Sperm Morphological Features Associated with Chronic Chagas Disease in the Semen of Experimentally Infected Dogs

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Abstract. The presence of trypanosomatids in the reproductive systems of different mammals (causing genital lesions in the acute stage of the disease) may predispose the animals to low semen quality. However, there are no studies examining the alterations in the sperm morphological features in the chronic stage of *Trypanosoma cruzi* infection. Knowledge of these aspects is important to understand the other ways of transmission of the Chagas disease. Progressive motility, mass motility, concentration, and sperm morphology of 84 ejaculates of dogs that were chronically infected with *T. cruzi* were evaluated. Most of the findings were consistent with the reference values and with those obtained from healthy control dogs. The scrotal circumference was not correlated with spermatozoa concentration in the infected animals. In conclusion, the *T. cruzi* Ninoa (MHOM/MX/1994/Ninoa) strain does not cause significant alterations in the semen quality of dogs experiencing chronic Chagas disease (at concentrations of 5×10^4 to 1×10^6 parasites per animal).

INTRODUCTION

Chagas disease (CD) is caused by *Trypanosoma cruzi* (*T. cruzi*), a hemoflagellate protozoan. Although CD was a Latin America-endemic disease at first, in recent decades, this disease has been observed most often in the United States, Canada, many European countries, and others in the western Pacific. This spread is observed mainly because of population mobility between Latin America and the rest of the world. The CD is now considered to be the sixth leading cause of death among Latin American adults. Treatment is usually successful when the infection is detected in the acute stage of the disease or when the patient suffering from chronic CD is < 15 years of age. The 30% of the people who are in the chronic stage develop cardiac damage and die.¹ Globally, 10 million people are infected, and more than 25 million are at risk of acquiring the disease.² A wide variety of mammalian hosts can also be infected and/or act as wild or domestic reservoirs. In Mexico, the seroprevalence in the dog population ranges between 1.6% and 21% depending on the geographical region of the country. Estado de México, Puebla, Chiapas, Morelos, Campeche, and Yucatán are the only states (6 of 32) where dogs infected with *T. cruzi* have been reported.^{3–6} Therefore, a notably large number of dogs act as reservoirs and as sources of bug blood meals, thereby creating a public health problem.

Trypanosoma cruzi is characterized by chronic chagasic cardiomyopathy and digestive and nervous manifestations. Although sexual transmission has been suggested since the discovery of Chagas disease, it has not been shown to date. The presence of trypanosomatids, such as *Trypanosoma vivax*, *Trypanosoma brucei*, *Trypanosoma congolense*, *T. cruzi*, and *Leishmania* sp., has been reported in the reproductive systems of a wide variety of mammals. Many authors have shown inflammation and parasite colonization in the genital tissues of such animals. These researchers have also

reported the shedding of parasites in the semen accompanied by several reproductive disorders, such as low semen quality, reduced libido, testicular degeneration, impaired spermatogenesis, and alterations in the extracellular matrix of the lamina propria of the seminiferous tubules.^{7–24} The presence of trypanosomatids in the semen of infected dogs indicates the possibility of venereal transmission of CD. Therefore, the use of chagasic dogs as sires should be avoided. The studies of semen quality or damage in the reproductive organs of individuals with CD will help to evaluate the hypothesis that CD affects sexual transmission. This can provide important epidemiological data to increase our knowledge about the natural history of the disease. The aim of this study was to evaluate the semen quality of chronically *T. cruzi* infected dogs.

MATERIALS AND METHODS

In this study, eight male dogs (5 Beagle and 3 mongrel), aged 2.5 (± 0.35) years, weighing 13.3 (± 6.2) kg were used. The animals were inoculated with a well-characterized Mexican *T. cruzi* Ninoa strain^{25,26} (MHOM/MX/1994/Ninoa [*T. cruzi* I])²⁷ by intraperitoneal injection at different times and with different doses of infection (Table 1). The establishment of acute infection was evaluated by examining freshly isolated blood samples that were collected every third day. Two hundred to 400 parasites/mL were observed (as the limits of detection) between day 21 and day 50 post infection only in those animals that were infected with doses of 2×10^5 , 5×10^5 , and 1×10^6 parasites per animal (Table 2). All of the dogs were monitored clinically with general physical examinations and electrocardiographic studies. The infected animals were serologically positive for *T. cruzi* based on standardized enzyme-linked immunosorbent assays and indirect immunofluorescence that were performed using methods described previously.²⁸

The animal handling followed the established guidelines of the International Guiding Principles for Biomedical Research involving Animals and the Norma Oficial Mexicana: Technical Specifications for the Care and Use of Laboratory

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TABLE 1
Physical features, times, and doses of infection of chronic chagasic experimental dogs

Dog no.	Breed	Age (months)	Weight (kg)	Time of infection (months)	Metacyclic trypomastigotes* by intraperitoneal injection (mt/IP)
1	Beagle	30	11.0	Control healthy	–
2	Beagle	24	12.6	Control healthy	–
3	Beagle	26	15.0	24	1×10^6
4	Beagle	26	15.0	24	5×10^5
5	Beagle	26	10.0	24	5×10^5
6	Mongrel	34	27.0	32	2×10^5
7	Mongrel	34	7.6	32	5×10^4
8	Mongrel	34	8.0	32	5×10^4

*The *Trypanosoma cruzi* Ninoa (MHOM/MX/1994/Ninoa) strain that was used was maintained by serial passage in reduviid vectors. Metacyclic trypomastigotes were obtained from urine and feces of triatomines and resuspended in physiological saline solution.

Animals.²⁹ The experimental protocol was approved by the Bioethics Committee of the Instituto Nacional de Cardiología, Ignacio Chávez.

General physical examinations evaluating the mental state and respiratory patterns of the dogs were performed. Vital signs, including rectal temperature, capillary refill time, beats per minute, and exploration for enlarged lymph nodes were recorded.

The male reproductive tract was examined by inspection and palpation of the scrotum, epididymis, testes, penis, prepuce, and prostate gland. The size, symmetry, content, consistency, skin thickness, lesions, anatomic abnormalities, and cryptorchidism were evaluated.

Testicular measurements were made while each dog was restrained in a standing position, and the measurements were obtained by a single person (E.P.-M). The testes were aligned side by side and pushed down into the distal part of the scrotum at the level of the spermatic cord. The measurement was recorded at the widest point with the help of a flexible measuring tape.

The male dogs were trained for a month before the start of the experiment such that their semen could be collected by digital manipulation. Twelve ejaculates were collected from the control and the infected dogs using a routine protocol with intervals ranging from 7 to 12 days.³⁰

The second fraction of the ejaculate, corresponding to the sperm-rich fraction, was collected in a separate tube using a pre-warmed plastic funnel attached to a graduated 15-mL Falcon conical tube (Becton Dickinson, Franklin Lakes, NJ), although taking care to avoid contamination with the two other (pre-spermatic and prostatic) fractions. The sperm-rich fraction was kept at 37°C and was examined immediately after harvest to determine the appearance, pH, volume, mass

motility, progressive motility, sperm concentration, percentages of viable sperm, and morphologically abnormal and immature sperm using routine methods.^{30–32} Briefly, the pH of the semen was measured using pH test strips. The progressive motility (percentage of linear and forward movement of spermatozoa) and the mass motility (scores 0–5; 0 = absence of movement, 1 = lesser speed, and 5 = faster speed or vigorous movement) were visually evaluated by a single person (E.P.-M) with an optical microscope (Leitz, Wetzlar, Germany) under 400× magnification using a small drop of semen between the coverslip and a warmed slide glass. The concentration of sperm in the semen was determined by diluting 20 µL of semen in 1.0 mL of saline formaldehyde solution and by hemacytometer counts (number of sperm cells/mL of semen). The number of sperm cells/ejaculate was obtained by multiplying the number of sperm cells/mL by the ejaculate volume. The percentages of viable sperm were estimated by counting actively motile sperm on a warmed slide glass and by the eosin-nigrosin staining method. The sperm morphology (percentage of the sperm defects) was examined by the eosin-nigrosin staining method using phase-contrast microscopy (Leitz, Germany) under 1,000× magnification. Spermatozoa with a cytoplasmic droplet attached to the mid-piece were counted as immature sperm.

The dogs were euthanized using sodium pentobarbital (Barbital, Holland Animal Health, Mexico) as a general anesthetic at a dose of 30 mg/kg applied intravenously, and a lethal dose of intravenous 15% potassium chloride was subsequently administered. Testis sections were fixed in 10% buffered formalin solution for 24 h, dehydrated in absolute ethanol, cleared in xylene, and embedded in paraffin for histological examination. Sections (5 µm) were stained with hematoxylin and eosin and evaluated by light microscopy (Carl Zeiss, K7, Jena, Germany). Images were obtained through a Bio-Doc-It Imaging System image analyzer (UVP, LLC, Upland, CA).

Statistical analyses were performed using the SPSS 13.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL). The differences between the means were analyzed statistically using analysis of variance or two-way analysis of variance followed by the Tukey test and were considered to be significant when $P \leq 0.05$. Regression analysis was used to correlate the testicular size (scrotal circumference) with the sperm-concentration data from semen analysis (calculated using Excel software [Microsoft 2007, Redmond, WA]).

Electrocardiograms and echocardiograms performed on month eight post-inoculation generated data consistent with ischemia, local conduction abnormalities, hypertrophy, diminished ejection fraction, left ventricular dilation, right bundle

TABLE 2

Parasitemia detection in experimentally infected dogs with *Trypanosoma cruzi*

Dog no.	Days after inoculation						
	10–15	16–20	21–25	26–30	31–35	36–40	41–45
1 (Beagle)							
2 (Beagle)							
3 (Beagle)			++				
4 (Beagle)				+		++	++
5 (Mongrel)					+	++	+
6 (Mongrel)					+	+	+
7 (Mongrel)				ND	ND	ND	ND
8 (Mongrel)				ND	ND	ND	ND

+ Detection of 1 or 2 parasites per blood sample of fresh drop examination.
ND = not determined.

TABLE 3
Semen quality* from chronically *Trypanosoma cruzi*-infected dogs (means $\pm$ SD)

Dog no.	Semen 2nd fraction volume(mL)	pH	Mass motility (0-5)	Progressive motility (%)	Sperm concentration/mL ($\times 10^6$)	Normal sperm (%)	Primary abnormalities† (%)	Secondary abnormalities‡ (%)	Viable sperm (%)
1 (Beagle)	1.3 $\pm$ 0.7	6.5 $\pm$ 0.3	2.8 $\pm$ 1.1	89.4 $\pm$ 5.9	173.3 $\pm$ 86.2	89.6 $\pm$ 3.6	4.8 $\pm$ 3.3	5.6 $\pm$ 1.3	89.9 $\pm$ 3.5
2 (Beagle)	2.0 $\pm$ 0.5	6.5 $\pm$ 0.4	4.2 $\pm$ 0.9	92.6 $\pm$ 3.7	224.2 $\pm$ 112.2	92.8 $\pm$ 3.6	3.0 $\pm$ 2.2	4.3 $\pm$ 2.3	90.9 $\pm$ 5.6
Means $\pm$ SD	1.6 $\pm$ 0.5	6.5 $\pm$ 0.0	3.5 $\pm$ 0.9	91.1 $\pm$ 2.4	198.8 $\pm$ 35.9	91.2 $\pm$ 2.2	3.9 $\pm$ 1.3	4.9 $\pm$ 0.9	90.4 $\pm$ 0.7
3 (Beagle)	2.3 $\pm$ 0.3	6.7 $\pm$ 0.3	4.1 $\pm$ 1.0	91.7 $\pm$ 3.3	310.8 $\pm$ 110.7	92.8 $\pm$ 3.2	2.6 $\pm$ 2.1	4.6 $\pm$ 2.8	89.6 $\pm$ 4.2
4 (Beagle)	1.8 $\pm$ 0.9	6.7 $\pm$ 0.3	2.5 $\pm$ 1.1	89.6 $\pm$ 4.5	158.3 $\pm$ 81.5	91.7 $\pm$ 3.2	3.8 $\pm$ 2.1	4.6 $\pm$ 2.1	89.9 $\pm$ 4.2
5 (Mongrel)	ND	ND	ND	ND	ND	ND	ND	ND	ND
6 (Mongrel)	2.0 $\pm$ 0.7	6.8 $\pm$ 0.5	3.2 $\pm$ 1.2	86.3 $\pm$ 10.7	194.2 $\pm$ 94.6	88.8 $\pm$ 4.2	3.8 $\pm$ 2.5	7.4 $\pm$ 3.6	85.8 $\pm$ 5.6
7 (Mongrel)	2.0 $\pm$ 1.0	6.6 $\pm$ 0.2	4.0 $\pm$ 0.9	92.9 $\pm$ 3.3	211.7 $\pm$ 131.9	92.58 $\pm$ 3.75	3.3 $\pm$ 1.9	4.1 $\pm$ 2.6	92.3 $\pm$ 3.0
8 (Mongrel)	1.0 $\pm$ 0.3	6.8 $\pm$ 0.3	4.1 $\pm$ 1.0	93.6 $\pm$ 2.8	239.2 $\pm$ 62.6	86.7 $\pm$ 7.2	2.0 $\pm$ 0.9	11.4 $\pm$ 7.7	93.5 $\pm$ 2.5
Means $\pm$ SD	1.8 $\pm$ 0.5	6.7 $\pm$ 0.1	3.6 $\pm$ 0.7	90.8 $\pm$ 3.0	222.8 $\pm$ 57.3	90.5 $\pm$ 2.7	3.1 $\pm$ 0.8	6.4 $\pm$ 3.1	90.2 $\pm$ 2.9

*Twelve semen collections were performed at intervals of 7–12 days.

†The primary abnormalities are those originating during spermatogenesis, such as micro- and macrocephalic sperms; elongated, pyriform, and double heads; double, swollen, bent, or attached midpieces; and, double and bent tails.

‡The secondary abnormalities are those that occur during transit from the testes to the epididymis or as a result of the handling of semen, such as detached heads, midpieces, and tails; proximal or distal cytoplasmic droplets; and coiled or bent sperms.

No significant differences were observed ($P > 0.05$). ND = not determined.

branch blocks, or ventricular premature complexes.^{28,33,34} However, mortalities were not observed during the study.

RESULTS

All of the control and experimental dogs appeared to be reproductively healthy. Only dog #5 was excluded (Table 3) because of a lack of libido, and dog #4 always needed the olfactory and visual stimulus of a teaser bitch (in any stage of the estral cycle). Both testicles and epididymis were normal on palpation. The total scrotal widths of the infected dogs averaged 15.0 ± 1.6 cm. The mean of this parameter was not different from that of healthy control dogs ($P > 0.05$). Rectal palpation of the prostatic glands showed no anomalies: the glands were smooth, bilobed, symmetrical, non-painful, and easily movable.

The appearance of the collected semen was whitish or milky for all of the dogs. The ejaculate characteristics of the control and experimental dogs are presented in Table 3. There were no statistically significant differences in the quality of the semen from two healthy dogs and five infected males. Almost all of the sperm quality parameters were in the normal ranges, except that the ejaculate volume of the second fraction was slightly higher and the mass motility was slightly lower in both groups of analyzed dogs. The ranges of the examined samples of the infected group were as follows: semen second-fraction volume between 1.8 and 2.0 mL, pH from 6.6 to 6.8, mass motility between 2.5 and 4.1, percentage of progressively motile sperm between 86.3% and 93.6%, sperm concentration from 158 to 239×10^6 cells/mL, live proportion between 85.8% and 93.5%, and morphologically normal spermatozoa from 86.7% to 92.8%.

In this study, a correlation of $r = 1.0$ was observed between the scrotal circumference (14.5 ± 2.12 cm) and the sperm concentration ($198.75 \pm 35.95 \times 10^6$ cells/mL) for control healthy dogs. In the chronically infected dogs, the scrotal circumference (15 ± 1.58 cm) was not correlated with the sperm concentration ($222.83 \pm 57.27 \times 10^6$ cells/mL), as determined by regression analysis ($r = 0.101$).

A comparison of semen parameters was performed among the five dogs that were experimentally infected with different concentrations of metacyclic trypomastigotes. The data analysis showed no significant differences in the macro and microscopic seminal features of these animals. A comparison was made

between the ejaculates of healthy and infected Beagle dogs (excluding mongrel dogs). The results showed no significant differences in semen characteristics, with mean values that are very similar to those observed in Table 3.

FIGURE 1. Micrographs of testicular structures of chronically *Trypanosoma cruzi*-infected dogs. (A) Seminiferous tubules (H & E $\times 400$), (B) epididymal tubes filled with sperm (H & E $\times 100$). Four hundred fields per dog were analyzed.

All the sections of testes showed seminiferous tubules with abundant spermatogonia at different stages of maturation. The epithelium of seminiferous tubules was stratified into 10–14 layers and abundant mitosis indicating active maturation was observed. In the lumen of the seminiferous tubules were observed spermatids, precursors of spermatozoa. The lumen of the epididymal tubes were filled with sperm. No evidence of inflammation related to the *T. cruzi* infection was detected; neither amastigote nests nor trypomastigotes were found (Figure 1).

DISCUSSION

The pathological effects that are associated with trypanosomatid infections in the genitalia of male domestic, laboratory, and wild animals have been studied and reported.^{7–10,12,15,16,18–24} However, information is scarce and controversial regarding *T. cruzi* infections (that affect the reproductive system) and the direct transmission of such infections through coitus.^{11,13,14} The latest report by Carvalho and others¹⁷ showed that *T. cruzi* can colonize different cells, including myoid cells, and suggested the possibility of parasite migration to the seminal fluid in case of a rupture of these cells. Therefore, the CD could potentially be transmitted through sexual intercourse.

In this study, changes did not occur in the testes or prostate glands of the chronically infected animals. However, it has been reported that total scrotal width or scrotal circumference (in addition to testicular volume) is highly correlated with testicular size. Some authors have reported that these are useful predictors for daily spermatozoa production and, consequently, for fertility in bulls, rams, dogs, and other species including humans.^{35–37} In this study, it was shown that the sperm counts of healthy animals but not of chronic chagasic dogs are dependent on the scrotal perimeter. This finding suggests that the scrotal circumference could be useful for predicting the production and ejaculation of sperm only in healthy males. These data are similar to those obtained by Olar and others³⁵ who found a high correlation ($r = 0.75$) between testicular volume and sperm ejaculated daily in dogs. However, the data are discordant with those of England³⁸ and Cortez and others,³⁹ whose results showed low correlations between the parameters.

The mass motility parameter showed means of 3.5 and 3.6 in healthy and chronically infected chagasic dogs, respectively. Both values were slightly lower than those reported by others (who obtained averages between 4.0 and 4.6).^{40,41}

There are no reports available that mention if the pH is affected by the trypanosomatid infection. The pH values reported for the semen of healthy dogs vary from 6.3 to 6.7.³⁹ In this study, the pH values of the semen in healthy and chronically infected dogs were within the reported range, 6.5 and 6.7, respectively. The progressive motility, sperm concentration, abnormalities of spermatozooids, and viability exhibited means (in both healthy control and chronically infected dogs) that are in accordance with the reference values.^{30,39,40}

These results indicate that chronic infection by *T. cruzi* had no effect on the reproductive parameters of male dogs. This result differs from the findings reported by other authors who carried out experiments in *Leishmania chagasi*-infected dogs,²⁰ *T. congolense*-infected New Zealand White rabbits,²¹ and *T. brucei*-infected West African dwarf bucks,¹⁸ or red fronted gazelles²² during the acute stage of infection.

These differences are attributable to the variety of the animal species that were analyzed and are observed because those authors conducted their evaluations during the acute stage of infection: 5 weeks,²¹ 7 weeks,²² and 10 weeks¹⁸ post infection, when both hyperthermia and inflammation are present. It is well known that elevated body temperature adversely affects spermatogenesis, suggesting the presence of testicular degeneration that involves thrombosis of the testicular vessels leading to ischemic necrosis. Thermal degeneration is experienced especially at the peak of hyperthermia and anoxia.¹⁸ These findings suggest that the reproductive vitality is reestablished once the acute stage has passed or when appropriate drugs are administered, such as diminazene aceturate^{9,21,22} or combined dexamethasone and gonadotropin-releasing hormone (GnRH).¹²

It is likely that the infective parasitic form used, the doses used, and the route of administration performed also influence the pathological effects of CD on the reproductive system. The doses of blood trypomastigotes used in other studies^{18,21,22} vary from 4.8×10^5 to 10×10^6 , and differ from our study by more than an order of magnitude. The routes of inoculation were intraperitoneal,^{18,22} intravenous,²² and a natural infection.²⁰ In this study, the metacyclic trypomastigotes (the natural infective form) were inoculated by intraperitoneal means (which simulate the natural route of infection by the transmission vector) at doses of 5×10^4 to 1×10^6 .

The abnormalities in the sperm morphology during the acute phase of the disease, the shedding of *T. cruzi* through the semen of acute infected dogs, the parasitic colonization in the reproductive organs, and the effects on the reproductive system of the different strains, inocula and/or routes of administration all require further elucidation. In conclusion, the *T. cruzi* Ninoa (MHOM/MX/1994/Ninoa) strain did not cause significant alterations in dog semen quality during the chronic phase of the CD at doses of 5×10^4 to 1×10^6 metacyclic trypomastigotes per animal (intraperitoneally inoculated).

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