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THERAPEUTIC USE OF MESENCHYMAL STEM CELLS MAY BE A MEANS OF TRANSMITTING LEISHMANIASIS IN DOGS

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ABSTRACT

The aim of this case report is to record the finding of leishmaniasis-causing parasites in mesenchymal stem cells from dogs from a non-endemic area, which were negative when submitted to the IFAT and ELISA tests, a fact that had not been documented until now. This demonstrates that this widely distributed and lethal zoonosis can escape traditional means of diagnosis and that stem cell therapy has the potential to be a source of transmission of the disease. Although the relative safety of stem cell therapy is presumed, the possible risks associated with its use, especially the risk of parasite transmission, cannot be ignored. It is therefore essential to develop rigorous screening and testing protocols to ensure the safety of stem cell therapy.

Keywords: Leishmaniasis. Cellular Therapy. Zoonosis.

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INTRODUCTION

Leishmaniasis is one of the neglected tropical diseases of greatest medical and veterinary importance and is a public health problem in several countries [1,2]. According to data published by the World Health Organization, 94% of new cases have occurred in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. These countries share problems such as frequent epidemics, humans and animals acting as reservoirs and the presence of the transmitting flies [3].

The disease is transmitted through the bite of sand flies of the genera Phlebotomus and Lutzomyia. It can manifest itself in cutaneous or visceral form, depending on which cell type is infected and if left untreated, the mortality rate in developing countries can reach 100% in 2 years [2,3].

Mesenchymal stem cells (MSCs) are multipotent cells that can be isolated from different tissues such as bone marrow [4], umbilical cord, adipose tissue [5], peripheral blood, tooth root and liver [6]. Stem cell therapy has emerged as a promising approach for treating a wide range of diseases and injuries [7,8]. Stem cells have the ability to differentiate into various types of cells and can be used to replace or repair damaged tissue [9]. However, the safety of stem cell therapy has been a major concern due to the possible risks associated with its use, including the possibility of introducing parasites into the host [10].

Stem cells have recently been shown to function as a protective niche for pathogens, such as Mycobacterium tuberculosis [11]. Studies suggest that these cells protect intracellular pathogens from the action of the immune system and from the action of drugs, as they reside in immunoprivileged sites in the bone marrow and mesenchymal stem cells do not trigger effector functions of cytotoxic T lymphocytes as they do not have major histocompatibility complex (MHC) II and their MHC I molecules are functionally inactive [12,13,14].

Leishmania spp. has developed survival mechanisms such as modulating the activities of macrophage phagolysosomes [15], neutrophils, increasing their lifespan in tissues and delaying their apoptosis, thus benefiting the multiplication of Leishmania spp., and even the mechanism known as the "trojan horse" [16]. In vitro studies have reported the infection of mesenchymal stem cells by different species of Leishmania, which may suggest a mechanism used by Leishmania spp. to evade the immune system, present asymptomatic patients, and reactivate the disease [17].

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Studies have shown that Leishmania spp. parasites are capable of infecting bone marrow-derived mesenchymal stem cells and altering their differentiation potential, leading to the development of an immunosuppressive microenvironment that supports the parasite's survival [18,19]. Another study reported the presence of Leishmania spp. parasites in umbilical cord derived MSCs, suggesting that the parasites may be present in the stem cell preparation itself [20].

The main objective of this case report is to document the unprecedented accidental isolation of amastigote forms of Leishmania spp. in mesenchymal stem cell cultures from dogs from a region not endemic for the disease. It is noteworthy that this is the first report of its kind in the world, and its purpose is to raise awareness of the need to establish strict selection and screening criteria for stem cell donors.

MATERIALS AND METHODS

Three healthy male dogs of mixed breed, two years old and with an average body mass of 18 ± 2 kg, were received at the Small Animal Experimentation Center of the Universidade Federal de Minas Gerais (UFMG) Veterinary School. The dogs were not client owned; they had been made available by the research institution to be used in another high-level study. The animals underwent a clinical examination and blood samples were taken for a blood count and serology to diagnose Leishmaniasis using the Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Immunofluorescence (IFAT) methods. No changes were observed in the clinical examinations or in the blood count, and the ELISA and IFAT results were negative for all three animals. The following week, the dogs underwent adipose tissue biopsy to isolate and culture mesenchymal stem cells. The right gluteal region was trichotomized and prepared for aseptic surgery. The cephalic vein was catheterized, and the animals were given propofol (Fresofol, Fresenius Kabi, Brazil) 3mg/kg, intravenous (IV) for intubation and anesthetic maintenance. As an analgesic and anti-inflammatory, meloxicam (Maxicam, Ouro Fino, Brazil) 0.2mg/kg, intramuscular (IM) was administered immediately after induction of anesthesia.

The adipose tissue samples were taken surgically from the subcutaneous gluteal region just above the greater trochanter. Approximately 1cm3 of adipose tissue was taken from each animal, totaling 3cm³ of the final sample. Immediately after collection, the adipose tissue samples were sent to the Stem Cell Center of the UFMG Veterinary

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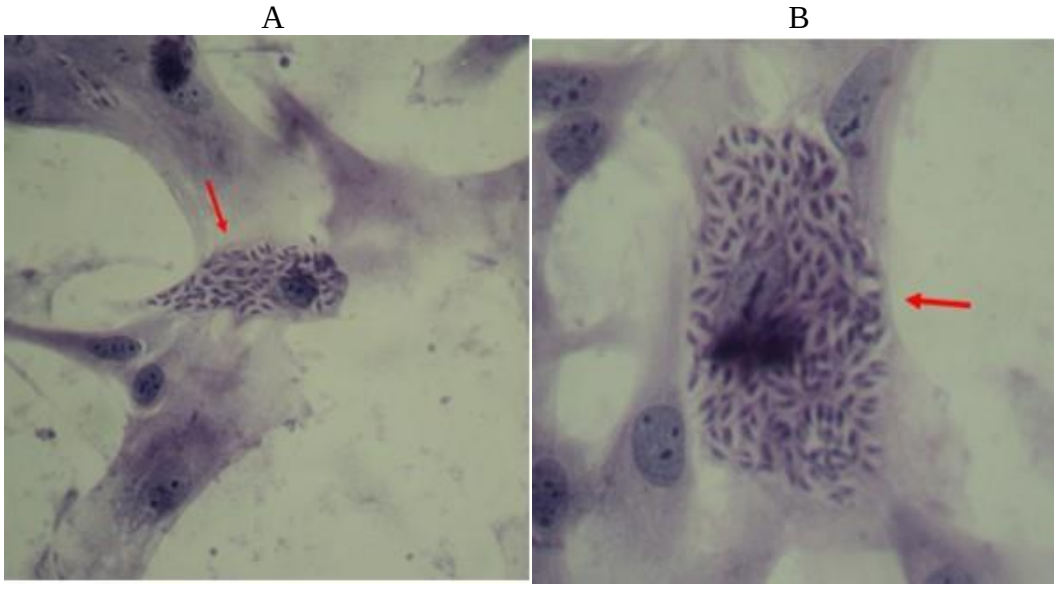
School for isolation and cultivation of Mesenchymal Stem Cells (MSCs) according to the established protocol described below [9]. The adipose tissue samples were washed with 0.15 molar phosphate-buffered saline (PBS) and subjected to a digestion protocol using a 0.1% mass/volume (M/V) collagenase B solution (Roche Applied Science, Germany). After processing, the stromal fraction was cultured in T75 bottles kept in an oven at 37 Cº and 5% CO2 with DMEM (Gibco, USA), enriched with gentamicin (60 µg/L), penicillin 100 UI/mL, streptomycin 100 µg/mL, amphotericin 25 µg/mL (PSA, Sigma-Aldrich, USA) and 10% fetal bovine serum (Sorali, Brazil). The culture medium was changed every 4 days and when cell confluence reached 80 - 90%, the cells were repotted into other T75 bottles. In the fourth passage, the cells were subjected to phenotypic characterization by flow cytometry to assess the expression of CD90, CD29, CD45 and CD34. Low expression of hematopoietic cell markers CD45 (1.54%) and CD34 (0.88%) and high expression of stem cell markers CD90 (60.94%) and CD29 (77.08%) were observed. For morphological assessment, the cells were plated at a density of 1x104 cells/cm2 in six-well plates (Techno Plastic Products in Trasadingen, Germany) containing sterile coverslips (Coverslips, Sarstedt, USA). After seven days of culture at 37o C and 5% CO2, the coverslips containing the MSC were fixed with 70% alcohol, stained with hematoxylin and eosin, and evaluated under light microscopy.

RESULTS

Fig. 1 Photomicrograph of adipose-derived mesenchymal stem cells from dogs infected with Leishmania spp. Note the cells' cytoplasm filled with amastigotes. Images "A and B" Hematoxylin-Eosin (H&E) staining, 40x 60x objective.



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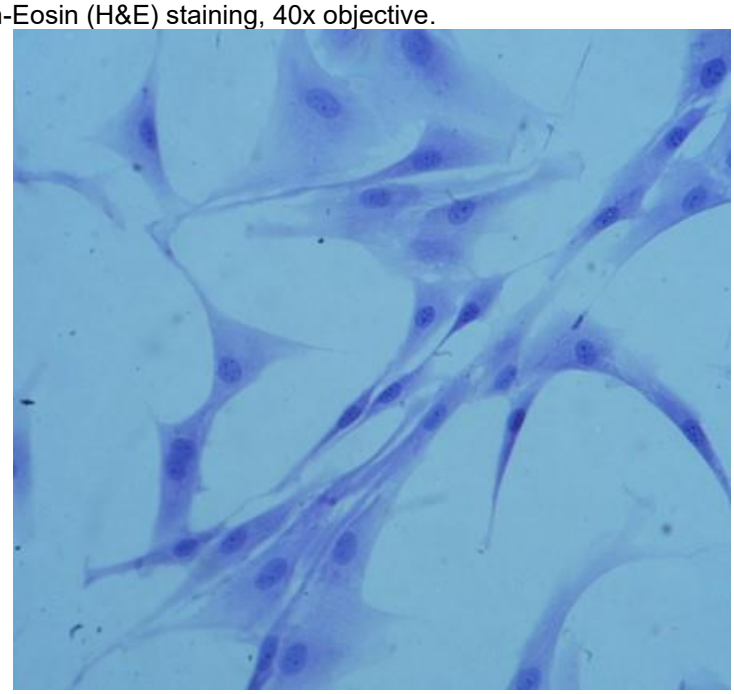
Unexpectedly, amastigote forms of Leishmania spp. were observed inside the cells (Fig. 1). All the cells were discarded, and the three dogs were retested by IFAT and ELISA and this time two animals were positive in both tests, and one was negative. The animals were also positive in the parasitological examination of the bone marrow. The two positive dogs were euthanized with an anesthetic overdose (Thiopental sodium 100 mg/Kg IV) followed by the application of potassium chloride (Potassium chloride solution 19.1% 20 ml IV).

These samples were meant for a study [9] into the isolation and culture of mesenchymal stem cells derived from adipose tissue and bone marrow of dogs, thus the aim was to obtain uncontaminated samples of MSCs (Fig 2).

Fig. 2 Photomicrograph of adipose-derived mesenchymal stem cells from healthy dogs. Note the cells' clear cytoplasm. Hematoxylin-Eosin (H&E) staining, 40x objective.



DISCUSSION

To the authors knowledge, this is the first documented report of the accidental isolation of Leishmania in dog mesenchymal stem cell (MSC) cultures. This unprecedented finding highlights the urgent need to establish rigorous standards in the selection of MSC donors. Once inoculated into the host, Leishmania spp. invades any cell of the mononuclear phagocytic system [21]. However, other cells have already been reported to be susceptible to Leishmania spp. Such as amniotic epithelial cells,

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fibroblasts [22], hepatocytes [23] and mesenchymal stem cells derived from adipose tissue [17].

The findings of this report highlight the limitations of laboratory tests for detecting leishmaniasis in dogs, especially recent infections. The use of serological tests such as indirect immunofluorescence (IFI) and enzyme-linked immunosorbent assay (ELISA) was not effective in detecting Leishmania spp. in the animals in this report. Although these tests are recommended by the Ministério da Agricultura, Pecuária e Abastecimento - MAPA (the Brazilian regulatory body) as screening tests, they were probably carried out at a time when the animals had not yet seroconverted. It is expected that during canine visceral leishmaniasis (CVL), symptomatic dogs and those with high parasitism are associated with an increase in immunoglobulins IgG, IgG2, IgM, IgA and IgE, while asymptomatic dogs and those with low parasitism are associated with an increase in IgG1 [24]. The chronic course of the disease, the delay in seroconversion, cross-reactions in the tests are all known mechanisms that hinder diagnosis and can result in the disease being detected several weeks after infection [24,25].

The parasitological method is highly specific and the identification of just one amastigote form is enough to determine the test as positive. The serological diagnostic methods of enzyme-linked immunosorbent assay and indirect immunofluorescence reaction are recommended by the MAPA, but they can cross react and do not differentiate between current and past infections. Even so, the dogs in the study were able to evade these diagnostic methods at the time of the first test. Currently, PCR-based techniques are the main diagnostic approach due to their high sensitivity, as well as allowing the assessment of the parasite load and the identification of the Leishmania species [26].

As an alternative to improve the chances of identifying a dog infected with Leishmania spp., the MAPA highlights the use of Polymerase Chain Reaction (PCR) tests. PCR is a molecular technique capable of detecting Leishmania spp. DNA in blood or tissue samples, providing higher specificity and confirming the infection. Additionally, the culture of Leishmania spp. from tissue samples, such as bone marrow or skin, can also be performed to validate the presence of the infection. Biopsies of affected tissues, such as the skin, can be taken to confirm the presence of the parasite. However, there are indications that for more effective screening there are tests that can

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detect parasite infection earlier, such as ELISAp (ELISA with promastigote-coated plates) and especially Western blotting [25].

One of the main challenges of stem cell therapy is ensuring its safety. Although stem cells have shown remarkable therapeutic potential in preclinical studies, their use in humans remains largely experimental.

Concerns about the safety of stem cell therapy include the risk of tumorigenesis, immunogenicity, and the possibility of transmission of infectious diseases, including parasites. These risks are particularly relevant in the context of allogeneic stem cell transplantation, in which stem cells from a donor are used to treat a patient [27].

Stem cell therapy is gaining popularity as a possible treatment for various conditions in dogs, including osteoarthritis, autoimmune diseases, and spinal cord injuries [8,10,28,29]. The use of autologous mesenchymal stem cells (MSCs) has been shown to be safe and effective in reducing pain and inflammation, improving mobility and quality of life, and promoting tissue repair in dogs [28]. However, stem cell therapy is not without its risks. One potential risk is the development of tumors or other adverse effects resulting from the uncontrolled growth or differentiation of transplanted cells [29]. Another risk is the possible transmission of infectious diseases, including viruses and bacteria, using contaminated stem cell preparations [17].

The application of amphotericin cannot be considered a limitation of the study because even though the drug has a known anti-leishmanial effect, the dose used is considered lethal to only 50% of the Leishmania spp. parasites [30], so it was not able to eliminate all the parasites of the species in the samples.

Despite the risks, the use of stem cells in veterinary medicine has intensified and in some places is even available to the public. Examples include the Animal Medical Center in New York City, USA, which offers stem cell therapy for a variety of diseases in dogs and cats, including osteoarthritis, intervertebral disc disease and chronic kidney disease; the Veterinary Specialty Hospital of the Carolinas in North Carolina, USA and the Veterinary Regenerative Medicine Center in Ontario, Canada.

This study has limitations such as the lack of identification of the species of Leishmania that was isolated along with the mesenchymal stem cells, as well as the absence of more comprehensive details about the parasite. In retrospect, it is crucial to point out that this incident occurred unexpectedly during a previous study. At the time, all the material was discarded since the finding of any parasite made it inappropriate for

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the study which it was initially acquired for. It was only many years later that we realized the importance of what had happened, when it was no longer possible to carry out additional tests to identify more in-depth details about the parasite.

CONCLUSION

The finding of amastigote forms of Leishmania spp. in these dogs again raises questions about the presumed safety of stem cell therapy. Therefore, there is an urgent need to develop standardized protocols for the characterization and quality control of stem cells used in clinical practice, as well as to establish clear guidelines for conducting clinical studies that evaluate the safety and efficacy of stem cell therapy. These efforts will be key to ensuring that stem cell therapy can be used safely and effectively to improve the lives of patients with various diseases and injuries.

AUTHOR CONTRIBUTIONS

All authors contributed equally to all parts of the project. All authors have read and agreed to the published version of the manuscript.

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· Conflict of interest/Competing interests: The authors declare that they have no conflict of interest, are scholars of stem cell therapy and are in favor of its clinical use if it is judicious and within what the legislation allows.

· Ethics approval and consent to participate: The findings of this work were observed during the conduct of the study: CAMARA, B.O.S.; OCARINO, N.M.; BERTASSOLI, B.M.;

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· The dogs were not client owned; they had been made available by the research institution to be used in another high-level study.

· Consent for publication: All authors have read and agreed to the published version of the manuscript.

· Data Availability Statement: Data sharing not applicable to this article as no datasets were generated or analyzed during the current study. However, it is important to note that the aim of this article is to register an undocumented accidental finding which occurred while conducting another work was being conducted. The findings of this work were observed during the conduct of the study: CAMARA, B.O.S.; OCARINO, N.M.; BERTASSOLI, B.M.; MALM, C. ; ARAÚJO, F.R. ; REIS, A.M.S. ; JORGE, E.C. ; ALVES, E.G.L. ; SERAKIDES, R. . Differentiation of canine adipose mesenchymal stem cells into insulin-producing cells: comparison of different culture medium compositions. DOMESTIC ANIMAL ENDOCRINOLOGY, v. 74, p. 106572, 2021. Available at https://www.sciencedirect.com/science/article/abs/pii/S0739724020301399?via%3Dihub

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· Code availability: Not applicable

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