

Adipose Tissue-Derived Mesenchymal Stem Cells as a New Host Cell in Latent Leishmaniasis

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Abstract. Some protozoan infections such as *Toxoplasma*, *Cryptosporidium*, and *Plasmodium* can be transmitted through stem cell transplantations. To our knowledge, so far, there is no study about transmission of *Leishmania* parasites in stem cell transplantation and interactions between parasites and stem cells *in vitro*. Therefore, the aim of this study was to investigate the interaction between different species of *Leishmania* parasites and adipose tissue-derived mesenchymal stem cells (ADMSCs). ADMSCs have been isolated, cultured, characterized, and infected with different species of *Leishmania* parasites (*L. donovani*, *L. major*, *L. tropica*, and *L. infantum*). Infectivity was examined by Giemsa staining, microculture, and polymerase chain reaction methods. As a result, infectivity of ADMSCs by *Leishmania* parasites has been determined for the first time in this study. According to our findings, it is very important that donors are screened for *Leishmania* parasites before stem cell transplantations in regions where leishmaniasis is endemic.

INTRODUCTION

Although parasitic diseases affect millions of people worldwide, only 5% of more than 340 known infectious diseases have been reported to be detected during transplantation.¹ The fact that several protozoa (*Toxoplasma*, *Cryptosporidium*, and *Plasmodium* types) could also create a risk factor in stem cells transplantations has been reported in several studies.^{2–4} The most important reason that these protozoa could be risk factors is the relapse of the latent infection in the recipient.⁵ Therefore, the detection of these protozoan diseases in donors and recipients within endemic regions before transplantation is crucial. However, in latent infections, it is not always possible to diagnose the existence of a parasite causing the disease with known diagnostic procedures.⁶

Leishmaniasis is one of the most important protozoan diseases that cause latent infections.⁷ In one of our previous studies, it was shown that *Leishmania* infection could be transmitted during pediatric organ transplantations.⁸ However, there is no report that has been published on the existence of *Leishmania* parasites in stem cell transplantations. Distinct from other infections, the lack of clinical evidence related to *Leishmania* infections in stem cell transplantations could be because of the facts that leishmaniasis is difficult to diagnose and that it sometimes leads to a misdiagnosis.⁹ Also, leishmaniasis is often neglected or underestimated from the clinical point of view.¹⁰

After infecting the host, *Leishmania* promastigotes are engulfed with receptor-dependent phagocytosis by skin macrophages. Phagocytosed promastigotes turn into amastigote forms, proliferate, and are released by bursting the host cell. These amastigotes again infect other macrophages and settle in reticuloendothelial system organs such as the spleen, liver, and bone marrow.¹¹

It has been reported that parasites infect not only professional phagocytic cells such as macrophages but also numerous other cell types such as amniotic epithelial cells, human epithelial cells, and fibroblast cells.¹²

Stem cells have the ability of self-renewal and differentiate into a variety of other cell types in a given tissue.¹³ Bone marrow-derived mesenchymal stem cells have become a major stem cell source in both research and therapeutic studies for

years. Mesenchymal stem cells from other sources such as umbilical cord and adipose tissue also gained interest because of several advantages over bone marrow-derived counterparts.¹³ Especially the adipose tissue, mainly because of the ease of the procedure, low morbidity, and increased aspirated volume, has become one of the primary mesenchymal stem cell sources. Recent reports also show that adipose tissue-derived mesenchymal stem cells (ADMSCs), when combined with autologous fat or various biomaterials, can be a novel and effective therapeutic source in stem cell-based therapies.¹⁴ Although increased numbers of reports have been published on the effectiveness of ADMSCs in animal models and several phase III clinical trials, similar disease transmission risks can also be applicable in stem cell transplantations using adipose tissue and its cellular derivatives.¹⁵

Although the phagocytic property of ADMSCs has been reported in one study,¹⁶ to our knowledge, there have not been any studies that report the interaction between ADMSCs and *Leishmania* parasites. For this reason, in this study, the interaction between ADMSCs and the different types of *Leishmania* parasites have been investigated *in vitro*. The infectivity status of mesenchymal stem cells by *Leishmania* parasites has also been tested with different methods.

MATERIALS AND METHODS

Parasite culture. *L. tropica* (MHOM/TR/99/EP39),¹⁷ *L. donovani* (HOM/IN/83/AG83), *L. major* (HOM/IL/81/Friedlin), and *L. infantum* (MCAN/TR/2005/EP126) promastigotes were kindly provided by K. P. Chang (Chicago Medical School, Chicago, IL). Promastigotes were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with L-glutamine (Sigma Chemical Co., St. Louis, MO), 10% fetal calf serum (FCS) (Sigma Chemical Co.), and gentamicin (80 µg/mL) (Sigma Chemical Co.) at 27°C. Cultures were passaged after 4 days incubation.¹⁸ The growth of promastigotes was monitored daily using an inverted microscope (Olympus CKX 41).

Parasite counting. The parasites were counted using a hemocytometer under standard light microscopy with 200× magnification. Before the parasite count, the parasites were fixed with formalin 2% (v/v) in phosphate-buffered saline (PBS) solution.¹⁸

Isolation and cultivation of ADMSCs. ADMSCs were isolated by enzymatic digestion of adipose tissue after lipoaspiration. Human lipoaspirate samples and corresponding written

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consent forms were obtained by three donors undergoing cosmetic surgery procedures. This study was approved by the Institutional Review Board (IRB) committee of Yildiz Technical University Bioengineering Department. After transferring the lipoaspirate samples to the laboratory, tissue pieces were washed three to four times with PBS and suspended in Dulbecco-modified Eagle medium (DMEM) (Mediatech Inc., Manassas, VA) supplemented with Glutamax, antibiotics, and 2 mg/mL collagenase type 2 (Sigma Chemical Co.) pre-warmed to 37°C. The tissue was placed in a water bath at 37°C for 60 minutes, placed in hemolysis buffer for 5 minutes, and centrifuged at 1,200 rpm for 10 minutes. The pellet was harvested into DMEM supplemented with Glutamax, antibiotics (100×), and 10% FBS and incubated in the condition of 5% CO₂ humidity at 37°C. This initial passage of the primary cell culture was referred to as passage 0 (P0).¹⁹

Differentiation and phenotypic characterization of ADMSCs.

For adipogenic differentiation, confluent cultures of primary ADMSCs were induced to undergo adipogenesis by replacing the stromal media with adipocyte induction medium composed of 43.2 mL DMEM, 5 mL FBS, 0.5 mL dexamethasone (Sigma-Aldrich, St. Louis, MO), 0.05 mL insulin (Sigma), 2 mL indomethacin (Sigma), 0.05 mL isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 0.5 mL penicillin/streptomycin (Sigma), and 0.5 mL L-glutamine (Sigma). Cells were maintained in culture for 2 weeks, and media was replaced every 3 days. After incubation, cultures were washed with PBS three times, fixed in a 10% solution of formaldehyde in PBS for at least 1 hour, washed with 60% isopropanol, stained with Oil red O solution (in 60% isopropanol) for 10 minutes followed by repeated washing with water, and destained in 100% isopropanol for 15 minutes.²⁰

Osteogenic and chondrogenic differentiations have been performed according to previously published protocols.^{19,21}

Phenotypic characterization has been performed by flow cytometric analysis using ADMSC-specific cell surface markers. First, cells were cultured in control medium before analysis. Cells were labeled with the following antihuman antibodies: CD34-PE, CD73-PE, CD90-FITC, and CD105-PE (Becton Dickinson, San Jose, CA). More than 10,000 labeled cells were acquired and analyzed using a CellLab Quanta (Beckman Coulter).²²

Infection of ADMSC with *Leishmania* promastigotes.

ADMSCs (25,000/mL) were harvested in a 12-well plate that contains a coverslip in every well. *Leishmania* promastigotes, in stationary phases, were washed two times with PBS by centrifugation at 1,500 rpm for 5 minutes; 2.5×10^5 parasites/mL were added into a 12-well plate containing ADMSCs and incubated in 5% CO₂ humidity at 37°C. After 4 hours, ADMSCs were washed with PBS to move the unattached parasites away. After washing, the culture was incubated in DMEM with 10% FBS and incubated in 5% CO₂ humidity at 37°C.

Giemsa staining. The coverslips were taken out from the wells for Giemsa staining after 24 hours, 7 days, 14 days, 21 days, and 28 days. They were washed two times with PBS and fixed in methyl alcohol for 3–5 minutes. After fixation, the coverslips were washed with tap water. The coverslips were stained in Giemsa dye for 25–30 minutes. Finally, the coverslips were washed with tap water and dried at room temperature. Dried coverslips were observed under a light microscope with 1,000× magnification using by immersion oil.²³ Amastigotes were counted for grading their density according to the method of Chulay and Bryceson.²⁴

Microcapillary culture method. ADMSC culture was trypsinized with 1.5 mL 10× trypsin and incubated in 5% CO₂ humidity at 37°C for 10 minutes; 5 mL DMEM with 10% FCS were added on to the unattached cells and centrifuged (1,500 rpm for 5 minutes). Supernatant was removed, and the pellet was resuspended; 50- to 60-μL cell suspensions were injected into the microcapillary tubes with 1-mL injectors. The terminal ends were sealed. Microcapillary tubes were incubated at 27°C atmospheric conditions. After 4 days, stem cell–parasite culture was observed under inverted microscope (Olympus CK2) with 200× magnification. Microcapillary culture was prepared every 7 days from ADMSCs culture infected by *Leishmania* parasites.^{25–27} Detected motile promastigotes were counted and evaluated according to the method of Chulay and Bryceson²⁴ with slight modifications: Grade 0 = 0 parasites/all fields; Grade 1+ = 1–5 parasites/all fields; Grade 2+ = 6–10 parasites/all fields; Grade 3+ = 11–25 parasites/all fields; Grade 4+ = 26–50 parasites/all fields; Grade 5+ = 51–100 parasites/all fields; Grade 6+ > 100 parasites/all fields.

DNA extraction. DNA extraction was performed using the phenol-chloroform-isoamyl alcohol method. Cells being infected with *Leishmania* parasites were cultured for 30 days and cryopreserved. Before DNA extraction, cells were removed from liquid nitrogen and washed with 3 mL PBS; 100 μL lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium dodecyl sulfate [SDS]) and 100 μg/mL proteinase K (Invitrogen Life Technologies, Paisley, UK) were added into the sample and incubated at 56°C for overnight. After incubation, 800 μL phenol-chloroform-isoamyl alcohol (25:24:1) were added, and the mixture was gently vortexed for 30 seconds. The mixture was centrifuged at 12,000 rpm at 4°C for 10 minutes, and the supernatant was collected. Two volumes of chloroform were added to one volume of supernatant, and they were mixed up and down and kept at 4°C for 30 minutes. The mixture was centrifuged at 12,000 rpm at 4°C for 15 minutes. After this centrifugation, supernatant was transferred to a new collection tube and treated with 1 mL ice-cold ethanol. The mixture was gently mixed (5–10 times) and kept at –20°C overnight. The sample was centrifuged at 13,000 rpm for 10 minutes, and the pellet was washed with cold 70% ethanol and then dissolved in 20 μL Tris EDTA (TE) buffer 10/0.1 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).²⁸

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed for the amplification of the *Leishmania*-specific gene using DNA extracted from ADMSCs that were previously infected with this parasite.

PCR was set up in a final volume of 25 μL with the 2× Master Mix (Fermentas, St. Leon-Rot, Germany); 100 pM for each primer was used for the detection and identification of *Leishmania* parasites (primers Fme/Rme: 5'-TATTGGTATG CGAAACTTCCG-3' and 5'-ACAGAACTGATACTTAT ATAGCG-3', respectively). This primer pair amplifies a 427-bp DNA fragment specific for *L. major*, a 389-bp DNA fragment specific for *L. donovani*, a 403-bp DNA fragment specific for *L. tropica*, and a 434-bp DNA fragment specific for *L. infantum* parasites. The reaction mixture consisted of 1 μL template DNA. Forty-five cycles were performed in a thermocycler (Techne). Each cycle consisted of 94°C initial denaturation for 5 minutes, 94°C denaturation (1 minute), 54°C annealing (1 minute), 72°C elongation (1.5 minutes), and 72°C post-elongation (7 minutes). In all assays, positive controls containing

Leishmania promastigotes DNA from *in vitro* culture and a negative control without DNA were included; 10 μ L reaction mixture were visualized by 2% agarose gel electrophoresis.²⁹

RESULTS

Primary culture of human ADMSCs. After the enzymatic digestion, cells isolated from the lipoaspirate sample adhered to the tissue culture flask and proliferated. Nonadherent cells, such as red blood cells, were removed by replacing the medium. The initial adherent cells grew into spindle- or satellite-shaped cells, which then developed into visible colonies 2–3 days after the initial plating. The cells began to proliferate quickly and were passaged by trypsinization two times per week. After the second passage, ADMSCs appeared to have a fibroblast-like shape similar to bone marrow stromal cells (Figure 1).

Phenotypic characterization of ADMSCs. The immunophenotype of undifferentiated human ADMSCs at passage 3 has been examined by flow cytometry. Similarly, we characterized the ADMSC population according to its Cluster of Differentiation (CD) marker profile using flow cytometry. ADMSCs were found to express CD73 ($94.2 \pm 4.12\%$), CD90 ($95.97 \pm 3.67\%$), and CD105 ($70.02 \pm 5.58\%$), but they did not express the expected hematopoietic lineage markers such as CD34 ($1.06 \pm 0.32\%$).

Differentiation of ADMSCs *in vitro*. For adipogenic differentiation, ADMSCs were cultured in control medium, and then, culture media was replaced by adipogenic medium for 14 days. Cells in adipogenic medium acquired expanded cell morphology, and a time-dependent increase in intracellular lipid vacuoles was observed (Figure 2). These lipid vacuoles became visible after 4 days of adipogenic induction, and the cell size and number of lipid vacuoles increased incessantly afterwards. Osteogenic and chondrogenic differentiation potentials of ADMSCs were detected by Alizarin red S and Alcian blue staining, respectively (data not shown).

Detection of intracellular amastigotes by Giemsa method. After the ADMSC cultures were infected by four different *Leishmania* species, detection of amastigotes was performed by Giemsa method. According to the results, amastigotes of the four different *Leishmania* species could be detected

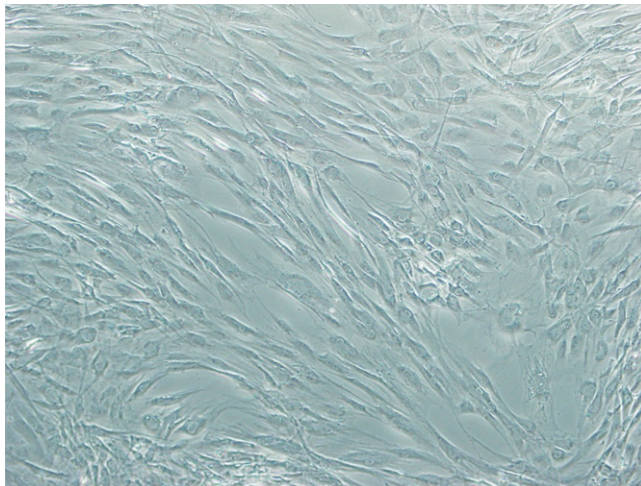


FIGURE 1. Human ADMSCs isolated from human lipoaspirate tissue and cryopreserved from passage 3 (P3). Original magnification was 40 \times .

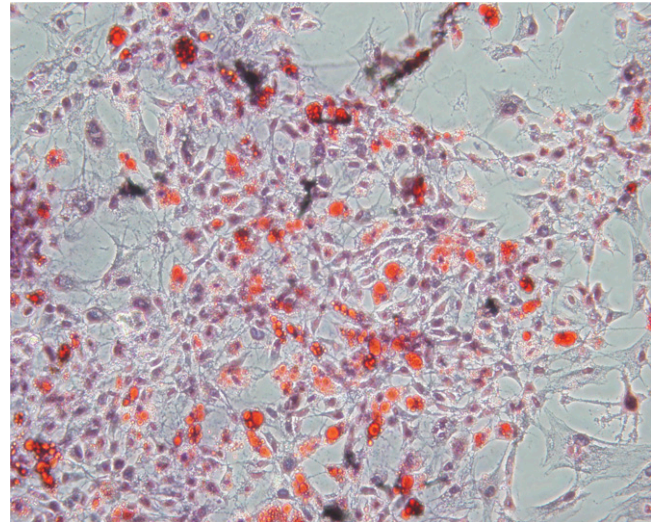


FIGURE 2. Adipogenic differentiation of ADMSCs *in vitro*. ADMSCs at P4 were stained for oil red O. Original magnification was 40 \times .

microscopically only after 24 hours (Figure 3). The presence of amastigotes in infected ADMSCs cultures could not be detected on days 7, 14, 21, and 28 of the experiment. Moreover, the density of the amastigotes after 24 hours was, in fact, very low, and no parasites could be detected during long-term culture (Table 1).

Detection of extracellular promastigotes by microculture method. The presence of parasites in infected ADMSC culture was also investigated by microculture method (MCM) on days 1, 7, 21, and 28 of the experiment. MCM is based on the facts that intracellular amastigotes can turn into extracellular promastigotes in microaerophilic environments and that these motile promastigotes can be detected under inverted microscope. In our experiment, promastigotes were still detected microscopically by MCM 28 days after infection. Infectivity results of ADMSC with different species of *Leishmania* parasites are shown in Table 1. According to the results, motile promastigotes were observed at all time points. Likewise, the

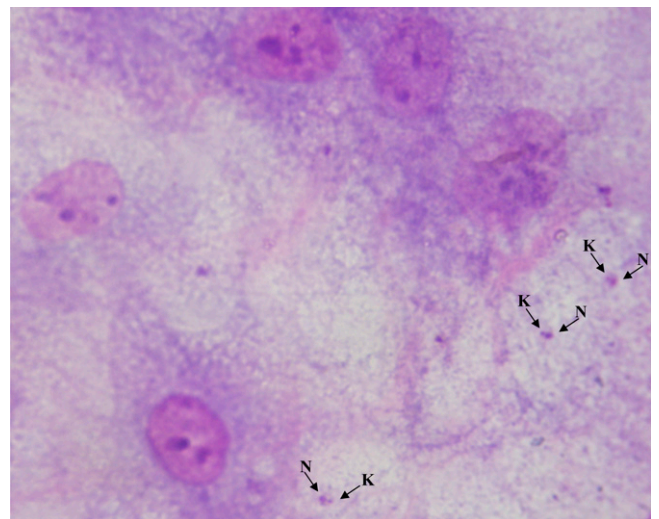


FIGURE 3. Infected ADMSCs with *L. infantum* promastigotes at day 1. K = kinetoplast; N = nucleus.

TABLE 1
Average density of different species of *Leishmania* amastigotes in smears and promastigotes in microculture

Day	Grading of <i>Leishmania</i> amastigotes in smears*				Grading of <i>Leishmania</i> promastigotes in MCM			
	<i>L. major</i>	<i>L. tropica</i>	<i>L. donovani</i>	<i>L. infantum</i>	<i>L. major</i>	<i>L. tropica</i>	<i>L. donovani</i>	<i>L. infantum</i>
1st	1+	1+	1+	1+	3+	2+	3+	3+
7th	0	0	0	0	1+	1+	1+	1+
14th	0	0	0	0	1+	2+	2+	1+
21st	0	0	0	0	2+	2+	2+	2+
28th	0	0	0	0	1+	1+	2+	2+

* Ref. 24.

number of promastigotes has been shown to change with respect to the time points in all *Leishmania* species (Table 1).

Detection of intracellular amastigotes by PCR. The presence of parasite-specific DNA amplification in infected ADMSCs was observed at all time points and is shown in Figures 4 and 5.

DISCUSSION

The presence of leishmaniasis, being one of the important protozoan diseases, has been reported in organ transplantations such as kidneys, pancreas, liver, lungs, and heart tissue.^{1,8} Although information on other protozoan infections is available, there are no reports where *Leishmania* infections were investigated in stem cell transplantations. In this study, the interaction between different types of *Leishmania* parasites and ADMSCs has been investigated *in vitro*. To our knowledge, this study is the first that documents such a relation between stem cells and leishmaniasis.

Leishmania parasites can survive in different tissues and organs for decades, even after treatment. In several studies, it has been found that intracellular parasites can persist and keep their viability, especially in fibroblast cells, as latent inactive forms.³⁰ Similarly, in our study, *Leishmania* parasites stayed viable as inactive forms in ADMSCs *in vitro* (Figure 3). These results can indicate that, after the treatment, parasites can stay inactive not only in fibroblasts but also in mesenchymal stem cells. Likewise, stem cells in an organism usually stay dormant unless an exogenic stimulus (growth factor expression, physical trauma, etc.) is present. This unique property of stem cells could also imply that these cells could be perfect host systems for *Leishmania* parasites. Therefore, especially for the stem cell transplantations to be performed within endemic regions, performing a screening that is specific for *Leishmania* parasites is an important issue. It is simply because of the fact that reactivation of *Leishmania* parasites after immunosuppressive treatment in organ transplantations has been reported in several studies.^{1,8}

Although several screening methodologies can be performed to detect *Leishmania* parasites in stem cell transplantations,

one of the most important problems is the diagnosis of the leishmaniasis disease. For the diseases that cause latent infections such as *Leishmania*, the diagnosis become very difficult.^{9,31} In our study, although we have used several diagnosis approaches such as PCR, Giemsa staining, and MCM, latent infection in ADMSC culture environment could only be detected by microculture approach and PCR. In microscopic Giemsa staining, parasites were only detected after 24 hours of the experiment, albeit in very low density. In our previous studies, it has been shown that MCM is more sensitive than the other classical culture approaches in the diagnosis of visceral and cutaneous leishmaniasis.²⁵⁻²⁷

It is already known that *Leishmania* parasites have developed a survival mechanism in which they stay viable in phagolysosome after infecting macrophages by receptor-based phagocytosis.¹¹ In the literature, it has been shown that parasites infect not only professional phagocytotic cells such as macrophages but also amnion epithelial cells, fibroblasts, kidney cells, and dendritic cells in mammalian hosts.^{11,12} Furthermore, it is documented that parasites enter these cells by binding different cell surface receptors. Although there are many studies that investigate cellular and molecular characteristics of mesenchymal stem cells, the information about the phagocytic properties of these cells is scarce. One study reported that ADMSCs show properties similar to macrophages in wounds.¹⁶ In another study, it was shown that pre-adipocytes can develop macrophage-like phagocytic characteristics.³² In our study, although the data are preliminary and it only confers the *in vitro* results, it has been shown, for the first time, that visceral and cutaneous leishmaniasis-causing parasite types can infect ADMSCs. Additional clinical studies on ADMSCs from infected patients are required to indicate the actual pathogenesis and transmission mechanisms of the parasite. However, these results are important, and infectivity of mesenchymal stem cells by *Leishmania* parasites can, in fact, be considered as a very important *in vitro* experimental model system for the analysis of interaction between stem cells and other infectious agents.

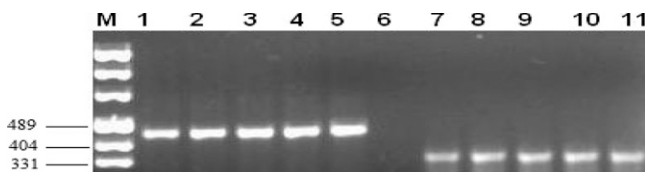


FIGURE 4. PCR products of infected ADMSC isolates. M = pUC mix marker 8. Lanes 1–5, *L. major* (lane 1, 7th day; lane 2, 14th day; lane 3, 21st day; lane 4, 28th day; lane 5, positive control). Lane 6, negative control. Lanes 7–11, *L. donovani* (lane 7, 7th day; lane 8, 14th day; lane 9, 21st day; lane 10, 28th day; lane 11, positive control).

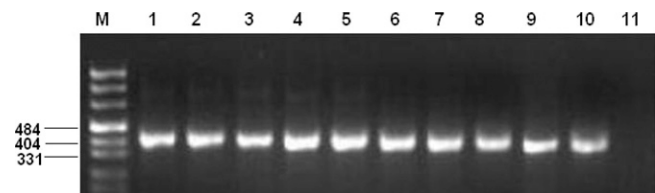


FIGURE 5. PCR products of infected ADMSC isolates. M = pUC mix marker 8. Lanes 1–5, *L. infantum* (lane 1, 7th day; lane 2, 14th day; lane 3, 21st day; lane 4, 28th day; lane 5, positive control). Lanes 6–10, *L. tropica* (lane 6, 7th day; lane 7, 14th day; lane 8, 21st day; lane 9, 28th day; lane 10, positive control). Lane 11, negative control.

In summary, we document, for the first time, the infectivity of ADMSCs by *Leishmania* parasites in *in vitro* settings. Our results strongly indicate that screening for *Leishmania* parasites before stem cell transplantations in endemic regions can be very important and deserves additional clinical investigation.

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