Congenital Transmission of Experimental Leishmaniasis in a Hamster Model

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Abstract. Little information is available on transplacental transmission of *Leishmania* spp. We determined the frequency and impact of congenital infection caused by *Leishmania panamensis* or *L. donovani* in experimentally infected hamsters. A polymerase chain reaction showed that congenital transmission occurred in 25.8% (24 of 93) of offspring born to *L. panamensis*-infected hamsters and 14.6% (11 of 75) offspring born to *L. donovani*-infected hamsters. Mortality during lactation was higher in offspring born to *L. panamensis*-infected hamsters and offspring born to *L. donovani*-infected hamsters than controls, and lymphoproliferation to *Leishmania* was more frequent in offspring born to *L. panamensis*-infected hamsters (17.4%, 11 of 63) than in offspring born to *L. donovani*-infected hamsters (8.5%, 3 of 35). After weaning, only offspring born to *L. donovani*-infected hamsters had lower weight gain ($P < 0.001$) and hematocrit levels ($P = 0.0045$) than controls. Challenge of offspring born to *L. panamensis*-infected hamsters with *L. panamensis* showed no differences in lesion evolution, and offspring born to *L. donovani*-infected hamsters were more susceptible to *L. donovani* challenge than controls. Consequently, prenatal exposure of hamsters to *L. donovani* significantly increased the mortality risk and susceptibility to secondary homologous infection.

INTRODUCTION

The impact of congenital transmission of *Leishmania* has not been studied in persons in disease-endemic regions and consequently epidemiologic data are lacking. Although transplacental infection with other species of the family *Trypanosomatidae* such as *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, resulted in 1,136 cases during 1994–2001,^{1,2} in the particular case of *Leishmania donovani*, only 11 cases of congenital transmission have been documented.³ In these cases, the possibility of vector transmission to the newborn was ruled out because the mothers were living in non-endemic regions free of phlebotomine sand flies.^{4,5} The difficulty in diagnosing congenital leishmaniasis is attributed to the lack of evident pathologic changes in newborns or length of the pre-patent period of visceral leishmaniasis (VL), which could be similar to that reported for primary infections in disease-endemic areas.^{6,7} Cutaneous leishmaniasis (CL) is also a systemic disease that seems to disseminate mainly through the lymphatic system, but the possibility of hematogenous circulation also has been documented in animals and humans.^{8–11} However, no attempts have been made to evaluate the feasibility of congenital transmission upon infections with *Leishmania* of the *Viannia* subgenus.

Experimental infections focused on congenital transmission of VL are scarce, and culture techniques used in earlier studies either failed to show transplacental transmission to offspring from infected hamsters or found markedly low transmission rates.^{12,13} Nevertheless, more recent studies in BALB/c mice and dogs by using polymerase chain reaction (PCR) to detect parasite DNA indicated that congenital transmission of VL could be more frequent than previously suspected.^{14,15}

Although congenital infections with VL have been reported in humans and dogs, the frequency of its occurrence is still unclear.^{16–18} The purpose of this study was to explore the feasibility of transplacental transmission of New World CL and determine the frequency of congenital VL in a hamster

model. For this purpose, we used the Syrian golden hamster, which is susceptible to most *Leishmania* species, including *L. donovani* and *L. panamensis*.^{19–21} Also, we determined the impact of *in utero* infection or exposure to leishmanial antigens on the newborn health and its susceptibility to a subsequent *Leishmania* challenge.

MATERIALS AND METHODS

All experimental protocols involving hamsters followed the international guidelines for animal experimentation and were approved by the Institutional Animal Care and Use Committee of Centro Internacional de Entrenamiento e Investigaciones Medicas according to the Guiding Principles for Biomedical Research Involving Animals (Council for International Organizations of Medical Sciences) and the Colombian Law 84 of 1989, resolution #0084300 of 1993.

Offspring born to infected female hamsters. Offspring born to hamsters with chronic CL or VL (CHR-offspring), were obtained from 3–4-month-old female hamsters infected with *L. (Viannia) panamensis* or *L. (Leishmania) donovani* one month before mating. To obtain offspring born to female hamsters pregnant during the acute phase of infection (AC-offspring), females were mated during a one-week period and then infected with the corresponding *Leishmania* species.²² This infection protocol assumed that female infection occurred between the second and sixth day of gestation. Infected female hamsters that did not become pregnant were excluded from the study, and groups of pregnant, non-infected hamsters were used as sources of control offspring (CTR-offspring). Females remained alone in the cage during gestation (the normal length of pregnancy is 16–18 days) and subsequently with the litter until weaning at day 21 of birth.

Female hamsters were infected intradermally in the snout with 1×10^4 cultured promastigotes of *L. panamensis* (MHOM/COL/84/1099) harvested at the stationary phase of growth (sixth day of culture) as described.⁸ Cultures were initiated from recent isolates obtained from infected hamsters to ensure strain pathogenicity. Cultured promastigotes of *L. donovani* (MHOM/IN/DD8/1968) also were harvested from the stationary phase of culture, and anesthetized female hamsters

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were infected with 1×10^6 parasites through the intraperitoneal route. Acute or chronic phases of infection represented distinct time points that could potentially lead to different congenital transmission rates and offspring immune responses.

Clinical evaluation of offspring born to infected mothers.

Offspring were maintained with the infected or uninfected mothers until weaned at 21 days of birth, which is the standard lactation period. The growth rate of the offspring based on change in body weight in grams and mortality was recorded every 15 days from the seventh to 45th day of age. Mortality was recorded in all the experimental groups, which were distributed as follows: 183 offspring born to females infected with *L. panamensis* (CHR-offspring, n = 79; AC-offspring, n = 104);

156 offspring born to females infected with *L. donovani* (CHR-offspring, n = 47; AC-offspring, n = 109); and 116 age-matched controls born to uninfected females (CTR-offspring). Hematocrits were evaluated at 45 days of age in *L. donovani* offspring (n = 92) and control offspring (CTR-offspring) (n = 20) by using heparinized capillary tubes. At the end of this clinical observation, animals were distributed into different experimental groups as shown in Figure 1.

Identification of congenital transmission. The frequency of congenital transmission was evaluated in offspring born to mothers with chronic infection (CHR-offspring) or born to mothers with acute infection (AC-offspring). Offspring between one and two months of age born to mothers infected

FIGURE 1. Experimental design of the study. Offspring born to female hamsters pregnant during the acute phase of the infection (AC-offs) or chronic phase of the infection (CHR-offs) with *Leishmania panamensis* or *L. donovani* were subjected together with offspring born to uninfected female controls (CTR-offs) to clinical evaluations (weight and mortality) and distributed in subgroups to evaluate **A**, congenital transmission by means of culture (*L. panamensis*, AC-offs, n = 40, CHR-offs, n = 33; *L. donovani*, AC-offs, n = 40, CHR-offs, n = 38) and polymerase chain reaction (PCR) (*L. panamensis*, AC-offs, n = 33, CHR-offs, n = 60; *L. donovani*, AC-offs, n = 37, CHR-offs, n = 38), **B**, lymphoproliferative response to the homologous leishmanial antigen or delayed-type hypersensitivity (DTH) reaction (*L. panamensis*, AC-offs, n = 22, CHR-offs, n = 14; *L. donovani*, AC-offs, n = 20, CHR-offs, n = 10, CTR-offs, n = 48), and **C**, acquired resistance to homologous challenge determined by clinical parameters in groups of male and female offspring (*L. panamensis*, male AC-offs, n = 14, female AC-offs, n = 12; male CHR-offs, n = 13, female CHR-offs, n = 12; male CTR-offs, n = 12; female CTR-offs, n = 13; *L. donovani*, male AC-offs, n = 20, female AC-offs, n = 20, CHR-offs = not done because animals were not available, male CTR-offs, n = 10, female CTR-offs, n = 10). Evaluations of congenital transmission, DTH response, and resistance to challenge were conducted in different subsets of animals. Mo. = months; ConA = concanavalin A; CL = cutaneous leishmaniasis; VL = visceral leishmaniasis.

either with *L. panamensis* (n = 140) or *L. donovani* (n = 78) were humanely killed, and samples of retropharyngeal lymph node, spleen, and liver were placed in Seneckije's culture medium, incubated at 24°C, and inspected for parasites weekly for one month. For PCRs, tissue samples and serum were collected by using new sampling materials for each animal to avoid potential cross-contamination, and were subsequently stored at -20°C (n = 93, offspring born to mothers infected with *L. panamensis*; n = 75, offspring born to mothers infected with *L. donovani*).

Two PCR methods were used. For conventional PCR-hybridization, DNA from lymph node, spleen, and liver was isolated to detect amplified *Leishmania* kinetoplast DNA (kDNA) with a biotin-labeled probe as described.²³ For real-time PCRs, DNA from lymph node, spleen, or serum was purified (NucleoSpin; Macherey-Nagel, Düren, Germany) and amplified with primers specific for a 120-basepair kDNA fragment of *L. donovani* (JW12, forward: 5'-GGGTAGGGGCGT TCTGCGAAA-3'; JW11, reverse: 5'-CCTATTTTACACC AACCCCAAGT-3'),²⁴ or a 140-basepair kDNA minicircle region of *L. panamensis* (B4, forward: 5'-AATCGTACC ACCCGACATGC-3'; 13B, reverse, 5'-ATATTACACAA CCCCTAATTGTGCA-3'). DNA was denatured (95°C for 10 seconds) and annealed (40 cycles at 60°C for 10 seconds) in a master mixture containing 20 µL of Fast Star DNA Master SYBR Green I (Hoffmann-La Roche Ltd., Basel, Switzerland), 750 nM of primers, and 100 ng or 500 ng of DNA (*L. panamensis* or *L. donovani*, respectively). Amplification curves and melting temperatures were obtained in the LightCycler 2.0 (Hoffmann-La Roche Ltd.). Specific products were identified by a melting temperature of 82.3°C for *L. donovani* and 83.5°C for *L. panamensis*. Standard curves prepared from tissue spiked with different parasite concentrations showed a sensitivity of 0.1 or 1 parasite of *L. donovani* or *L. panamensis*, respectively. The specificity of the amplification product was confirmed by electrophoresis and sequencing of the purified product (Wizard SVG PCR Clean-Up; Promega, Madison, WI) in one congenital case of infection with *L. donovani* and one congenital case of infection with *L. panamensis*.

Cellular immune response. Cellular immune response of the offspring was assessed by lymphoproliferation of retropharyngeal lymph node lymphocytes and delayed-type hypersensitivity (DTH) reaction (Figure 1). Lymphocytes (5×10^5) of offspring born to mothers infected with *L. panamensis* (n = 63), offspring born to mothers infected with *L. donovani* (n = 35), and CTR offspring (n = 30) were stimulated *in vitro* over a two-day period with 2 µg/mL of concanavalin A or over a three-day period with 5×10^3 thawed-frozen *L. panamensis* or *L. donovani* promastigotes per well as described.²⁵ The blastogenic response was expressed as the stimulation index (ratio of incorporation of tritiated thymidine over an eight-hour period of antigen-stimulated lymph node cells to that of non-stimulated cells of the same animal). A different group of offspring was used to evaluate sensitization to leishmanial antigens by DTH; these offspring were not subjected to PCR or lymphoproliferative studies because Montenegro antigen used in the test contains a large number ($10^7/50$ µL) of formalin-fixed promastigotes that could potentially give false-positive results in the PCR or lymphoproliferation assays. One and two months after weaning, Montenegro antigen was injected intradermally in the right foot of offspring born to

mothers infected with *L. panamensis* (n = 36), offspring born to mothers infected with *L. donovani* (n = 20), and controls born to uninfected mothers (n = 48); the vehicle alone was injected in the left foot as a control. After 72 hours, induration in the foot was measured by using a digital caliper, and the DTH results were expressed as the difference between the diameter of the right and left foot.

Challenge of offspring born to infected and uninfected mothers. *Leishmania panamensis*-infected offspring. Female and male offspring born to mothers infected with *L. panamensis* were challenged intradermally in the snout with 1×10^6 luciferase-transfected *L. panamensis*²⁶ on the 45th day of birth (CHR-offspring, n = 25; AC-offspring, n = 26; CTR-offspring, n = 25). Development of cutaneous lesions in all groups was followed every 15 days until the end of the experiment on the 75th day post-challenge. At this point, the parasite burden was determined in the lesion and draining lymph node by using luminometry as described.²²

Leishmania donovani-infected offspring. Forty-five days after birth, groups of female (n = 20) and male (n = 20) juvenile hamsters born to mothers infected during pregnancy with *L. donovani* were subjected to homologous intracardial challenge with 1×10^6 *L. donovani* promastigotes, and evolution of infection was compared with CTR-offspring born from uninfected mothers (n = 10 females, n = 10 males). Progression of VL was evaluated as follows: body weight, every 15 days up to 3 months post-challenge; hematocrit levels, monthly from the first to the third month post-challenge; cachexia, starting at three months post-challenge and up to the end of the experiment and at six months post-challenge. Cachexia was defined as clinically evident dehydration (lack of skin elasticity of the back identified as failure to return to its normal position after pulling) and severe weight loss (> 10%). Offspring mortality was recorded every 15 days up to 6 months post-challenge, and the spleen weight as an indicator of splenomegaly was determined at the end of the experiment. Offspring born to mothers infected before pregnancy with *L. donovani* were not available for this set of experiments because of the small number of animals produced by these females.

Statistical analysis. Statistical analysis was performed by using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, Inc., La Jolla, CA). The statistical test and number of animals used in each experiment is specified in the corresponding tables and figure legends.

RESULTS

Clinical evaluation of offspring born to infected female hamsters. The growth rate of offspring born to mothers infected with *L. panamensis* (n = 146) was equivalent to that of CTR-offspring (n = 162), as determined during the 21 days of lactation. Similarly, during the same period of lactation, no clinical manifestations of VL were observed in offspring from mothers infected with *L. donovani* (Figure 2A). However, the mortality rate up to the 45th day of age was significantly higher in offspring born to infected mothers than in offspring born to uninfected hamsters (Figure 2B). Analysis of the time at which mothers were infected showed that mortality was higher in offspring born to females with acute infection with both *Leishmania* species (*L. panamensis* or *L. donovani*) than in control offspring (CTR-offspring)

FIGURE 2. Clinical evolution from the 7th to 45th day after birth of offspring born to hamster mothers infected at the chronic phase of the infection with *Leishmania donovani* (CHR-offs) or born to mothers infected during pregnancy (AC-offs) compared with offspring born to uninfected mothers (CTR-offs). **A**, Body weight at the end of lactation (21 days of birth) and after weaning at 45 days of age; CHR-offs and AC-offs had body weights similar to CTR-offs during lactation; after weaning, CHR-offs ($n = 96$) weighed significantly less than AC-offs ($n = 68$) or CTR-offs ($n = 55$) (** $P < 0.001$ each, by Tukey-Kramer multiple comparisons test). **B**, Mortality of AC-offs and CHR-offs infected with *L. panamensis* (L.p.) or *L. donovani* (L.d.) was higher than that of controls (CTR-offs) (*L. panamensis* AC-offs, $n = 104$, $P = 0.001$; *L. panamensis* CHR-offs, $n = 79$, $P = 0.0014$; *L. donovani* AC-offs, $n = 109$, $P < 0.0001$; *L. donovani* CHR-offs, $n = 47$, $P = 0.019$; CTR-offs, $n = 162$, by Fisher's exact test). **C**, Hematocrit at 45 days of age; *L. donovani* CHR-offspring ($n = 52$) had significantly lower hematocrit than *L. donovani* AC-offs ($n = 40$; *** $P < 0.0001$, by Mann-Whitney test) or CTR-offs ($n = 20$) (** $P = 0.0045$, by Mann-Whitney test).

(Figure 2B). The mortality rate in offspring born to chronically infected mothers was also significantly higher than in controls (Figure 2B). Despite higher mortality of offspring born to infected females, evaluations made on surviving offspring after weaning at 45 days of age indicated that the growth rate of offspring from *L. panamensis*-infected mothers was similar to that of controls. Conversely, CHR-offspring of mothers infected with *L. donovani* were clinically less fit, as demonstrated by lower weight gain and lower hematocrits than for CTR-offspring (Figure 2A and C) ($P < 0.001$ and $P < 0.0001$).

Congenital transmission. A low proportion of 140 (0.71%) offspring born to female hamsters infected with *L. panamensis* was positive when spleen samples were cultured at one month of age (Table 1), and none of the *L. donovani* cultures were positive (Table 2). However, molecular diagnosis by PCR dramatically increased detection of congenital transmission: 24 (25.8%) of 93 offspring born to mothers infected with *L. panamensis* and 11 (14.6%) of 75 offspring from mothers infected with *L. donovani* were positive (Tables 1 and 2).

TABLE 1

Frequency of congenital transmission to offspring from female hamsters infected intradermally with 10^4 promastigotes of *Leishmania panamensis**

Group	No. positive offspring/no. evaluated (%)		
	Culture	PCR†	P‡
CHR-offspring	1/30 (3.3)	11/60 (18.3)§	0.045
AC-offspring¶	0/70 (0)	13/33 (39.4)	
Total	1/140 (0.7)	24/93 (25.8)	

* Offspring were born to females in the chronic phase of the infection (CHR-offspring) or born to females infected during pregnancy (AC-offspring). PCR = polymerase chain reaction.

† CHR-offspring were evaluated by PCR-hybridization or real-time PCR.

‡ P value of Fisher's exact test, congenital cases in CHR-offspring versus AC-offspring.

§ Seven (23.3%) of 30 were positive by conventional PCR-hybridization and 4 (13.3%) of 30 were positive by real-time PCR.

¶ The gestational period of hamsters is 16–18 days; weaning took place 21 days after birth. Hamsters were evaluated between one and two months after birth by real-time PCR.

Occurrence of congenital transmission of CL was higher in offspring born to females during the acute phase of the infection (AC-offspring, 39.4%) than in offspring born to females during the chronic phase of infection (CHR-offspring, 18.3%) ($P = 0.045$) (Table 1). An opposite trend was observed in *L. donovani* (AC-offspring, 8%, CHR-offspring, 21%) (Table 2). Consequently, under the experimental conditions used in this set of experiments, congenital transmission of *L. donovani* tended to be lower than that of *L. panamensis*.

We obtained samples from lymph nodes, whole blood, and serum from offspring born to mothers infected with *L. panamensis*. We found that lymph nodes were more frequently positive than other tissues: 20 (25%) of 79 for lymph nodes, 2 (2.9%) of 69 for serum, and 0% of 12 for whole blood. To evaluate congenital transmission of *L. donovani*, we obtained samples from bone marrow, whole blood, and serum. In these samples, parasite DNA was detected in 5 (13%) of 38 bone marrow samples, 1 (2.8%) of 35 whole blood samples, and 5 (8%) of 62 serum samples. Although we

TABLE 2

Frequency of congenital transmission to offspring from female hamsters infected intradermally with 10^6 promastigotes of *Leishmania donovani**

Group	No. positive offspring/no. evaluated (%)		
	Culture†	PCR‡	P§
CHR-offspring	0/38 (0)	8/38 (21)	0.19
AC-offspring¶	0/40 (0)	3/37 (8)	
Total	0/78 (0)	11/75 (15)	

* Offspring were born to females in the chronic phase of the infection (CHR-offspring) or to females infected during pregnancy (AC-offspring). PCR = polymerase chain reaction.

† Samples from lymph node, spleen, and bone marrow were evaluated by culture.

‡ Samples from serum, blood, or bone marrow were obtained between one and two months after birth and evaluated by real-time PCR.

§ P value of Fisher's exact test, CHR-offspring versus AC-offspring.

¶ AC-offspring were born to female hamsters infected with *L. donovani* at 7–11 days of pregnancy.

TABLE 3
Cellular immune response of offspring born to hamsters infected with *Leishmania panamensis* or *L. donovani**

Offspring group		No. positive offspring/no. evaluated (%)		
		Lymphoproliferation†		DTH‡
		Con A	<i>Leishmania</i>	
<i>L. (Vianna) panamensis</i>	CHR-offspring	24/37 (64.9)§	7/37 (18.9)	4/14 (28.6)¶
	AC-offspring	11/26 (42.3)#	4/26 (15.3)	1/22 (4.5)
<i>L. (Leishmania) donovani</i>	CHR-offspring	0/18 (0)**	0/20	ND
	AC-offspring	13/15 (87)	3/15 (20.0)	2/20 (10)††
CTR-offspring		22/30 (73.3)	1/30 (3.3)‡‡	0/48

*T cell proliferation and delayed-type hypersensitivity (DTH) reaction was determined in offspring born to female hamsters in the chronic phase of the infection (CHR-offspring) or born to females infected during pregnancy (AC-offspring) compared with those born to uninfected female controls (CTR-offspring). ND = not done; low numbers of offspring BP were designed for parasitologic evaluations by polymerase chain reaction.

†Lymphoproliferation of lymph node lymphocytes were obtained from offspring at 1–4 months of age, stimulated with concanavalin A (Con A) and specific *Leishmania* antigens (thaw-frozen parasites). Positive lymphoproliferative response was defined as a stimulation index > 5.0 (stimulation index = counts per minute stimulated cells/counts per minute nonstimulated cells).

‡Positive DTH was defined as a ≥ 0.3 mm difference in footpad diameter between the test (*Leishmania* antigens) and the control foot injected with vehicle (mean DTH + 3 SD of control offspring).

§ $P = 0.05$.

¶ $P = 0.0019$.

$P = 0.022$.

** $P = 0.0001$.

†† $P = 0.08$.

‡‡ Statistical analysis of data indicated that the control offspring had a positive result could not be considered an outlier.

did not intend to compare results of PCR-hybridization with those of real-time PCR, we found that both methods yielded similar positive results for *L. panamensis* samples: 7 (23%) of 30 were positive by PCR-hybridization and 17 (25%) of 63 were positive by real-time PCR (this comparison was not made for *L. donovani*-infected samples).

Lymphoproliferation and DTH in offspring of infected and uninfected females. To evaluate the immune response of offspring born to infected mothers, we test the proliferation of lymphocytes from retropharyngeal lymph nodes to the mitogen concanavalin A and *Leishmania* antigens. Eleven (17.4%) of 63 offspring from mothers infected with *L. panamensis* and 3 (8.5%) of 35 offspring from mothers infected with *L. donovani* showed positive blastogenic response to *Leishmania* antigens (Table 3). Although 1 of 30 offspring born to uninfected hamsters (3.3%, CTR-offspring) responded non-specifically to *Leishmania* antigens (stimulation index > 5), a higher proportion of offspring born to infected hamsters responded to leishmanial antigens (Table 3). Notably, the lack of blastogenic response to concanavalin A or *Leishmania* antigens in *L. donovani* CHR-offspring suggested that the cellular immune response of this group was severely impaired. The DTH test for a similar subgroup of hamsters showed that 5 (13.9%) of 36 offspring from mothers infected with *L. panamensis* and 2 (10%) of 20 offspring from mothers infected with *L. donovani* had positive reactions, and none of 48 CTR offspring showed a false-positive result (Table 3).

Analysis of *L. donovani* CHR-offs demonstrated that positive cellular immune responses to *Leishmania* antigens were less frequent than parasitologic positivity (0 of 20 positive lymphoproliferation results versus 8 of 38 positive PCR results) ($P = 0.041$). Non-significant differences between lymphoproliferation and parasite PCR positivity were found in other groups (Tables 2 and 3). We did not relate lymphoproliferation and parasite status in most animals, and no comparisons were made for *L. donovani* infections. However, data from *L. panamensis* CHR-offspring indicated that 3 of 10 PCR-positive animals also showed positive results in the lymphoproliferation assay. In AC-offspring, 2 of 10 animals also showed positive results by PCR and lymphoproliferation.

Nevertheless, the small number of animals in these groups prevented us from drawing any conclusion.

Offspring susceptibility to a homologous challenge. We evaluated the outcome of a subsequent homologous challenge in offspring because this result could be a plausible scenario in humans inhabiting disease-endemic foci. We found no differences up to three months post-challenge regarding development of dermal lesions in offspring challenged intradermally in the snout with 1×10^6 *L. panamensis* promastigotes (mean evolution index of lesion size, observed value – initial baseline value/initial baseline value $\pm$ SD): AC-offspring, 1.25 ± 0.49 , $n = 26$; CHR-offspring, 1.16 ± 0.39 , $n = 26$; CTR-offspring, 1.24 ± 0.5 , $n = 25$). No differences in parasite burden in the lesion or draining lymph node were found at the end of the experiment at three months post-challenge.

The influence of prenatal exposure to *L. donovani* upon a subsequent homologous challenge infection was evaluated only in offspring born to females during the acute phase of *L. donovani* infection (AC-offspring) because few offspring born to mothers during the chronic phase of VL were available and consequently were used to evaluate other parameters of the study (Table 3). We found that male and female AC-offspring responded differently to *L. donovani* challenge. Body weight at three months post-infection was similar in female AC-offspring and their matched female CTR-offspring (Figure 3A). However, male AC-offspring ($n = 20$) had significantly lower body weights after *L. donovani* challenge than their respective male CTR-offspring ($n = 10$) (Figure 3B) ($P < 0.001$). From the third to the sixth month post-challenge, both sexes of AC-offspring were more frequently cachectic than corresponding sex-matched, challenged CTR-offspring ($P = 0.05$, by Fisher's exact test, Figure 2C, and $P = 0.008$, by Fisher's exact test, Figure 3D).

Survival of challenged female AC-offspring was similar to that of female CTR-offspring (Figure 3E) as opposed to that of male AC-offspring, which was significantly lower than their CTR-offspring (Figure 2F) ($P = 0.009$, by log-rank Mantel-Cox test). Despite the similar survival rate of female AC-offspring, they exhibited marked splenomegaly compared with female CTR-offspring (Figure 3G and Figure 4) ($P = 0.013$). The same trend was observed in male AC-offspring when

FIGURE 3. Susceptibility of offspring born to *Leishmania donovani*-infected female hamsters to a homologous challenge than offspring born to uninfected females. Offspring born to mothers infected during pregnancy with *L. donovani* were challenged intraperitoneally with 1×10^6 stationary phase promastigotes at one month of age. **A** and **B**, Body weight at three months post-infection. No differences in body weight were found from 1–3 months after the challenge of female AC-offs ($n = 20$) compared with female CTR-offs ($n = 10$) (**A**); in contrast, the body weight of male AC-offs ($n = 20$) was significantly lower than male CTR-offs ($n = 10$) (** $P < 0.001$, by Tukey-Kramer test) (**B**). **C** and **D**, Cachexia. After three months post-challenge, overt disease was manifested and the percentage of female AC-offs ($n = 20$) (**C**) and male AC-offs ($n = 20$) (**D**) with cachexia (dehydration and severe weight loss) was significantly higher than in sex-matched CTR-offs ($n = 10$ females, $n = 10$ males) (males $P = 0.0088$ and females $P = 0.0562$, by Fisher's exact test). **E** and **F**, Kaplan-Meier survival curves. Female AC-offs ($n = 20$) had similar survival curves than female CTR-offs ($n = 10$) up to six months post-challenge (end of the experiment) (**E**); Survival of male AC-offspring ($n = 20$) was lower than that of male CTR-offspring ($n = 10$), $P = 0.005$, by log-rank Mantel-Cox test) (**F**). **G** and **H**, Splenomegaly. At the end of the experiment, surviving female AC-offs ($n = 7$) developed larger splenomegaly than female CTR-offs ($n = 11$, * $P = 0.013$, by *t*-test) (**G**) and the same trend was observed in male AC-offs ($n = 8$) compared with male CTR-offs ($n = 9$) (**H**). Splenomegaly is depicted as the percentage of spleen weight relative to body weight. AC-offs = mothers infected during pregnancy; CTR-offs = offspring born to uninfected mothers.

compared with male CTR-offspring, but the difference was not statistically significant, probably because male AC-offspring with the largest spleens had already died, leaving only offspring with relatively smaller spleens available for evaluation (Figure 3H).

DISCUSSION

This study focused on the potentially negative effects that congenital infection could have on offspring. Our findings suggest that both *Leishmania* species tested could be transmitted *in utero*, and more importantly, that prenatal exposure to *L. donovani* modulates the immune response of the offspring and increases the risk for mortality or susceptibility to subsequent leishmanial infections.

As expected, PCRs were more sensitive than culture techniques in detecting congenital infections, demonstrating that approximately 25% of offspring born to infected mothers harbored *L. panamensis*. To our knowledge, this is the first time that congenital infection of a *Leishmania* species producing dermal leishmaniasis is reported. A small percentage of offspring showed DTH compared with PCR positivity, suggesting that the parasite or its antigens sensitized a low proportion of fetuses *in utero*.

No epidemiologic data for humans are available concerning congenital transmission of CL, but mucosal metastasis and isolation of parasites from lymph nodes and blood and diffuse CL in patients indicate that *Leishmania* species producing dermal pathologic changes are also disseminated systemically^{11,27} and may reach placental tissues. Moreover, PCR screening of dog populations suggested that *Leishmania* (*Viannia*) spp. circulate in the blood of asymptomatic dogs,¹⁰ and experimental infections in hamsters indicated that hematogenous dissemination is feasible in this animal model.⁹ Thus, the feasibility of congenital transmission of *Leishmania* (*Viannia*) spp. in humans requires further exploration.

We speculated that the phase of *Leishmania* infection of the mother (acute or chronic) could determine the frequency of congenital transmission. We found that congenital transmission of *L. panamensis*, as determined by PCR, was more frequent during an acute infection of the mother. Different factors could have contributed to this result. Hamsters infected with *Leishmania* (*Viannia*) sp. showed development of chronic but controlled infections in which the number of parasites tends to plateau or diminish with time post-infection.¹⁹ *Leishmania* (*Viannia*) sp. disseminates principally to draining lymph nodes, and circulates in low numbers in the blood of hamsters,⁹ decreasing the likelihood of transplacental transmission in females infected before pregnancy. Conversely, considerable numbers of promastigotes could have accessed the peripheral blood by spill over from intradermal infections of pregnant females (acutely infected females), thereby increasing hematogenous contact of *L. panamensis* with placental tissues. We expected to see a higher rate of congenital transmission in females chronically infected with *L. donovani* because of the progressive nature of the disease in the hamster model. However, these differences did not show statistical significance.

Previous studies using culture as diagnostic tool demonstrated either leishmanial antigen sensitization or low infection rates of offspring born to hamsters infected with

FIGURE 4. Splenomegaly of offspring born to infected relative to uninfected hamster mothers upon challenge with *Leishmania donovani*. A marked splenomegaly was found in surviving female AC-offs (right) compared with CTR-offs (left) at six-months post-challenge. AC-offs = mothers infected during pregnancy; CTR-offs = offspring born to uninfected mothers.

L. donovani.^{12,13} In our study, we detected by PCR prenatal infection in 15% ($n = 75$) of the litters of female hamsters infected with *L. donovani*. In humans, there are few reports regarding transplacental infection of *L. infantum* or *L. donovani* from symptomatic or asymptomatic women.^{28,29} Nevertheless, in some of these cases, health of the newborns was severely affected.³⁰ In Saudi Arabia³¹ and Pakistan,³² 5–14% of cases occur in children before they are one year of age. In Brazil, 28% of VL cases are diagnosed in children less than four years of age,³³ and in Colombia 85% of VL cases were found in children less than two years of age, including three-month-old babies.³⁴ Although high biting rates of infected sand flies, parasite virulence, and childhood malnutrition play a significant role in the early appearance of overt disease, the contribution of vertical transmission still needs to be defined.

Placentation in rodents is similar to that of humans (hemocorial), i.e., the chorionic cells are in direct contact with maternal blood, making the observations related to congenital infections amenable to extrapolation. However, in animals with other placentation types (endotheliochorial) in which the blood of the mother is not in intimate contact with chorionic cells, such as in carnivores, congenital infection is also possible, suggesting that *Leishmania* spp. have greater capacity to reach the fetus than commonly believed. Transplacental infection of *L. infantum* in dogs has been reported in experimental infections and, more importantly, in natural infections in which 32% of 52 fetuses showed PCR-positive results for different tissues.¹⁵ The epidemiologic implications of transplacental transmission in the principal domestic reservoir of VL are still unknown and should be clarified.

We found that prenatal exposure to both *Leishmania* species led to increased mortality during the early phase of lactation. Accordingly, studies in human populations suggested that even subclinical infections have a negative effect on the general health of humans, as shown by the decreased growth rate of asymptomatic, Montenegro skin test-positive children.³⁵

Interestingly, we found sex-associated differences in hamster susceptibility to challenge infections after prenatal exposure to *L. donovani*. After homologous parasite challenge, males showed decreased weight gain and significantly higher mortality rates at any given time point than female or control male offspring. These results are consistent with those of previous studies with hamsters in which male susceptibility to *L. panamensis* or *L. guyanensis* was comparatively greater than that of females.²⁰ Of potential epidemiologic relevance is the concept that *in utero* exposure to *L. donovani* resulted in increased susceptibility to a homologous parasite challenge, characterized by marked weight loss, splenomegaly, and mortality. From the standpoint of reservoir hosts and transmission dynamics, we speculate that puppies born to infected bitches also may have higher susceptibility to VL and become polysymptomatic earlier or more often, and therefore highly infective to sand flies.^{36,37}

Offspring born to females during the chronic stage *L. donovani* infection had impaired cellular responses to *Leishmania* antigens and the mitogen concanavalin A, suggesting an *in utero* modulation of the immune response. A plausible explanation for these results is the development of specific T regulatory cell populations that induced tolerance and higher susceptibility during the first years of age, as reported for malaria^{38,39} and *Wuchereria bancrofti* infection.⁴⁰ The low

proportion of offspring born to females chronically infected with *Leishmania* that responded to leishmanial antigen contrast with the frequent T cell responses observed in dogs congenitally infected with *L. infantum*.¹⁸ Nevertheless, additional studies are necessary to define the parasitologic and host variables that conditioned the cellular immune response to *L. donovani* in the hamster model.

This, experimental study suggested that CL and VL could lead to congenital transmission resulting in symptomatic or asymptomatic infections. These experimental infections suggest that humans and reservoir hosts exposed *in utero* to *L. donovani* may have an increased mortality risk early in life or marked susceptibility to a secondary homologous infection. It could contribute to the maintenance of the transmission cycle and may influence future vaccination strategies. A closer look at vertically acquired infections aimed at defining their relative epidemiologic importance may help to adopt more knowledge-based prevention and control measures.

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