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CLINIC AND SURGERY

Clinical evaluation of allogeneic adipose tissue-derived stem cells for the treatment of osteoarthritis secondary to hip dysplasia in dogs

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ABSTRACT: This study assessed the efficacy of an intra-articular injection of allogeneic adipose tissue-derived from mesenchymal stem cells (AD-MSCs) for the treatment of hip dysplasia in dogs. The study group included 12 otherwise healthy dogs of different breeds, ages, weights, and degrees of hip dysplasia diagnosed using radiography. An orthopedic assessment was performed on all dogs before and at 30, 60, and 90 days after infusion of AD-MSCs(2×10^6cells). On the same days, each dog's owner answered a questionnaire based on theHelsinkiChronic Pain Index. The data were converted to ordinal data based on the score for each variable, and the Friedman test for multiple comparisons was used to verify the results. Compared with the corresponding values on day 0, orthopedic and gait assessments and owners' reported pain indexes improved over the 90-day observation period. These results suggested that treatment with allogeneic AD-MSCs significantly reduced the clinical signs associated with hip dysplasia during the study period. However, long-term studies are needed to determine the optimal therapeutic protocol for routine clinical use of AD-MSCs in hip dysplasia.
Key words: degenerative joint disease, osteoarthritis, chronic pain, cell therapy.

Avaliação da eficácia clínica do uso de células-tronco mesenquimais alógenas derivadas de tecido adiposo no tratamento da osteoartrite secundária a displasia coxofemoral em cães

RESUMO: O objetivo deste estudo foi avaliar a eficácia clínica da aplicação intra-articular de células-tronco mesenquimais alógenas derivadas do tecido adiposo (AD-CTM) no tratamento de cães portadores de osteoartrite secundária a displasia do coxofemoral (DCF). Doze cães de ambos os sexos, diferentes raças, idades e peso, portadores de graus variados de DCF comprovada em radiografia e livres de quaisquer outras alterações clínicas ou ortopédicas, foram utilizados no estudo. Todos os cães foram submetidos a avaliação ortopédica nos dias 0, 30, 60 e 90 após aplicação de AD-CTM na dose de 2×10^6. Além disso, os tutores preencheram a um questionário, baseado no Índice de dor crônica de Helsinque (IDCH) nos mesmos intervalos. Em comparação com o dia 0, observou-se melhora significativa na avalição em locomoção e físico-ortopédica assim como na avaliação dos tutores pelo IDCH ao longo dos 90 dias. Os resultados permitem inferir que as AD-CTM alógenas contribuíram significativamente para a redução dos sinais clínicos comumente associados a DCF durante o período de estudo. Entretanto, há necessidade de estudos de longo prazo para melhor determinação de protocolos terapêuticos baseados no uso de AD-CTM na rotina clínica.
Palavras -chave: doença articular degenerativa, osteoartrose, dor crônica, terapia celular.

INTRODUCTION

Hip dysplasia (HD) is a hereditary disease characterized by abnormal development, instability, and varying degrees of looseness in the hip joints (GENEVOIS et al., 2020, SCHACHNER & LOPEZ, 2015). It is a limiting condition that causes pain and physical disability, which over time, leads to wearing of the joint surfaces, progressive remodeling of the hip structures, and subsequent development of osteoarthritis (OA) (GINJA et al., 2010).

Although, dogs of all sizes are susceptible to HD, itoccurs more frequently in medium and large breeds. The multifactorial cause, involving genetic and environmental factors, limitsits control and treatment (GENEVOIS et al., 2020). Diagnosis of HD is based on anamnesis, clinical symptoms, and an orthopedic clinical examination and is confirmed using imaging (GINJA et al., 2010).

The choice between conservative or surgical treatment depends on the patient's age and disease severity. Conservative treatment, which

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includes exercise restriction, weight loss, analgesics, and chondroprotective agents, focuses on controlling pain and delaying degenerative changes. However, its effectiveness is controversial (SCHACHNER & LOPEZ, 2015). Surgical treatment is performed when OA is present, and procedures include hip replacement, acetabular denervation, and femoral head and neck excision. However, these procedures are costly and have varying degrees of success (GINJA et al., 2010, BERGH & BUDSBERG, 2014, SCHACHNER & LOPEZ, 2015).

Therefore, alternative treatments for HD have been pursued to minimize clinical symptoms and improve quality of life (VILAR et al., 2014, CUERVO et al., 2014). Cellular therapy has recently emerged as an alternative for managing OA resulting from HD (BLACK et al., 2007; VILAR et al., 2014).

Mesenchymal stem cells (MSCs) can differentiate into various tissues and have anti-inflammatory, immunomodulatory, and immunosuppressive effects. They have the potential to regenerate and maintain joint cartilage, which can reduce pain and increase joint functionality (WHITWORTH & BANKS, 2014).

This study evaluated the clinical effect of allogeneic adipose tissue-derived MSCs (AD-MSCs) for the treatment of HD-associated OA in dogs. Efficacy was evaluated through assessments of locomotion and a physical-orthopedic examination as well as a questionnaire, the Helsinki Chronic Pain Index (HCPI), aimed at evaluating each owner's perception of their dog's condition.

MATERIALS AND METHODS

A total of 12 adult dogs, 1.5–9 years of age and weighing 17–48 kg, were selected for the study. The group consisted of four females and eight males of various breeds, all of whom had confirmed secondary OA due to bilateral HD, as determined by digital radiography according to the method of the International Canine Federation (FCI). During selection, animal data, including body condition score (1–9), were recorded. The dogs underwent clinical, orthopedic, and neurological examinations to rule out any concurrent disease or alteration. Blood tests, renal and liver function tests, echocardiography, electrocardiography, and non-invasive blood pressure measurement were also performed. Any parallel conservative treatment was suspended.

To investigate the clinical effect of AD-MSCs, subjective evaluations were conducted at 0, 30, 60, and 90 days post-injection using established clinical parameters (POLLMEIER et al., 2006, BLACK et al., 2007). A numerical score was assigned to each clinical parameter. The evaluation was divided into two parts: (1) during locomotion and (2) physical-orthopedic examination (Figure 1).

Each owner was asked to answer an 11-item questionnaire to assess their dog's pain, called the HCPI (Figure 2), which has been validated (HIELM-BJORKMAN et al., 2009, MATSUBARA et al., 2019).

AD-MSCs were applied on day 0 after confirming the normality of all tests and an 8h fast. The dogs were anesthetized with 0.2% acepromazine (Acepram, 0.04 mg/kg, SC; Vetnil, Brazil) as a pre-anesthetic, followed by 1% propofol (Provive, 4.0 mg/kg, IV; União Química, Brazil) as an anesthetic. Isoflurane (Isoflurano, BioChimico, Brazil) was used for anesthetic maintenance in a closed-circuit anesthesia system with constant oxygen flow (2.5 L/min).

The allogeneic AD-MSCswere obtained through a partnership with Regenera Stem Cells Laboratory, which is authorized and approved by the Ministry of Agriculture, Livestock, and Supply (approval number 00001759/2017 MAPA). Cryotubes containing 2×10^6 cells were kept in liquid nitrogen until thawed for use.

For AD-MSC application, after rigorous pre-surgical handling, arthrocentesis of the carpal joints was performed using a 16G needle. The AD-MSCs were diluted in 1 mL of 0.9% NaCl and injectedinto each joint immediately after collecting 1 mL of synovial fluid.

The obtained data were transformed to ordinal data based on the score for each variable. The Friedman test with multiple comparisons was used to verify the obtained responses. Statistical significance was set at $P < 0.05$. Raw data tabulation and statistical calculations were performed using GraphPad Prism software, version 7.0.

RESULTS

Twelve dogs of both sexes with varying grades of HD classified according to the FCI guidelines were used in the study (two were classified as grade C, four as grade D, and six as grade E).

The animals' mean weight, age, and body condition score were 33.34 kg, 4.9 years, and 7.2, respectively. The parameters used to evaluate the hind limbs during locomotion and in the physical-orthopedic examination on days 0, 30, 60, and 90 are presented in table 1.

Compared to the parameters on day 0, there were no significant differences in the parameters of

CLINICAL PARAMETERS	SCORES				
CLAUDICATION ON WALKING	Not detected (1)	Intermittent (2)	Discrete and continuous (3)	Moderate and continuous (4)	Severe (5)
CLAUDICATION ON TROTTING					
RANGE OF MOTION	No limitations (1)	Moderate decrease in range of motion (2)	Slight decrease in range of motion (3)		Severe decrease (4)
FUNCTIONAL CAPACITY	Normal activity (1)	Slightly stiff gait only noticeable by running (2)	Stiff gait, noticeable difficulty in walking or running (rabbit gait) (3)	Very stiff gait, dog does not want to walk or run unless stimulated (4)	Reluctant to walk even when stimulated (5)
CLINICAL PARAMETERS	SCORES				
PAIN ON CRANIAL EXTENSION	Absent (1)	Moderate (2)	Discrete (3)	Severe (4)	Very severe (5)
PAIN ON CAUDAL EXTENSION					
FLEXION PAIN					
PAIN ON EXTERNAL ROTATION					
PAIN ON ABDUCTION					
MUSCULAR ATROPHY					
JOINT CREPITATION					
BIPED STATION TEST	No change (1)	Able to express discomfort (2)			Incapable (3)

Figure 1 - Clinical parameters evaluated during locomotion and physical-orthopedic examination at 0, 30, 60, and 90 days after application of allogenic adipose tissue-derived allogeneic mesenchymal stem cells (AD-MSCs) in dogs with hip dysplasia.

lameness when walking (for both the right and left hind limbs), lameness when trotting (left hind limb), and range of motion (left hind limb) on day 30. However, there were significant positive differences in all parameters evaluated during locomotion on days 60 and 90.

The clinical evaluation of pain during cranial extension did not show a significant difference at 30 days in either limb. However, compared to this parameter on day 0, there were differences in the right hind limb ($P < 0.0002$) at 60 and 90 days and in the left hind limb ($P < 0.0004$) at 90 days. Analysis of pain during caudal extension revealed significantly less severe pain in the right hind limb ($P < 0.0004$) at 30 and 60 days and in the left hind limb ($P < 0.0001$) at all time points evaluated (30, 60, and 90 days) than at 0 days.

Analysis of pain during flexion did not show a significant change in left hind limb ($P < 0.05$) at the evaluated time points. However, there was a significant difference in right hind limb ($P < 0.0004$) at 60 and 90 days of age. Significant improvements were observed for pain during external rotation of the right hind limb ($P < 0.0001$) at 60 and 90 days and the left hind limb ($P < 0.0001$) at all evaluated time points (30, 60, and 90 days). Improvement of pain in both limbs was noted throughout the evaluation period for abduction. Parameters related to muscle atrophy, bipedal stance test, and joint crepitus did not change significantly ($P > 0.05$) at any of the evaluated time points (30, 60, and 90 days) compared to those at the initial evaluation (on day 0).

Results related to the Helsinki Chronic Pain Index, which assesses the overall condition of the patient, are presented in Table 2. At the 30-day follow-up, compared to the initial assessment on day 0, significant improvements were observed in the patient's (1) mood ($P < 0.0008$), (3) frequency of pain vocalization ($P < 0.0001$), (4) willingness to walk ($P < 0.0001$), (6) willingness to gallop ($P < 0.0001$), (7) willingness to jump ($P < 0.0001$), (9) ease of getting up from a lying position ($P < 0.0001$), (10) ease of moving after a prolonged period of rest ($P < 0.0001$), and (11) ease of moving after physical or intense effort ($P < 0.0001$).

Compared to the baseline assessment (day 0),scores for parameters (3), (4), (5), (6), (7), (9), (10), and (11) differed at 60 days. Willingness to trot (5) differed significantly ($P < 0.0004$) at 60 days but

1.Mood
(0) very alert (1) alert (2) neither alert nor indifferent (3) indifferent (4) very indifferent
2.Willingness to participate in play
(0) very willing (1) willing (2) reluctant (3) very reluctant (4) does not play
3.Frequency in which the dog vocalizes complaints audibly
(0) never (1) hardly ever (2) sometimes (3) frequently (4) very frequently
4.,5., 6., and 7.Willingness of the dog to: walk, trot, gallop, and jump, respectively
(0) very willing (1) willing (2) reluctant (3) very reluctant (4) does not walk, trot, gallop, or jump, respectively
8.Ease of the dog in lying down
(0) very easily (1) easily (2) neither easily nor with difficulty (3) with difficulty (4) with great difficulty
9.Ease of the dog in getting up from a lying position
(0) very easily (1) easily (2) neither easily nor with difficulty (3) with difficulty (4) with great difficulty
10.Ease of the dogin moving around after a long rest
(0) very easily (1) easily (2) neither easily nor with difficulty (3) with difficulty (4) with great difficulty
11.Ease of the dog in moving after intense or heavy exercise
(0) very easily (1) easily (2) neither easily nor with difficulty (3) with difficulty (4) with great difficulty

Figure 2 - Questions based on the Helsinki Chronic Pain Index asked to owners on at 0, 30, 60, and 90 days after treatment with AD-MSCs. Adapted from HIELM-BJÖRKMAN et al., 2009.

not at 30 days. Similarly, significant differences were observed for parameters (1), (2), (3), (4), (5), (6), (7), (9), (10), and (11) at 90 days. However, no significant difference was observed for parameter (8),ease of lying down, at any time point when compared to before the treatment (day 0).

DISCUSSION

Although, there are various treatments available for HD, current modalities for managing secondary OA due to HD have limitations, making it challenging in clinical practice (BERGH & BUDSBERG, 2014, SCHACHNER & LOPEZ, 2015). Several studies have shown the benefits of using MSCs for treating HD (WHITWORTH & BANKS, 2014, FILARDO et al., 2016, MARKOSKI, 2016); however, a well-designed methodology is still lacking, as described in a systematic review by OLSSON et al. (2021). Questions related to the origin and quantity of MSCs applied, as well as the number of injections and the delivery system, remain unanswered. Other issues, including the use of co-stimulatory factors such as platelet-rich plasma, hyaluronic acid, or growth factors, and the cell source (autologous or allogenic) require further investigation (ZHANG et al., 2021).

This study was the first to test the use of a lower dose (2×10^6 cells) for the treatment of secondary OA caused by HD. This dose is lower than those used in most studies, e.g., HUŇÁKOVÁ et al. (2020):5×10^6, VILAR et al. (2016): 15×10^6, CUERVO et al. (2014): 30×10^6, VILAR et al. (2014): 15×10^6, TSAI et al. (2014): 5×10^6, VILAR et al. (2013): 15×10^6, and BLACK et al. (2007): 5×10^6 cells.

In this study, the clinical signs commonly associated with HD, as evaluated during locomotion and physical-orthopedic examination (Table 1), improved after application of AD-MSCs. The owners' evaluations obtained through a questionnaire also showed improvement (Table 2).

Similar results were obtained in a previous randomized, blinded, placebo-controlled clinical trial conducted by BLACK et al. (2007).They treated dogs with chronic OA with an intra-articular injection of adipose tissue-derived stromal vascular fraction (SVF) into the cranial cruciate ligament (CCL). The methodology also included the assessment of clinical parameters related to locomotion and orthopedic-physical aspects as well as a questionnaire given to the owners. The authors reported significant improvements in dogs treated with SVF throughout the study. Compared to the values in the control group, improvements were observed in lameness during trotting, range of motion, and pain during manipulation at 30, 60, and 90 days after stem cell application. However, in contrast to the previous study, we observed significant differences in lameness and range of motion

Table 1 - Parameters evaluated in both limbs during locomotion and physical-orthopedic examination at 0, 30, 60, and 90 days after application of allogeneic AD-MSCs.

Parameter Evaluated	----------0 Days----------		--------30 Days--------		----------60 Days----------		----------90 Days----------	
	RHL	*LHL*	*RHL*	*LHL*	*RHL*	*LHL*	*RHL*	*LHL*
Claudication on walking	3.5	3.542	2.3 ns	2.5 ns	2.1*	2.083*	2.1*	1.875**
Claudication on trotting	3.9	3.708	2.4*	2.5 ns	1.85**	1.833**	1.85**	1.958**
Range of motion	3.8	3.75	2.2*	2.583 ns	2**	1.833***	2**	1.833***
Functional Capacity	3.95	3.958	2.15**	2.208**	1.95**	1.958***	1.95**	1.875***
Pain on cranial extension	3.6	3.417	2.3ns	2.417 ns	2.15*	2.25 ns	1.95*	1.917 *
Pain on caudal extension	3.7	3.833	2.1*	2.208*	1.75**	2.042**	2.45ns	1.917***
Pain on flexion	3.6	3.292	2.4 ns	2.333 ns	2*	2.208 ns	2*	2.167 ns
Pain on external rotation	3.9	3.958	2.6 ns	2.25**	1.75***	2 ***	1.75***	1.792**
Pain on abduction	3.9	3.667	2.25*	2.375*	1.85**	1.917***	2**	2.042***
Muscle atrophy	2.55	2.5	2.55ns	2.5 ns	2.35 ns	2.5 ns	2.55 ns	2.5 ns
Biped test station	2.8	2.75	2.4 ns	2.417 ns	2.4 ns	2.417 ns	2.4 ns	2.417 ns
Joint crepitus	2.85	2.667	2.65ns	2.667 ns	2.25 ns	2.5 ns	2.25 ns	2.167 ns
Pain on cranial extension	3.6	3.417	2.3ns	2.417 ns	2.15*	2.25 ns	1.95*	1.917*
Pain on caudal extension	3.7	3.833	2.1*	2.208*	1.75**	2.042**	2.45ns	1.917***
Pain on flexion	3.6	3.292	2.4 ns	2.333 ns	2*	2.208 ns	2*	2.167 ns
Pain on external rotation	3.9	3.958	2.6 ns	2.25**	1.75***	2 ***	1.75***	1.792 **
Pain on abduction	3.9	3.667	2.25*	2.375*	1.85**	1.917***	2**	2.042***
Muscle atrophy	2.55	2.5	2.55ns	2.5 ns	2.35 ns	2.5 ns	2.55 ns	2.5 ns
Biped test station	2.8	2.75	2.4 ns	2.417 ns	2.4 ns	2.417 ns	2.4 ns	2.417 ns
Joint crepitus	2.85	2.667	2.65ns	2.667 ns	2.25 ns	2.5 ns	2.25 ns	2.167 ns

RHL - Right hind limb; LHL - Left hind limb.
ns - not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

only after 60 days. Additionally, functional capacity improved considerably in this study; however, it was not significantly different. Furthermore; although, the owners' answers to the questionnaire were not significantly different, the International Veterinary Scoring System used in this study contributed significantly to the treatment evaluation.

VILAR et al. (2014) evaluated the effect of combining MSC therapy with intra-articular injection of platelet-rich growth factors (PRGF) in the CCL in dogs with severe OA. Dogs were evaluated using a force plate. Although, this methodology differed from what we used in the present study, the treatment group showed less lameness than the control group at 6 months, which supports the findings of the present study.

Studies have demonstrated the effectiveness of stem cells for treating OA in various other animal species, including horses (SEO et al., 2013), goats (MURPHY et al., 2003), sheep (KANDEL et al., 2006), guinea pigs (SATO et al., 2012), and rabbits (DESANDO et al., 2013). It is worth noting that unlike the present study, most of these studies were focused on the knee joint. Nevertheless, most reported positive outcomes.

Findings from studies on the use of MSCs in humans with hip joint alterations also support our results. One study demonstrated the outcomes of intra-articular injections of autologous bone marrow-derived MSCs (BM-MSCs) in combination with hip arthroscopy in patients with femoroacetabular impingement (FAI). The results showed improvements in quality of life and functional scores in patients with FAI and cartilage injuries (MARDONES et al., 2016). Similarly, successful use of autologous BM-MSCs in combination with core decompression in the femoral head has been reported for human patients with aseptic necrosis of the femoral head (XU et al., 2017).

Our observations regarding the improvement of animal symptoms after injection of AD-MSCs may be associated with their reported anti-inflammatory, immunomodulatory, immunosuppressive, angiogenic, chemotactic, and antiapoptotic effects (ZHANG et al., 2021; MARKOSKI, 2016; WHITWORTH & BANKS, 2014; DA SILVA MEIRELLES et al., 2009). In OA, excess production of destructive substances and inflammation mediators leads to the breakdown of joint cartilage. Interleukin (IL)-1β and tumor necrosis

Table 2 - Data obtained from owners based on the Helsinki Chronic Pain Index at 0, 30, 60, and 90 days.

Parameter evaluated	0 days	30 days	60 days	90 days
1	3.5	2.125*	2.25 ns	2.125*
2	3.417	2.375 ns	2.25 ns	1.958*
3	3.833	2.25**	1.958*	1.958**
4	3.583	2.292*	2.208*	1.917**
5	3.458	2.542 ns	1.958*	2.042*
6	3.792	2.5*	2.0**	1.708***
7	3.958	2.5*	1.75***	1.792***
8	2.833	2.458 ns	2.208 ns	2.5 ns
9	3.958	2.167**	2.0***	1.875***
10	3.958	2.208**	1.792***	2.042***
11	3.958	2.333**	1.667***	2.042***

(1) Mood; (2) Willingness to participate in play; (3) Frequency in which the dog vocalizes complaints audibly; (4) (5) (6) (7) Willingness of the dog to: walk, trot, gallop, and jump (4 items), respectively; (8) Ease of the dog in lying down; (9) Ease of the dog in getting up from a lying position; (10) Ease of the dog in moving from after a long rest; and (11) Ease of the dog in moving after intense or heavy exercise.
ns - not significant; * P <0.05; ** P <0.01; *** P <0.0001.

factor (TNF) are known contributors to degeneration of the joint matrix (KAPOOR et al., 2011).

Other animal studies also support these observations. HUŇÁKOVÁ et al. (2020) analyzed the use of allogeneic AD-MSCs in conditioned medium for treating bilateral OA in the elbow joints of Labrador retrievers. Analysis of synovial fluid showed a reduction in the pro-inflammatory cytokines IL-6 and TNF and the metalloproteinases TIMP and MMP-3, resulting in improved functional capacity.

In recent years, the field of cellular therapy has seen significant advances, leading to several commercial laboratories exploring the use of MSCs for various applications (MARKOSKI, 2016). In parallel, the International Society for Cellular Therapy (ISCT) has established minimal criteria for the characterization of MSCs (DOMINICI et al., 2006). The AD-MSCs used in this study were obtained from a commercial company chosen due to their ease of procurement and handling, possibility of immediate availability, and compliance with basic ISCT requirements.

Our choice of AD-MSCs was driven by their potential effectiveness and other factors, such as ease of collection (KERN et al., 2006), high number of cells (WEBB et al., 2012), and high proliferation rate (KERN et al., 2006).

Body weight is widely recognized as one of the most significant factors in the development of HD and subsequent OA (FIRMINO et al., 2020). Based on their body condition index, the animals in our study were overweight, following the increasing global trend in weight. Overweight animals may experience exacerbated clinical signs, which could explain the lack of significant results in the bipedal stance test parameter.

Despite some variations, the dogs in this study showed significant improvement in the evaluation of pain during caudal extension, abduction, and external rotation. These results demonstrated the positive effect of allogenic AD-MSCs in dogs with HD. Furthermore, the findings agree with previous findings regarding pain, which was primarily perceived during the evaluation of these parameters (GINJA et al., 2010).

According to ESSNER et al. (2017), evaluating pain in dogs with joint conditions through the owner offers the advantages of prolonged analysis in the animal's typical environment by someone who intimately knows its daily routine and is able to identify subtle behavioral changes. The HCPI questionnaire (Table 2) confirmed the results of the clinical evaluation and proved to be a practical tool for assessing pain caused by HD in the animals.However, a difficulty was identified in the owners' evaluation of "ease of lying down," which may explain the lack of significance for this parameter. However, this allows for reflection on the importance of this item in the evaluation. Similarly, some analyzed items, such as the dog's mood and willingness to play, have a higher degree of subjectivity than the other items and may be influenced by other factors, such as the animal's age and disposition, which may explain the variability in the results for these items.

In addition to the information obtained with the HCPI, various other specific improvements were reported. For example, in two male animals, there was a return to the characteristic male urination position (with one leg lifted), an improvement in disposition for specific activities, such as jumping over obstacles (a gate at the residence), an improvement in getting in and out of cars, and the ability to mount a female (without reproductive purpose).

CONCLUSION

Based on the results obtained from the mobility assessment, physical-orthopedic examination, and HCPI questionnaire, it can be inferred that administration of allogeneic AD-MSCs, at a dose of 2×10^6, significantly reduced the clinical signs commonly associated with HD. This intervention led to an improvement in the dogs' quality of life during the study period. However, long-term studies are needed to determine the optimal therapeutic protocols for the use of AD-MSCs in clinical practice.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

After obtaining approval from the Ethics Committee on Animal Use of the Federal Rural University of Pernambuco (CEUA/UFRPE) (approval number 23082.010283/2017-97), dogs with HD were selected from the Veterinary Hospital of UFRPE and private services in the Metropolitan Region of Recife.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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