi, 2014; De Francesco et al., 2015). Under inhaled general anesthesia, 20 grams of adipose tissue were taken from the base of the tail using aseptic surgical procedure. The adipose tissue was washed in saline solution to remove cellular and blood residue, macerated, and then exposed to hyaluronidase to promote enzymatic digestion of the tissue. Subsequently, the cells were filtered for selection of MSC, placed in culture flasks with Dulbecco's Modified Eagle's Medium (DMEM), and incubated at 37.5 °C with 5% CO₂. After 24 h, the media was discarded together with non-adherent cells, and fresh culture media was added to the flasks. The media was changed every three days until the cells reached 80% confluence, at which point they were trypsinized, counted in a Neubauer chamber, and frozen in straws (1 x 10⁶ cells / straw) with DMSO and fetal bovine serum, in liquid nitrogen, as described previously (De Rosa et al., 2009; Cui & Pu, 2010).

Characterization of the MSC was performed as specified by the International Society for Cell therapy (Dominici et al., 2006). Positive cell surface antibodies were rat anti-human CD29-RD1 (Beckman Counter, Fullerton, CA, USA), mouse anti-horse CD44-FITC (AbD Serotec, Raleigh, NC, USA), primary mouse anti-dog CD90 (Washington State University, Seattle, WA, USA) with secondary AF594-conjugated goat anti-mouse IgM (Thermo Sci., Rockford, IL, USA). The negative marker was mouse anti-human CD34-FITC (Invitrogen). MSC function was also tested by the presence of two specific cytoplasmic differentiation markers (SOX2 and OCT 3/4). These markers were analyzed by immunophenotyping using flow cytometry.

The differentiation capacity of the cells was confirmed by inducing osteogenesis, adipogenesis and chondrogenesis (De Francesco et al., 2015; Marx et al., 2015). Cells were evaluated for contaminants (bacteria, fungi and mycoplasma) by PCR (Veriti Thermal Cycler - ThermoFischer Scientific). Furthermore, cellular viability after thawing was evaluated by flow cytometry for annexin - Alexa Fluor 488 and propidium iodide (PI) (Thermo Fisher Scientific).

The treatment protocol involved three endovenous applications of allogeneic MSC at a dose of one million cells/kg of live weight, with 21 days between applications. The animals were evaluated, and the neurological signs documented through filming before and after treatment, with the aim of allowing a comparative analysis of the evolution of neurological manifestations. The analysis classified the clinical signs (frequency of epileptic crises, myoclonus and walking difficulties) as improvement, deterioration, or stability in any one of the three signs, with the scoring system of +1 (improvement), 0 (stability), and -1 (deterioration). According to this system, the neurological response after each treatment could be: deterioration (-1 to -3 points); no change (0 points), or improvement (1 to 3 points). The frequency of epileptic crises was a numerical value, as crises were determined as number per week. The degree of myoclonus was quantified according to the evaluations of the observer, per minute. Finally, walking difficulty was a subjective analysis, in which the observer noted improvement, stability or deterioration, between the films. The clinical files of the animals were also examined in order to detect any other adverse effect.

For statistical analysis, the neurological evaluation data (epileptic crises, myoclonus and walking difficulty) were transformed into numerical variables for determination of the improvement/deterioration after each application of MSC, and subsequently the data were analyzed for differences between ordered pairs with the Wilcoxon non-parametric test, using the SAS program (Cary, North Carolina, v.5.1).

3. Results

MSC from the donor animals were appropriately isolated and characterized. The flow cytometry results after thawing of cells showed that the MSC expressed CD29, CD44 and CD90, and did not express the hematopoietic marker CD34. There was also a high level of expression of SOX2 and OCT3/4. These results demonstrate that

the cells used for therapy were indeed mesenchymal stem cells with the expected functionality. Cell viability, as analyzed by flow cytometry, showed 80.6% viable cells two hours after thawing (data not shown), demonstrating high viability of the cells that were transplanted into the patients.

Of the 14 dogs evaluated, one animal was given only one MSC application, and two others were given only two applications, by choice of their owners. For the patient that received only one application, and one of those that received two applications, the clinical symptoms remained stable. In the other patient that received only two applications, there was a complete recovery of movement. Of the 14 treated animals that had presented with absence of movement at the pelvic and/or thoracic limbs, there was a recovery of unaided locomotive function in ten (71.4%). Of the 11 animals that received the complete treatment (three applications of MSC), nine fully recovered (81,8%) (Table 1).

Table 1. Grading of clinical symptoms on initial evaluation and at the different time points after allogeneic MSC therapy.

	Before therapy			After 1st application*			After 2nd application*			After 3rd application*			Final Classification**
	Epil	Myo	WD	Epil	Myo	WD	Epil	Myo	WD	Epil	Myo	WD	
1	No	Yes	Yes	0	1	1	0	1	1	0	0	1	III
2	No	Yes	Yes	0	0	-1	0	0	0	0	0	1	II
3	No	Yes	Yes	0	0	-1	0	0	0	0	0	0	I
4	No	Yes	Yes	0	0	0	0	0	0	-	-	-	II
5	No	Yes	Yes	0	0	0	-	-	-	-	-	-	II
6	Yes	Yes	Yes	1	1	1	0	1	1	0	1	1	III
7	Yes	Yes	Yes	0	1	1	1	0	1	1	0	1	III
8	No	Yes	Yes	0	1	1	0	0	1	0	1	1	III
9	No	Yes	Yes	0	0	1	0	1	1	0	0	1	III
10	No	No	Yes	0	0	1	0	0	1	-	-	-	III
11	No	No	Yes	0	0	1	0	0	1	0	0	1	III
12	No	Yes	Yes	0	1	1	0	0	1	0	1	1	III
13	No	Yes	Yes	0	1	1	0	1	1	0	1	1	III
14	No	Yes	Yes	0	1	1	0	1	1	0	1	1	III

Epil: Epileptic crises; Myo: Myoclonus; WD: Walking difficulties. *Evaluation of response to therapy: Improvement in any of the three symptoms: 1 point per symptom; Stability in any of the three symptoms: 0 points per symptom; Deterioration in any of the three symptoms: -1 point per symptom. **Final classification of neurological manifestations after therapy: I. deterioration: -1 to -3 points; II. Stable: 0 points; III. Improvement: 1 to 3 points.

Only two of the treated animals had presented with epileptic crises before MSC therapy, and for both of these there was a decrease in frequency of such episodes at the end of treatment, however, the data were insufficient to determine significance, as shown in Figure 1. These animals used anticonvulsive medication (phenobarbital) before, and continued after therapy, with no alteration in the dosage of the medication at any point.

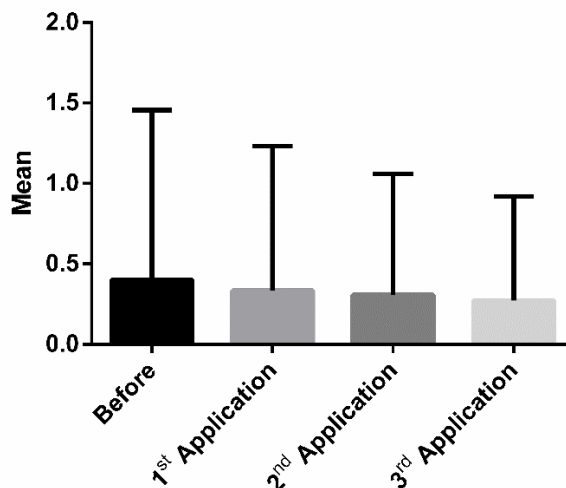


Figure 1. Figure showing the decrease in the frequency of epileptic crises along MSC applications.

Myoclonus was detected in 12 dogs, and a decrease in frequency throughout the treatment was seen in eight animals, representing a significant difference in comparisons Before/1st Application ($P = 0.0209$), Before/2nd Application ($P = 0.0056$) and Before/3rd Application ($P = 0.004$) (Figure 2).

Regarding walking difficulties, a progressive improvement was observed throughout the MSC application period, showing differences between evaluations: Before/1st Application ($P = 0.0063$), Before/2nd Application ($P = 0.011$) and Before/3rd Application ($P = 0.0007$).

The most significant improvement was observed on comparison between the first and second applications ($P = 0.0217$), and between the first and third applications ($P = 0.0017$), suggesting the MSC therapy protocol should involve a series of applications, even in cases where there is a stabilization of clinical symptoms after a single MSC application (Figure 3). Of the 14 animals included in the study that had presented walking difficulties, ten showed a recovery of ambulation, as seen in the accompanying videos.

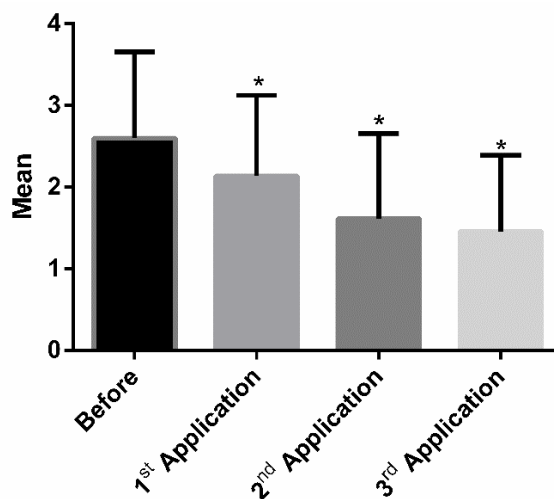


Figure 2. Decrease in the frequency of myoclonus during the MSC treatment period. * Indicates statistical difference.

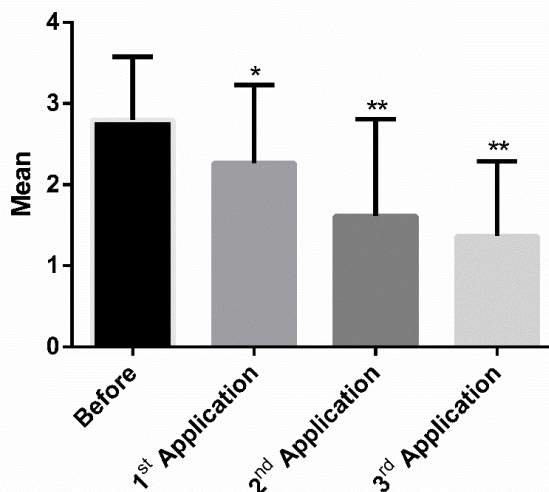


Figure 3. Decrease in walking difficulty throughout the treatment period. * $p < 0,05$; ** $p < 0,01$.

4. Discussion

In the era of regenerative medicine, stem cell transplants appear to be a promising option for treatment of a range of neurological diseases without specific or effective treatments, including neurological sequelae resulting from the canine distemper virus. Stem cell therapies can modify the physiopathology of neurodegenerative diseases (Karumbayaram et al., 2009), delay or interrupt the progression of diseases, and even improve neuromuscular function, possibly providing protective factors to adjacent cells, modulating the immune activity of the patient, inhibiting inflammation, or even stimulating substitution of damaged cells (Kim et al., 2010).

The improvement in clinical signs observed in the present study can be attributed to a possible neuroprotective and regenerative effect of the MSC, reducing the chance of glial scarring (Kim et al., 2016), as well as attracting endogenous neural multipotent cells, that can differentiate and promoting axonal regeneration (Kang et al., 2006; Jung et al., 2009). Furthermore, MSC mediate regeneration by substitution of dead cells, including oligodendrocytes (Escalhão et al., 2017), cells that are destroyed by the distemper virus, a process that promotes demyelination (Tipold et al., 1992).

The action of MSC, when applied *in-vivo*, is to release anti-inflammatory cytokines and neurotrophic factors, in other words, to combat inflammation and support neuronal differentiation (Jung et al., 2009). These effects also promote a decrease in nervous system degeneration, as described by Marcone et al. (2013) when they tested the effect of MSC in animals with induced nervous dysfunctions. These authors showed that the transplanted cells delayed motor deterioration by four to six weeks, and maintained the number of motor neurons, as well as regulation the level of glial neurotrophic factors in the spinal fluid. Such effects may also be present in the animals with neurological sequelae from distemper treated with MSC, since, on eliminating the viral degeneration effect, the neurotrophic factors possibly released by MSC may be acting to remake the myelin sheaths on axons, through regeneration of oligodendrocytes.

According to the evaluations, none of the animals presented any adverse reaction due to MSC treatment. This suggests that the use of allogeneic MSC may be performed safely, as these cells show low expression levels of the major histocompatibility complex class I (MHC I), and absence of MHC II on the cell surface (Le Blanc et al., 2003).

5. Final Considerationss

This study demonstrated the possibility of using allogeneic MSC from cell banks for treatment of neurological sequelae of distemper. Animals submitted to cell therapy presented less myoclonus and improvement in walking capability. As such, it is important to conduct new controlled studies in dogs, with a view to understanding the mode of action of transplanted MSC, and to determine the optimal dosages and application intervals.

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