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Study of the safety and therapeutic efficacy of multipotent adult progenitor cells in the treatment of perianal fistula in dogs

Estudo da segurança e eficácia terapêutica das células progenitoras adultas multipotentes no tratamento de fistula perianal em cães

Estudio de la seguridad y eficacia terapéutica de células progenitoras adultas multipotentes en el tratamiento de la fístula perianal en perros

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Abstract
Perianal fistula is a conical, progressive, and painful inflammatory lesion that presents with ulceration in the anal and perianal regions. Current treatments consist of cleaning the area and analgesics or a surgical approach. Currently, stem cell therapy has been used successfully in the treatment of various diseases that affect small and large animals. The aim of this study was to evaluate the safety and efficacy of multipotent adult progenitor cell therapy in the treatment of perianal fistula in dogs. In the present study, twelve dogs were selected that had a perianal fistula for at least six months, and were refractory to treatment with cyclosporine A. The patients underwent cell transplantation with allogeneic stem cells to determine their safety and therapeutic efficacy. The results obtained demonstrated that this new methodology can result in the recovery of patients' quality of life.
Keywords: Stem cells; Perianal fistula; Cellular therapy; Security; Efficiency.

Resumo
A fístula perianal é uma lesão inflámatória cônica, progressiva e dolorosa que apresenta um quadro de ulceração nas regiões anal e perianal. Os tratamentos atuais consistem na limpeza do local e analgêsicos ou uma abordagem cirúrgica. Atualmente a terapia com células-tronco vem sendo utilizada com sucesso no tratamento de diversas doenças que acomentem os pequenos e grandes animais. O trabalho tem por objetivo avaliar a segurança e eficácia da terapia com células progenitoras adultas multipotentes no tratamento de fistula perianal em cães. No presente estudo forma selecionados doze cães que apresentavam um quadro de fístula perianal a pelo menos seis meses, sendo refratários ao tratamento com ciclosporina A. Os pacientes foram submetidos ao transplante celular com células-tronco alogênicas de forma a determinar sua segurança e eficácia terapêutica. Os resultados obtidos demonstraram que esta nova metodologia pode resultar na recuperação da qualidade de vida dos pacientes.
Palavras-chave: Células-tronco; Fístula perianal; Terapia celular; Segurança; Eficácia.

Resumen
La fístula perianal es una lesión inflamatoria cónica, progresiva y dolorosa que cursa con ulceración en las regiones anal y perianal. Los tratamientos actuales consisten en limpieza de la zona y analgésicos o abordaje quirúrgico. Actualmente, la terapia con células madre se ha utilizado con éxito en el tratamiento de diversas enfermedades que afectan a animales pequeños y grandes. El objetivo de este estudio era evaluar la seguridad y eficacia de la terapia con células progenitoras adultas multipotentes en el tratamiento de la fístula perianal en perros. En el presente estudio se

seleccionaron doce perros que presentaban fístula perianal durante al menos seis meses, siendo refractarios al tratamiento con ciclosporina A. Los pacientes fueron sometidos a trasplante celular con células madre alogénicas para determinar su seguridad y eficacia terapéutica. Los resultados obtenidos demostraron que esta nueva metodología puede redundar en la recuperación de la calidad de vida de los pacientes.
Palabras clave: Células madre; Fístula perianal; Terapia celular; Seguridad; Eficiencia.

1. Introduction

Perianal fistulas, also known as perianal fissures or anal furunculosis, are characterized by chronic, painful, and progressive inflammatory lesions and ulceration located in the perianal region, anal and perirectal tissues. Perianal fistulas mainly affect middle-aged dogs, predominantly Labrador Retrievers, German Shepherds, Border Collies, Bulldogs, Old English Sheepdogs, Irish Setters, Beagles and Spaniels. Although perianal fistula affects both genders, the most common reports are in male dogs (Elkins, 2008). It is currently believed that the formation of a perianal fistula may be related to genetic predisposition, trauma, colitis, retention of the foreign body, poor conformation of the perianal region, autoimmune disease, a large number of sweat glands around the anus and/or keeping the tail low.

Clinical signs include tenesmus, self-mutilation, dyskinesia, constipation, weight loss, lethargy, increased frequency of defecation, pain on examination of the tail and perianal region, and perianal licking (Ellison, 1995; Mathews, 1997). The lesions can vary in appearance from superficial fistulas to large ulcers, sometimes extending deep into the perianal region.

Medical treatment consists of local cleansing and antiseptics, anti-inflammatories, and painkillers, although these are associated, at best, with only a temporary improvement in clinical signs. However, a surgical approach consisting of removing the affected tissue as well as the entire epithelial lining, amputation of the tail, cryosurgery, and chemical cauterization are also options in veterinary medicine.

Until the mid-1990s, surgical interventions were the main and often the only treatment for perianal fistula in dogs, since the use of antibiotics alone did not result in satisfactory healing of the perianal sinuses in dogs (Ellison, 1995). In 1997, Mathews and colleagues reported the results of oral administration of cyclosporine A (CsA) in dogs affected by perianal fistula (Mathews, 1997). In a controlled clinical trial involving twenty dogs, it was demonstrated that the treatment of canine perianal fistula with CsA is highly effective since all the dogs treated with cyclosporine and none of the dogs treated with placebo improved after 4 weeks. The data showed that the healing rate of animals treated with CsA over a period of sixteen weeks was 85 percent (Mathews, 1997). Subsequent studies have proven the efficacy of CsA, which has been used to treat dogs affected by perianal fistula (Doust, 2003; Hardie, 2005; House, 2006). On the other hand, apart from the high cost, CsA tends to cause undesirable effects, such as the formation of gingival hyperplasia and hair change with hypertrichosis. Gingival hyperplasia is due to the inhibition of collagen degradation and is reversible after reducing or changing the medication, while hair loss with hypertrichosis occurs because cyclosporine stimulates the keratinocytes in the hair follicles (Hardie, 2005; Elkins, 2008; Sancho, 2009). Other drugs such as ketoconazole, prednisone, azathioprine, and tacrolimus have also been used in veterinary medicine, although they all have side effects (Ettinger, 2004; Elkins, 2008; Stanley, 2009; Pieper, 2011).

Multipotent adult progenitor cells (MAPCs), also known in the scientific literature as mesenchymal stem cells, are spindle-shaped, long, flattened cells that adhere to the polymeric surfaces of tissue culture bottles/plates, capable of expressing specific surface markers, with the potential for proliferation, self-renewal (the ability to multiply generating cells equal to the original cell) and to differentiate into multilineage cells (Santos, 2018a). One of the characteristics of MAPCs is their ability to generate colonies when plated at low densities, growing from individual foci or colonies from a microscopic point of view, which have been called colony-forming units (Berger, 2020). MAPCs can be isolated from a variety of tissues, although the most commonly used sources for therapeutic purposes in veterinary medicine are bone marrow, adipose tissue, and umbilical vein/umbilical cord blood.

Although they were originally used clinically in the hope of harnessing their differentiation and proliferation potential, we know that MAPCs act in the tissue repair process in a paracrine way by modulating the immune response, angiogenesis, apoptosis, fibrosis, oxidation levels, migration, and/or stimulation of surrounding cells. Although they were originally used clinically in the hope of harnessing their differentiation and proliferation potential, we know that MAPCs act in the tissue repair process in a paracrine way by modulating the immune response, angiogenesis, apoptosis, fibrosis, oxidation levels, migration, and/or stimulation of surrounding cells.

Stem cell therapy in veterinary medicine has been applied in the treatment of various clinical conditions that affect both small and large animals (Santos, 2023a). These include neurological sequelae of distemper (Santos, 2019a), feline gingivitis stomatitis (Assis, 2017), inflammatory bowel lesions (Cristóbal, 2021), spinal cord aplasia (Santos, 2023b), eye lesions (Villatoro, 2015), kidney disease (Santos, 2020), dermatological lesions (Villatoro, 2018), tendon lesions (Smith, 2005), laminitis (Mendes, 2021), osteoarthritis (Black, 2007), spinal cord trauma (Mukhamedshina, 2019), feline asthma (Santos, 2024a) and canine bronchitis (Santos, 2024b). Currently, several companies around the world are making this new therapeutic approach available, including VetStem (United States), Stem Cell Vet (England), and CELLTROVET (Brazil), which shows that stem cell therapy is already a reality in the veterinary medical market.

In a study carried out by Ferrer et al., the effect of local stem cell transplantation in naturally affected dogs was reported. In this study, stem cells were injected around the perianal sinuses in six dogs affected by perianal fistula. The procedure was well tolerated and all the dogs were completely free of fistulae three months after the injection. Concomitant pharmacological treatment was maintained for at least thirty days after cell transplantation (Ferrer, 2016).

This study aims to determine the possible safety and therapeutic efficacy of allogeneic MAPCs derived from dog adipose tissue in the treatment of dogs affected by perianal fistula, to improve the quality of life of patients.

2. Methodology

Selection of adipose tissue donor animals

The five adipose tissue donor dogs were selected from patient populations at veterinary clinics in the city of São Paulo. The tissue was collected after the owners signed an informed consent form. The MAPCs used in this study were isolated from clinically and laboratory-healthy dogs up to six months old.

Selection of animals for the study

Twelve dogs were included in the study (two Labrador Retrievers, two German Shepherds, one Poodle, three Golden Retrievers, two Border Collies, and two SRDs), nine intact males and three intact females, ranging in age from 4 years and 3 months to 8 years and 9 months, weighing from 4 kg to 58 kg, vaccinated and wormed, affected by perianal fistula (Table 1). The pre-treatment patients were assessed using a liver profile (ALT and FA); renal function (urea, creatinine, albumin, sodium, potassium, and calcium), hemogram, chest X-ray, and abdominal ultrasound. All the dogs underwent a full physical examination and the number and size of perianal fistulas were recorded. The animals had been partially or completely refractory to oral cyclosporine A treatment for at least six months. Patients affected by other diseases and who had undergone surgery (cryosurgery, anal sac resection, chemical cauterization, or tail amputation) to treat anal fistulas were excluded from the study.

Table 1 - The dogs have been identified in the text as dogs and their respective numbers. The table shows breed, sex, age (Y = years and M = months) and weight.

	DOG 1	DOG 2	DOG 3	DOG 4	DOG 5	DOG 6	DOG 7	DOG 8	DOG 9	DOG 10	DOG 11	DOG 12
Breed	Labrador Retriever	Labrador Retriever	Pastor Alemão	Pastor Alemão	Poodle	Golden Retrievers	Golden Retrievers	Golden Retrievers	Border Collies	Border Collies	SRD	SRD
Sex	Male	Male	Male	Female	Male	Female	Male	Male	Male	Female	Male	Male
Age	4 Y 9 M	5 Y 1 M	7 Y 3 M	4 Y 3 M	7 A 4 M	8 Y 6 M	8 Y 9 M	7 Y 3 M	5 Y 2 M	6 Y 6 M	4 Y 1 M	5 Y 8 M
Weight	32 Kg	36 Kg	32 Kg	28 Kg	4 Kg	30 Kg	32 kg	34 kg	16 Kg	14 Kg	12 kg	10 kg

Source: Authors.

Molecular Analysis

To carry out the molecular analysis using the polymerase chain reaction (PCR) method, blood samples were taken from the patients. The amplification targeted the presence of *Canine herpes virus* (CHV), *Distemper virus* (CDV), *Ehrlichia canis* (ECA), *Babesia spp*. (BAB), *Hepatozoon spp.* (HEP), *Anaplasma platys* (ANP), *Bartonella spp.* (BAR), *Brucella spp*. (BRU), *Borrelia burgdorferi* (BBU), *Rickettsia app*. (RIC), *Leishmaniasis app* (LEI) and *Mycoplasma spp* (MYC). RNAs were extracted with Trizol (Invitrogen) and used for cDNA synthesis by reverse transcription with superscript II (Invitrogen). DNAs were extracted using DNAzol (Invitrogen). Positive reactions showed DNA fragments of 136 bp (HVC), 287 bp (CDV), 959 bp (ECA), bp (HEP), 551 bp (BAB), 574 bp (HEP), 504 bp (ANP), 379 bp (BAR), 280 bp (BRU), 262 bp (BBU), 549 bp (RIC), 120 bp (LEI) and 595 bp (MYC) (Table 2). Two extracted samples, positive and negative controls, were submitted for each study.

Table 2 - Primers used to amplify fragments from the target regions of *Canine herpes virus* (CHV), *Distemper virus* (CDV), *Ehrlichia canis* (ECA), *Babesia spp*. (BAB), *Hepatozoon spp.* (HEP), *Anaplasma platys* (ANP), *Bartonella spp.* (BAR), *Brucella spp.* (BRU), *Borrelia burgdorferi (*BBU), *Rickettsia app.* (RIC), *Leishmaniasis app* (LEI), and *Mycoplasma spp* (MYC).

	PRIMER FORWARD	PRIMER REVERSE	APLIFIED FRAGMENT	TARGET REGION
HVC	5' - ACAGAGGTTGATAGAAGAGGTATG - 3'	5' - CTGGTGTATTAAACTTTGAAGGCTTTA - 3'	136 pb	Glycoprotein B
CVD	5' - ACA GGA TTG CTG AGG ACC TAT - 3'	5' - CAA GAT AAC CAT GTA CGG TGC - 3'	287 pb	Nucleoprotein
ECA	5'- CATTATCATTTCAATACGTAACTC - 3'	5' - TTTTGATTTTCTTCTGACATAGTG - 3'	959 pb	16S rRNA
BAB	5' - CCGTGCTAATTGTAGGGCTAATACA - 3'	5' - GCTTGAAACACTCTARTTTTCTCAAAG - 3'	551 pb	18S rRNA
HEP	5' - GGTAATTCTAGAGCTAATACATGAGC - 3'	5' - ACAATAAAGTAAAAAACAYTTCAAAG - 3'	574 pb	18S Srna
ANP	5' - AAGTCGAACGGATTTTTGTC - 3'	5' - CTCTCCCGGACTCTAGTC - 3'	504 pb	16S rRNA
BAR	5' - GGGGACCAGCTCATGGtGG - 3'	5' - AATGCAAAAAGAACAGTAAACA - 3'	379 pb	*gltA*
BRU	5' - GTCGCGGATTCTACCTCACCT - 3'	5' - TAAGCAGGTAAGAGGCAATTT - 3'	280 pb	*virB2*
BBU	5′ - CCCCACCCAATTATACTAGC - 3′	5′ - GTCGCGTCACAAACATTAAG - 3′	262 pb	*flgE*
RIC	5' - CAGGGTCTTCGTGCATTTCTT - 3'	5' - GCTCTTCTCATCCTATGGCTATTAT - 3'	549 pb	*gltA*
LEI	5´ - GTG GGG GAG GGG CGT TCT - 3´	5´ - ATT TTA CAC CAA CCC CCA GTT - 3´	120 pb	Kinetoplast minicircle
MYC	5' - ATGTTGCTTAATTCGATAATACACGAAA - 3'	5' - ACRGGATTACTAGTGATTCCAACTTCAA - 3'	595 pb	16S do rRNA

Source: Authors.

Analysis of tumorigenicity

As part of establishing the safety of treatment with MAPCS-TACs, they were assessed for their potential for tumorigenicity. A single dose of $2x10^6$ MAPCs-TAC per 8-week-old nude mouse was injected intravenously. A subgroup was sacrificed 3 months (n = 3) or 6 months (n = 3) after the injection for histopathological assessment, and no evidence of masses or teratomas was found at either time point (Santos, 2019b).

Isolation and characterization of MAPCs-TACs

The adipose tissue obtained at the time of castration was sent to the CELLTROVET Laboratory where it was washed in PBS 1x (phosphate-buffered saline) to remove blood and debris. After washing, the tissue was kept for 30 minutes at 37ºC, 5% CO_2 in the presence of 0.075% collagenase type IV (Sigma-Aldrich®). 5 mL of basal medium was added, and the supernatant was removed and centrifuged for 5 minutes at 200xg. The precipitate was suspended and transferred to a 25 cm^2 culture bottle which was kept at 37ºC, 5% CO_2 for 48 hours in the presence of basal medium, when it was changed. Subsequent repiques were carried out by enzymatic action using 0.025% trypsin (Invitrogen®). The MAPCs-TAC were divided into $2x10^6$ aliquots, suspended in freezing medium (10% DMSO, 70% fetal bovine serum, and 20% basal medium), and stored in liquid nitrogen. The MAPCs-TAC were administered with less than 1 year of storage. To be applied to the dogs, the cells were thawed and the cryopreservation medium was removed (Santos, 2019b).

For the proliferative analysis, a colony of expanded MAPCs-TAC was isolated until it reached 70% confluence in a 25 cm^2 plate. The cells were removed by enzymatic action (0.025% trypsin, Invitrogen®) and distributed in triplicates on 60 cm^2 plates at a concentration of 10^5 cells. After 48 hours of cultivation, the cells were removed and replaced. The process was repeated until the 12^{th} passage. Cell viability was determined using trypan blue analysis, showing a rate of 96% viable cells (Santos, 2019b).

The osteogenic potential of MAPCs-TAC was demonstrated using *Von Kossa* staining after the cells had been kept in culture for 21 days in the presence of osteogenic differentiation medium ((Dulbecco's Modified Eagle's Medium - Low Glucose, Invitrogen®), 1% 10^{-5} M dexamethasone (Sigma-Aldrich®), 1% 5 mM ascorbic acid (Sigma-Aldrich®), 10% fetal bovine serum (HyCloneTM) and 1% penicillin-streptomycin (penicillin G 10.000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®). The medium was changed every 3 or 4 days. On the 10^{th} day, 1% of 200 mM β-glycerolphosphate (Sigma-Aldrich®) was added. After 21 days, the cells were washed twice with PBS and fixed for 24 hours in 4% paraformaldehyde at 4°C. *Von Kossa* staining was carried out the following day. For this, the cells were stained with 5% silver nitrate (Scytek, Logan, UT, USA) for 60 min with exposure to ultraviolet light, followed by 5% sodium thiosulphate (Scytek, Logan, UT, USA) for 2~3 min and then exposed to Nuclear Red stain (Scytek, Logan, UT, USA) for 5 min (Santos, 2019b).

To analyze the adipogenic differentiation potential, the MAPCs-TACs were cultured in adipogenic differentiation medium ((Dulbecco's Modified Eagle's Medium - Low Glucose, Invitrogen®), 10% fetal bovine serum (HyCloneTM), 1 mM dexamethasone (Sigma-Aldrich®), 100 mM indomethacin (Sigma-Aldrich®), 0.5 M isobutylmethylxanthine (Sigma-Aldrich®) + 10 µM insulin (Sigma-Aldrich®), and 1% penicillin-streptomycin (penicillin G 10. 000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®)), for 21 days. After three weeks, the cells were washed twice with PBS and fixed for 24 hours in 4% paraformaldehyde at 4°C. The following day, the cells were stained with *Oil Red O* (Sigma-Aldrich®) (Santos, 2019b).

The potential for chondrogenic differentiation was demonstrated using toluidine blue (Sigma-Aldrich®) staining after the MAPCs-TAC had been cultured for 21 days in the presence of chondrogenic differentiation medium ((Dulbecco's Modified Eagle's Medium - High Glucose, Invitrogen®) supplemented with 1% fetal bovine serum (HyCloneTM), 6.25 mM insulin (Sigma-Aldrich®), 0.1 mM dexamethasone (Sigma-Aldrich®), 1 mM sodium pyruvate (Invitrogen®), 10 ng/mL TGF-β1 (R&D System, LGC Biotechnology), and 1% penicillin-streptomycin (penicillin G 10. 000 IU/mL, streptomycin 10,000 µg/mL, Invitrogen®)). On day 21, the cell aggregate was fixed in 10% formaldehyde for 1 hour at room temperature, dehydrated in serial dilutions of ethyl alcohol (70%, 95%, and 100%), diaphanized in xylene and infiltrated with liquid paraffin. The paraffin blocks were sectioned using a microtome (4µm) and the slides were stained with *toluidine blue* (Santos, 2019b).

Transplantation of MAPCs-TAC

The patients underwent three transplants with the MAPCs-TAC with an average interval of 30 days between each procedure, using a dose of 6 x 10^6 MAPCs-TAC. The MAPCs-TAC were thawed in a water bath at 37°C for 2 minutes and transferred to a 15 ml falcon tube, adding physiological solution in a 1:1 ratio. The cell concentrate was homogenized and centrifuged at 210xg for 5 minutes at room temperature. The supernatant was discarded and 3 ml of saline solution was added. The cell precipitate was resuspended and homogenized, then centrifuged at 210xg for 5 minutes at room temperature. This procedure was repeated 2 more times. The MAPCs-TAC2 cells were then resuspended in 5 ml of saline solution and homogenized so that they could be resuspended in saline solution and transplanted intralesionally under local sedation (Dexmedetomidine Hydrochloride (5 µg/kg/IV)). The cells were injected with a 21 g needle around the perianal fistulas, and the area was previously drained. The viability of the MAPCs-TACs was analyzed after thawing using Triplan Blue staining, which showed a viability value of over 90%.

Monitoring of animals submitted to cell transplantation with the MAPCs-TAC strain

The dogs treated in this study underwent clinical and laboratory examinations. The tests were carried out on days 0, 15, 30, 45, 60, and 90, as well as 180 days after the third transplant. The dogs were thoroughly examined and the number, extent, and depth of the fistulas were recorded. A full blood count and serum chemistry analysis were carried out at each time point. The procedure was approved by the CELLTROVET Animal Use Ethics Committee under number 5/2022.

3. Results and Discussion

Escherichia coli, *Staphylococcus aureus* and β hemolytic Streptococcus are the bacteria most commonly found in canine perianal fistula cultures. Antibiotic treatment needs to be carried out according to the results of the antibiogram, with metronidazole, cephalexin, and amoxicillin with clavulanate being the most commonly used. Cyclosporine has been much discussed in the treatment of perianal fistulas, but one of the known forms of the disease, fistulous (small, deep wounds) does not respond very well to it. Another issue is that the cost of cyclosporine is high and it is better absorbed during fasting, causing some undesirable gastrointestinal damage, among other harms (Harvey, Horton, 2023; Asai, Sturion, 2014).

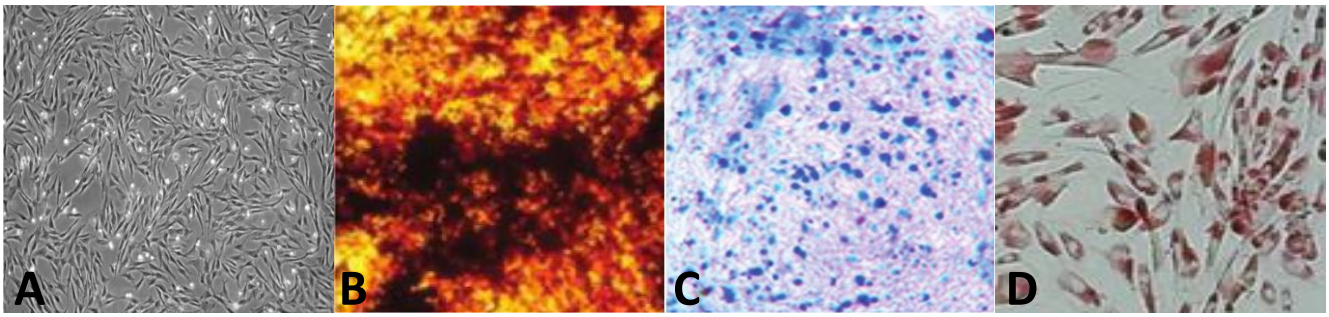
In recent years, stem cell therapy has gained increasing prominence in veterinary medicine as it has been used safely and effectively in the treatment of various diseases affecting small and large animals. This new therapeutic approach was first reported by Smith et al. when they used stem cells obtained from bone marrow to recover horses affected by tendinopathy. The stem cells were infused, guided by ultrasound, into the injured region of the superficial digital flexor tendon. After the procedure, the horses were kept on a regime of controlled upward exercise. Periodic ultrasound evaluations were carried out and showed rapid filling of the lesion (Smith, 2005). Two years later, Black and colleagues published the first randomized, double-blind, placebo-controlled clinical trial reporting the efficacy of stem cell therapy in dogs. This study assessed improvement in lameness, pain, and range of motion scores compared to control dogs. Stem cells were transplanted via the intra-articular route resulting in a significant improvement in lameness, reduced discomfort, and increased functional capacity of the patient compared to the control group (Black, 2007).

Five MAPCs-TAC strains were isolated from the adipose tissue of five young, healthy dogs and named MAPCs-TAC1, MAPCs-TAC2, MAPCs-TAC3, MAPCs-TAC4, and MAPCs-TAC5. The strains were characterized based on their ability to adhere to the plastic surface of the cell culture bottle, fibroblastoid morphology (Figure 1A), osteogenic differentiation capacity evidenced by the presence of calcium deposits in the differentiated osteocytes (Figure 1B), chondrogenic, shown by the increased deposition of cellular matrix produced by the differentiated chondrocytes (Figure 1C)

and adipogenic, shown by the presence of lipid vacuoles characteristic of adipogenic cells (Figure 1D), as well as the potential for cell proliferation (Figure 2). In this analysis, exponential cell growth was detected until the 9th passage, when a process of decay began in the proliferative analysis curve. In the in vivo study, carried out on nude mice, $2x10^4$ cells were infused into the animals which were sacrificed 3 months (n = 3) or 6 months (n = 3) after the injection for histopathological assessment. No evidence of masses or teratomas was found in the animals at either time point, suggesting that MAPCs-TAC are safe for in vivo transplantation.

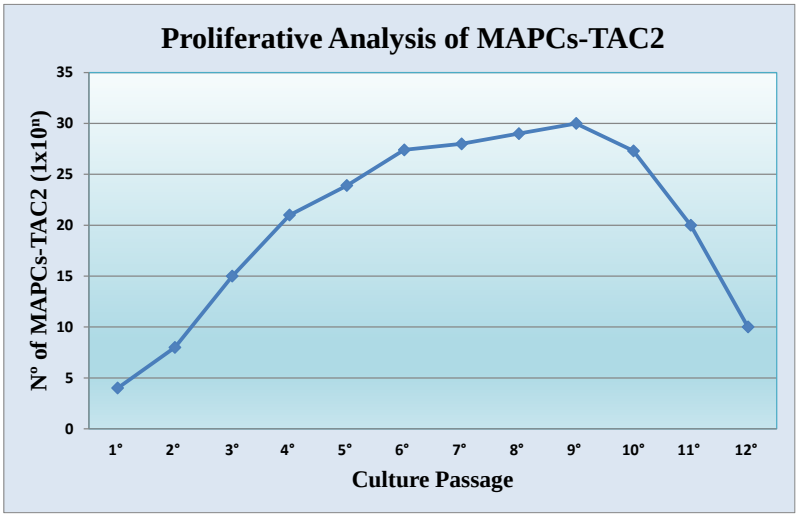
As this was an allogeneic treatment, the adipose tissue donor animals were tested for positive reactions to DNA fragments of 136 bp (HVC), 287 bp (CDV), 959 bp (ECA), bp (HEP), 551 bp (BAB), 574 bp (HEP), 504 bp (ANP), 379 bp (BAR), 280 bp (BRU), 262 bp (BBU), 549 bp (RIC), 120 bp (LEI) and 595 bp (MYC), and the presence of the pathogens was not detected. This prevents their possible transmission to recipient animals (Santos, 2019).

Figure 1 - Stem cells isolated from canine adipose tissue (MAPCs-TAC) show a fusiform, long, flat, and fibroblastoid morphological appearance, demonstrating the ability to adhere to the polymer surface of the cell culture bottle (A). Representative images of the *in vitro* differentiation tests carried out on MAPCs-TAC after being exposed to osteogenic, adipogenic, and chondrogenic differentiation media using *Von Kossa*, *Triplan Blue*, and *Oil Red O* stains, respectively. 10x (A), 20x (B, C) and 40x (D) objectives.



Source: Authors.

Figure 2 - Representative graph analyzing the proliferation potential of MAPCs-TAC2. The rate of cell growth was assessed at 72-hour intervals between replays using enzymatic action.



Source: Authors.

Although no comparative analysis was carried out with a control group (placebo) to assess the effectiveness of the therapy, the results demonstrate the therapeutic potential of MAPCs-TAC2 in the treatment of perianal fistula. The patients selected for the study were clinically analyzed for symptoms of dyschezia, constipation, tenesmus, weight loss, lethargy, self-mutilation, increased frequency of defecation, pain on examination of the tail and perianal region, and perianal licking (Ellison, 1995; Mathews, 1997). Patients affected by neoplasm, infection, hypertension, or hypotension were excluded from the study.

The dogs were sedated and the MAPCs-TAC2 strain was transplanted locally to test its safety and therapeutic efficacy. The dogs had active perianal fistulas and were being treated with CsA. The perianal fistula area was previously drained to remove the purulent secretion (pus), which had an extremely foul odor (Figure 3). No complications were detected during the procedure and no side effects such as nausea, vomiting, or changes in blood pressure were observed after the procedure, demonstrating that the transplant was well tolerated by the twelve dogs. The transplants were carried out on days zero, thirty, and sixty, followed by fortnightly monitoring of the patients. Follow-up did not reveal any pathology associated with the formation of abnormal tissue. Previous studies have shown that allogeneic MAPCs transplants are safe in terms of possible rejection by the recipient animal and do not require the use of immunosuppressive drugs (Santos et al., 2019a; Santos, 2019b; Santos, 2023b; Santos, 2024a; Santos 2024b).

Figure 3 - Purulent and fetid secretion was removed from the patient's perianal fistula before the MAPCs-TAC2 transplant procedure.

Source: Authors.

The first analysis of the patients was carried out 15 days after the first transplant of the MAPCs-TAC2. The clinical assessment showed that all the animals had improved appetite, were in a better mood, and had a marked improvement in constipation, although they still had a significant amount of fecal secretion. In the assessment carried out on day 30, the date of the second transplant of the MAPCs-TAC2, the patients continued to show improvements in appetite, mood, and constipation, with dogs 1, 4, and 11 also showing an improvement in the amount of secretion.

In the analysis carried out a fortnight after the second transplant of MAPCs-TAC2, the dogs maintained a stable picture of improvement, with dogs 1, 4, and 11 continuing to show improvement in the amount of secretion released. Dogs 2, 9, and 11 began to show a reduction in the amount of secretion. In the assessment carried out thirty days after the second transplant of the MAPCs-TAC2, the date on which the third transplant was carried out, the dogs continued to show improvement, with dogs 1, 4, and 11 releasing practically no secretion and all the other patients showing a significant improvement in secretion.

An analysis carried out ninety days after the first transplant showed that all the animals were eating and drinking water normally and were no longer showing constipation or secretions. One hundred and eighty days after the third MAPCs-

TAC2 transplant, the twelve dogs were re-evaluated and found to be clinically healthy, with no recurrence of perianal fistula in any of the patients.

Perianal fistula is a clinical condition characterized by a chronic and progressive inflammatory process that tends to increase in severity, with periodic exacerbations. Spontaneous healing of the perianal fistula is an extremely uncommon event, which makes conventional treatment necessary throughout the patient's life (Cain, 2019). The fact that fistulas heal completely after cell transplantation in dogs refractory to standard conventional treatments is a strong indication that MAPCs-TAC2 exert a healing process through immune modulation and/or tissue repair.

4. Final Considerations

The data obtained in this study, by transplanting the allogeneic MAPCS-TAC2 lineage, demonstrated the safety and therapeutic efficacy in the treatment of twelve dogs affected by perianal fistula. The results showed that cell transplantation was effective regardless of the breed, sex, or age of the dogs. This study, although preliminary, should encourage further research into the therapeutic potential of MAPCs in the treatment of perianal fistula, to improve the quality of life of animals.

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