Mesenchymal stem cells in dogs with demyelinating leukoencephalitis as an experimental model of multiple sclerosis

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ARTICLE INFO

Keywords:
Cell biology
Neuroscience
Mesenchymal stem cells
Canine distemper
Multiple sclerosis
Demyelination

ABSTRACT

Researchers have used dogs with neurological sequelae caused by distemper as an experimental model for multiple sclerosis, owing to the similarities of the neuropathological changes between distemper virus-induced demyelinating leukoencephalitis and multiple sclerosis in humans. However, little is known about the role of mesenchymal stem cells in treating such clinical conditions. Therefore, we investigated the use of mesenchymal stem cells in four dogs with neurological lesions caused by the distemper virus. During the first year after cellular therapy, the animals did not demonstrate significant changes in their locomotive abilities. However, the intense (Grade V) myoclonus in three animals was reduced to a moderate (Grade IV) level. At one year after the mesenchymal stem cell infusions, three animals regained functional ambulation (Grade I), and all four dogs started to move independently (Grades I and II). In two animals, the myoclonic severity had become mild (Grade III). It was concluded that the use of mesenchymal stem cells could improve the quality of life of dogs with neurological sequelae caused by canine distemper, thus presenting hope for similar positive results in human patients with multiple sclerosis.

1. Introduction

Demyelinating leukoencephalitis is the major aggravating factor and cause of mortality from canine distemper [1]. It commonly represents the neurological stage of this disease, where inflammation of the central nervous system, demyelination, and axonal injury occur [1, 2]. However, similar to other morbilliviruses, the canine distemper virus behaves as a lymphotropic and immunosuppressive agent, rendering the animals highly susceptible to opportunistic infections and resulting in a variety of clinical forms that characterize distemper [3]. Dogs that survive this stage sustain disabling sequelae that are often incompatible with life [4]. Furthermore, owing to the morphological similarities to the neuropathological changes associated with human multiple sclerosis, canine distemper represents one of the few spontaneous occurrences in animals that can be applied as a model for the study of the pathogenesis of myelin loss [2, 3].

Multiple sclerosis, a complex human disease with unknown etiology and pathophysiology, manifests primarily as a result of an aberrant response of the immune system cells to the autoantigens of the myelin sheath of neurons. This condition results in multiple areas of scarring (sclerosis) and is also characterized by inflammation, demyelination, and axonal degeneration, which occur in canine distemper as well [5]. Because multiple sclerosis and similar degenerative myelopathy in domesticated animals require treatments that aim to recover the myelin sheath and repair the damaged neuronal tissue, their therapy and cure remain major challenges in both the human and veterinary medical fields [1].

Several research groups have investigated the therapeutic use of mesenchymal stem cells (MSCs) for demyelinating diseases [5, 6, 7, 8]. 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) [9]. In addition, some clinical trials have shown promising results in the use of MSCs in multiple sclerosis [5, 10, 11, 12, 13, 14].

MSCs, which are considered a somatic stem cell line, are present in the perivascular regions of adult tissues that are responsible for cell regeneration and homeostasis [15]. These cells have already been isolated from a variety of tissues (e.g., bone marrow, umbilical cord blood, skin, dental pulp, etc.), among which adipose tissue stands out as a common source owing to a higher rate of isolation and yield [16]. Thus, the present study aimed to evaluate the therapeutic potential of MSCs in inducing the recovery from neurological sequelae in dogs naturally affected by demyelinating leukoencephalitis, assessing signs of neurological changes that may represent hope for human patients with multiple sclerosis.

2. Materials and methods

The dogs used in this study were from the Medical Clinic Sector of the Veterinary Hospital “Prof. Mário Dias Teixeira” at the Federal Rural University of Amazonia (UFRA). The animal protocol was approved by the UFRA Committee on Ethics in the Use of Animals (Protocol No. 053/2015).

2.1. Treatment protocol and evaluation parameters

We selected four dogs (designated c1, c2, c3, and c4) with evident signs of demyelinating leukoencephalitis. The diagnosis of distemper was confirmed from the clinical signs and through laboratory tests. After treatment of the multisystemic clinical symptomatology, neurological sequelae compatible with those caused by the disease still remained, however, without alterations in laboratory tests, including the polymease chain reaction (PCR) - negative for the distemper virus.

The dogs were given a complete neurological examination, which consisted of evaluations of their mental state, locomotion, cranial nerves, postural reactions, spinal reflexes, sensory perception, and muscle tone. For analytical purposes, the neurological record was rated as 0 for absence, 1 for decrease, 2 for normality, and 3 for increase of the evaluated signal. Two neurological scales that were created by Santos [17] were used for evaluating the sequelae of distemper. One scale was for locomotion, with the following grades: (I) functional ambulation; (II) ataxic animal – walks with incoordination; (III) tetraparetic animal – stays in station, but does not get up; (IV) tetraparetic animal – does not stay in station or stand up; and (V) tetraplegic animal – without deep pain and with signs of Grade IV. The other scale was for myoclonus, with the following grades: (I) absent; (II) only at moments of agitation; (III) present – mild; (IV) present – moderate; and (V) present – intense.

The MSCs were extracted from the flank adipose tissue of each canine patient of this study through enzymatic digestion according to the protocol of Zuk et al. [18]. Three separate doses of 1 × 10⁷ cells at passages P3 or P4 were injected into the dogs through the femoral artery at 30-day intervals, and monthly neurological examinations before each application as well as one final evaluation one year later were carried out.

2.2. Clinical conditions of the selected animals

Prior to MSC treatment, all animals were conscious and the neurological changes at the first visit were related to locomotion, postural reactions, spinal reflexes, muscle tone, and myoclonus. c1 presented with monoparesis of the right pelvic limb, with decreased conscious proprioception and hypertonia of this limb, besides motor incoordination and spontaneous falls. c2 presented with monoparesis of the right pelvic limb, with decreased conscious proprioception, patellar hyperreflexia, and hypertonia of this limb, as well as motor incoordination and spontaneous falls. c3 presented with functional deambulation, without changes in the neurological examination. c4 presented with tetraparesis, the absence of conscious proprioception, and hypertonia in the four limbs, as well as cervical stiffness. The four dogs had myoclonus of several muscular groups, with a noticeably greater incidence in the masticatory muscles. The myoclonus was classified as intense for c1, c2, and c4 and moderate for c3.

2.3. MSC cultivation, cryopreservation, and phenotype analysis

After isolation, the MSCs were maintained in cultures at 37 °C with 5% CO2 in growth media complete (Dulbecco’s modified Eagle’s medium, with 20% fetal bovine serum), with a medium change every 2–3 days. The cultures were cryopreserved at the P0 and P1 passages. After thawing, the viability of the cells at each passage was tested using the trypan blue exclusion dye (0.4%) test (Sigma, USA). For intra-arterial administration, the MSCs were thawed and maintained in culture for an average of 7 days for the expansion needed to reach the determined amount of cells (1 × 10⁷ cells).

For phenotype analysis by immunofluorescence, the cells were plated and incubated with primary anti-CD105 (1:25), anti-CD34 (1:100), and anti-CD45 (1:100) antibodies from Abcam (USA), and goat anti-CD73 (1:25) and anti-vimentin (1:25) antibodies from Santa Cruz Biotechnology (USA). Following further processing, they were analyzed under a Nikon 80i fluorescence microscope.

For phenotype analysis by flow cytometry, the cells were first incubated with the primary antibodies (CD105, CD73, CD90, CD34, CD45, and CD79) for 45 min at 4 °C. After washing in phosphate-buffered saline, they were incubated with phycoerythrin- or fluorocine isothiocyanate-conjugated secondary antibodies for 30 min. Following this, 10,000 events were acquired on the FACSCalibur flow cytometer and FlowJo software was used to analyze the data obtained.

2.4. Gene expression by RT-qPCR

Total RNA was extracted using TRIzol Reagent (Life Technologies, USA) and reverse transcribed into cDNA using SuperScript III (Invitrogen, USA), following the manufacturers’ protocols. The cDNA was then subjected to quantitative PCR (qPCR) using SYBR Green Supermix (Bio-Rad, USA). Each sample was run in triplicate. Primers for specific genes were synthesized using Primer3 software (v. 0.4.0) or were available from the Harvard Primer Bank online. The conditions of the PCR cycles were as follows: 30 s at 95 °C, 30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C for 50 cycles. Melting curve analysis was then conducted to verify the amplification specificity. All analyses were done by absolute quantification, with the levels of the target genes normalized to that of the GAPDH gene as a reference control, using standard curves.

2.5. MSC differentiation potential

To determine the osteogenic differentiation potential of the MSCs, 5 × 10³ MSCs/mL were cultured in osteogenic differentiation induction medium (STEMPRO Osteogenesis Kit; Gibco, USA), with a change of the medium on every other day for 14 days, according to the manufacturer’s recommendations. The cells were then stained with 2% Alizarin Red S (Sigma-Aldrich, USA) for 5 min.

For observation of their adipogenic differentiation potential, 1 × 10⁴ MSCs/mL were cultured in induction medium (STEMPRO Adipogenesis Kit; Gibco, USA), with a change of the medium on every other day for 14 days. The cells were then evaluated by staining with 1.25% Oil Red O (Sigma-Aldrich, USA) for 5 min.

For determination of their chondrogenic differentiation potential, the MSCs were cultured in a conical tube at high cell density (5.7 × 10³ cells/mL) in a micromass system. After centrifugation and disposal of the maintenance medium, chondrogenic differentiation induction medium (STEMPRO Chondrogenesis Kit; Gibco, USA) was added, followed by
homogenization and further centrifugation. The tube was maintained in an oven at 37°C with 5% CO₂, with change of the differentiation medium on every other day for 21 days, following the manufacturer’s recommendations. For fixation of the micromass cells, 4% paraformaldehyde was added. The cells were then dehydrated in serial dilutions of ethanol, embedded in paraffin blocks, and further processed according to routine histological protocols. The blocks were cut into 5-μm-thickness sections that were then stained with 1% Alcian Blue solution for 10 min.

2.6. Chromosomal stability analysis

The numerical chromosomal stability of cells of the P4, P6, and P8 passages was analyzed. To obtain the cells at metaphase, 100 μL of 0.016% colchicine solution (Gibco, USA) was added to 5 mL of the culture and the cells were kept in a 37°C oven for 1 h. The cells were then dissociated with trypsin and transferred to a conical tube for centrifugation at 556 g for 10 min. The supernatant was discarded and the cell pellet was resuspended in a 0.075 M hypotonic solution (KCl) and kept in a 37°C oven for 10 min. Subsequently, Carnoy’s fixative solution (methanol:acetic acid, 3:1) was added, and the mixture was homogenized and centrifuged; this process was repeated two more times. Finally, the pellet was resuspended in 2 mL of the fixative and the cells were stored under 6°C refrigeration. For visualization of the chromosomes, slides containing the cells were stained for 3 min with 10% Wright solution diluted in phosphate buffer (pH 6.8), and 15 metaphase cells of each passage were analyzed under a Leica DM1000 optical microscope.

2.7. Statistical analysis

Descriptive statistics were used as appropriate. The Friedman test at 5% significance was applied to determine the median treatment effect for the locomotion and myoclonus scores before and after three infusions and after one year.

3. Results

After 24 h, it was possible to observe the adherence of some of the MSCs to the plastic surface of the culture flask, and the cell confluence reached 80% after 48 h (Fig. 1A, B). After thawing, the cells propagated rapidly, maintaining a fibroblastic morphology in all the passages analyzed (P1–P8) (Fig. 1C), with a mean viability of 94.7% (Fig. 2A). The mean viability of the infused cells was 97.5% (P3) and 98.5% (P4).

In the immunocytochemical evaluation, the analyzed samples were positive for the mesenchymal labels CD105, CD73, and vimentin, and negative for the hematopoietic cell markers CD34 and CD45 (Fig. 1D–I).

In the flow cytometric analysis, the cells showed positivity for the CD105, CD73, and CD90 markers, whereas the CD79, CD34, and CD45 hematopoietic cell markers were undetectable (Fig. 2B).

Images of the best metaphase cells were captured using the GenASIs platform (Applied Spectral Imaging, USA), which is also used for karyotyping.
As determined by RT-qPCR, the analyzed samples did not express pluripotency-related genes (Nanog, Oct4, and Sox2), as expected. However, they showed CD44 and CD105 expression (mesenchymal cell markers) and had no expression of CD29 and CD45 (hematopoietic cell markers) (Fig. 2C).

With regard to the cell differentiation processes, the MSC culture produced an extracellular calcium matrix in the osteogenic differentiation medium, revealing cells with osteogenic characteristics (Fig. 1J). In the adipogenic differentiation medium, the cells had a rounded shape and accumulation of lipid droplets in their cytoplasm, indicative of adipogenic differentiation (Fig. 1L). Histologically, the micromass that had formed under the induction of the chondrogenic differentiation medium showed rounded cells surrounded by a glycosaminoglycan matrix (Fig. 1M).

The numerical chromosomal stability of the MSC cultures was successfully demonstrated. Cells in the analyzed passages (P4, P6, and P8) maintained the diploid number of 78 chromosomes for the domestic dog (Fig. 2D, E).

In the evaluation after the first autologous infusion of MSCs, the c1 and c4 animals presented changes in the neurological examinations when compared with the initial examination and at the time of admission of the canine patients to the study. That is, c1 presented normal locomotion of the right pelvic limb and the absence of both motor incoordination when walking and spontaneous falls. The c2 and c3 animals did not present changes in the neurological analysis, whereas the c4 animal remained at a moderate degree (Table 1).

Finally, the Friedman test was applied to compare the median treatment effect in the group for the locomotive and myoclonic degrees. The results revealed that there were no significant differences before and after the three infusions, and before and after one year of treatment (Fig. 3).

4. Discussion

We emphasize that depending on the virulence of the virus strain, and the age and immune status of the dog, distemper can be fatal in many cases [2]. This justifies the low number of animals used in this study, since we aimed to select only dogs with sequelae of neurological lesions (demyelinating leukoencephalitis) caused by the distemper virus, all of which had already been treated conservatively and conventionally but did not show recovery of their motor integrity. The dogs of this study did not present with multisystemic clinical symptoms and had no changes in their laboratory test findings, such blood counts and negative RT-qPCR for the virus, in accord with the animals recommended by Gebara et al. [19] and Nelson and Couto [20].
nerve dysfunctions, muscular atrophy, hyperesthesia, myoclonus, de
convulsions, ataxia, tetraparesis, tetraplegia, proprioception and cranial
demyelinating leukoencephalitis in distemper are behavioral disorders,
the evolution phase of the disease \[3\]. The neurological changes
20\]. Moreover, myoclonus is the most common sign of this condition,
curs in multiple sclerosis \[1, 2, 22, 23\]. Multiple sclerosis causes a de
associated with immune-mediated mechanisms, such as that which oc-
rally occurring model for the study of the pathogenesis of myelin loss
one of the selected animals. Demyelinating leukoencephalitis, the
of the neurological phase of distemper, has been suggested as a suit-
ult a consistent pattern of decreased clinical signs, increased weight,
ment of the disease and reductions of both demyelination and axonal loss \[5, 26\]. However, the mechanisms underlying the ther-
apic effects are still unknown and may involve one or more of the
following possibilities, according to Rivera and Aigner \[26\]:
transdifferentiation of MSCs in mature neurons and/or functional oli-
godendrocytes (plasticity); immunoregulatory effect on host-derived
mune reactive cells (immunomodulation); protective effect on the
survival of damaged neurons and/or oligodendrocytes (neuro-
tection); and induction of the differentiation and maturation of neural
 precursor cells or oligodendrocyte progenitor cells present at the lesion
ite (remyelination).

In this context, the literature highlights that the most evident signs of
demyelinating leukoencephalitis in distemper are behavioral disorders,
convulsions, ataxia, tetraparesis, tetraplegia, proprioception and cranial
nerve dysfunctions, muscular atrophy, hyperesthesia, myoclonus, deficits
or abnormal reflexes in the spine, and urinary incontinence, regardless of
the evolution phase of the disease \[3\]. The neurological changes
observed in the animals selected for this study were related to locomotion
(ataxia and monoparesis), postural reactions (absent or diminished pro-
ception), spinal reflexes (hyperesthesia), muscle tone (hypertonia), and
myoclonus of various muscle groups, in accord with the literature \[3,
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MSCs, both Brito [32] and Monteiro [33] reported a poor health status of some dogs during the study, which may be related to the acute stage of clinical symptomatology, which was different from that of our dogs. Similar to our study, Gonçalves et al. [34] recently used dogs with neurological sequelae only after treatment of the multisystemic symptoms of distemper, and performed three intravenous infusions of adipose tissue-derived MSCs, but theirs were allogenic. In that study, based on the numerical scale proposed by the authors for evaluation, attenuation of the clinical signs was observed after 15 days and was maintained throughout the 180 days of observation, but with statistical differences only for the urinary incontinence and fecal incontinence variables.

In our present uncontrolled clinical trial, the autologous use of adipose tissue-derived MSCs by the intra-arterial route on every 30 days, totaling three infusions, resulted in improvement of the neurological status of the animals, with one dog regaining functional ambulation and achieving a reduction of the myoclonic intensity to Grade IV (moderate). However, consistent improvements were observed in the evaluation after one year post treatment, where three animals regained functional ambulation (Grade I), all animals moved independently (Grades I and II), and two animals presented Grade III myoclonus (mild).

The route of MSC administration is an important variable that can define the success of a transplant by interfering directly with the efficient delivery of cells to the site of interest [35]. The venous system, being the least invasive route, has been the one most often used. However, in addition to a lack of knowledge regarding the actual cellular concentration required to reach the desired lesion area, studies have shown that MSCs accumulate rapidly in the lungs, spleen, and liver after administration [35, 36]. However, by bypassing the initial uptake by the lungs, administration through the arterial system results in a greater availability of cells to ischemic sites, but may lead to a greater probability of microvascular occlusions [37]. In our study, no side effects related to short-term and long-term intra-arterial MSC administrations were observed, and choice for the femoral artery was considered due to easier access compared to the carotid artery or intrathecal route.

We believe that the indiscriminate commercialization of stem cells as a form of “treatment” of various diseases (including for the recovery of canine distemper sequelae) is unacceptable in both veterinary medicine, since it cannot be stated categorically that this therapy does in fact lead to the healing of patients. This is a current concern in many countries owing to the lack of regulations and control for the clinical use of stem cells in veterinary medicine, allowing for the increasing offer of the service by private companies and resulting in the implementation of therapies that lack proven effectiveness either in vitro or in preclinical animal studies [38, 39]. The US Food and Drug Administration’s Center for Veterinary Medicine was the only legislative body to formally publish specific definitions and recommendations for stem cell use through guidelines [40], where cell-based products must follow the same legal requirements that apply to other animal drugs, forcing the industry to prove efficacy and manufacturing quality and safety prior to commercialization [38].

Despite the promising results regarding the alleviation of the severity of the disabling lesions of demyelinating leukoencephalitis caused by distemper (considered irreversible and often incompatible with animal life), our findings are considered limited because of the small sample size, and future studies should involve a greater number of animals. In addition, both in vivo and in vitro studies should be performed to determine the mechanisms underlying the therapeutic effects of MSCs in dog with neurological sequelae of distemper. In this context, the technology of induced pluripotent stem cells, from genetically modified and reprogrammed adult cells [41], would be a powerful tool in basic research, tissue differentiation research, and disease modeling, as well as being promising for future clinical applications.

5. Conclusions

Our results indicate that the strategy of three intra-arterial infusions of $1 \times 10^7$ MSCs, with a 30-day interval in between administrations, appears to be safe in dogs with demyelinating leukoencephalitis caused by the distemper virus, and presents moderate efficacy for the rehabilitation of neurological signs after recovery from a multisystemic infection, with considerable improvements in the neurological status of the animals after one year of cell therapy. However, further extensive investigations are needed for a better understanding of the mechanism of action of these MSCs on the injured nervous tissue and the time of recovery, in future studies that include a larger number of animals, placebo group and investigation of other routes of administration.

Declarations

Author contribution statement

Luane Lopes Pinheiro: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Ana Rita de Lima: Analyzed and interpreted the data.

Danielli Martinelli Martins, Michel Platini C. Souza, Carla Maria Figueiredo de Carvalho Miranda: Performed the experiments.

Elivaldo Herculano C. de Oliveira, Patrícia Cristina Baleeiro Beltrão-Braga, Fabiele Baldino Russo, Graciela Conceição Fignatari, Ednaldo da Silva Filho: Contributed reagents, materials, analysis tools or data.

Erika Branco: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil (Finance Code 001).

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2019.e01857.

Acknowledgements

The authors thank the Coordination of Improvement of Higher Education Personnel (CAPES) for financial support; the Laboratory of Tissue Culture and Cytogenetics of the Environment Section of the Evandro Chagas Institute; and the Veterinary Hospital “Prof. Mário Dias Teixeira” (UFRA) for supporting this study.

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