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 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 $5 \times 10^4$ to $1 \times 10^6$ parasites per animal).

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 $5 \times 10^4$ to $1 \times 10^6$ parasites per animal).

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 strainMHOM/MX/1994/Ninoa [T. cruzi I] 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 $2 \times 10^5$, $5 \times 10^5$, and $1 \times 10^6$ 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.

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
After harvest to determine the appearance, pH, volume, mass

Parasitemia detection in experimentally infected dogs with

Animals. The experimental protocol was approved by the Bioethics Committee of the Instituto Nacional de Cardiologia, 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.

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. 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, Wetzler, 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 hematocytometer 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).

Table 1

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Breed</th>
<th>Age (months)</th>
<th>Weight (kg)</th>
<th>Time of infection (months)</th>
<th>Metacyclic trypomastigotes* by intraperitoneal injection (mt/IP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beagle</td>
<td>30</td>
<td>11.0</td>
<td>Control healthy</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Beagle</td>
<td>24</td>
<td>12.6</td>
<td>Control healthy</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Beagle</td>
<td>26</td>
<td>15.0</td>
<td>24</td>
<td>1 × 10⁶</td>
</tr>
<tr>
<td>4</td>
<td>Beagle</td>
<td>26</td>
<td>15.0</td>
<td>24</td>
<td>5 × 10⁵</td>
</tr>
<tr>
<td>5</td>
<td>Beagle</td>
<td>26</td>
<td>10.0</td>
<td>24</td>
<td>5 × 10⁵</td>
</tr>
<tr>
<td>6</td>
<td>Mongrel</td>
<td>34</td>
<td>27.0</td>
<td>32</td>
<td>2 × 10³</td>
</tr>
<tr>
<td>7</td>
<td>Mongrel</td>
<td>34</td>
<td>7.6</td>
<td>32</td>
<td>5 × 10⁴</td>
</tr>
<tr>
<td>8</td>
<td>Mongrel</td>
<td>34</td>
<td>8.0</td>
<td>32</td>
<td>5 × 10⁴</td>
</tr>
</tbody>
</table>

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

Table 2

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Beagle)</td>
<td>10-15</td>
</tr>
<tr>
<td>2 (Beagle)</td>
<td></td>
</tr>
<tr>
<td>3 (Beagle)</td>
<td></td>
</tr>
<tr>
<td>4 (Beagle)</td>
<td></td>
</tr>
<tr>
<td>5 (Mongrel)</td>
<td></td>
</tr>
<tr>
<td>6 (Mongrel)</td>
<td></td>
</tr>
<tr>
<td>7 (Mongrel)</td>
<td></td>
</tr>
<tr>
<td>8 (Mongrel)</td>
<td></td>
</tr>
</tbody>
</table>

*pDetection of 1 or 2 parasites per blood sample of fresh drop examination. ND = not determined.
branch blocks, or ventricular premature complexes. 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 $\times$ 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 ± 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 ± 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.

### Table 3

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>2nd fraction volume (mL)</th>
<th>pH</th>
<th>Progressive motility (%)</th>
<th>Sperm concentration (x10^6/mL)</th>
<th>Normal sperm (%)</th>
<th>Primary abno. (%)</th>
<th>Secondary abno. (%)</th>
<th>Viable sperm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Beagle)</td>
<td>1.3 ± 0.7</td>
<td>6.5 ± 0.3</td>
<td>2.8 ± 1.1</td>
<td>89.4 ± 5.9</td>
<td>173.5 ± 86.2</td>
<td>89.6 ± 3.6</td>
<td>4.8 ± 3.3</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>2 (Beagle)</td>
<td>2.0 ± 0.5</td>
<td>6.5 ± 0.4</td>
<td>4.2 ± 0.9</td>
<td>92.6 ± 3.7</td>
<td>224.2 ± 112.2</td>
<td>92.8 ± 3.6</td>
<td>3.0 ± 2.2</td>
<td>4.3 ± 2.3</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>1.6 ± 0.5</td>
<td>6.5 ± 0.0</td>
<td>3.5 ± 0.9</td>
<td>91.1 ± 2.4</td>
<td>198.8 ± 35.9</td>
<td>91.2 ± 2.2</td>
<td>3.9 ± 1.3</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>3 (Beagle)</td>
<td>2.3 ± 0.3</td>
<td>6.7 ± 0.3</td>
<td>4.1 ± 1.0</td>
<td>91.7 ± 3.3</td>
<td>310.8 ± 110.7</td>
<td>92.8 ± 3.2</td>
<td>2.6 ± 2.1</td>
<td>4.6 ± 2.8</td>
</tr>
<tr>
<td>4 (Beagle)</td>
<td>1.8 ± 0.9</td>
<td>6.7 ± 0.3</td>
<td>2.5 ± 1.1</td>
<td>89.6 ± 4.5</td>
<td>158.3 ± 81.5</td>
<td>91.7 ± 3.2</td>
<td>3.8 ± 2.1</td>
<td>4.6 ± 2.1</td>
</tr>
<tr>
<td>5 (Mongrel)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6 (Mongrel)</td>
<td>2.0 ± 0.7</td>
<td>6.8 ± 0.5</td>
<td>3.2 ± 1.2</td>
<td>86.3 ± 10.7</td>
<td>194.2 ± 94.6</td>
<td>88.8 ± 4.2</td>
<td>3.8 ± 2.5</td>
<td>7.4 ± 3.6</td>
</tr>
<tr>
<td>7 (Mongrel)</td>
<td>2.0 ± 1.0</td>
<td>6.6 ± 0.2</td>
<td>4.0 ± 0.9</td>
<td>92.9 ± 3.3</td>
<td>211.7 ± 131.9</td>
<td>92.5 ± 3.7</td>
<td>3.3 ± 1.9</td>
<td>4.1 ± 2.6</td>
</tr>
<tr>
<td>8 (Mongrel)</td>
<td>1.0 ± 0.3</td>
<td>6.8 ± 0.3</td>
<td>4.1 ± 1.0</td>
<td>93.6 ± 2.8</td>
<td>239.2 ± 62.6</td>
<td>86.7 ± 7.2</td>
<td>2.0 ± 0.9</td>
<td>11.4 ± 7.7</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>1.8 ± 0.5</td>
<td>6.7 ± 0.1</td>
<td>3.6 ± 0.7</td>
<td>90.8 ± 3.0</td>
<td>222.8 ± 57.3</td>
<td>90.5 ± 2.7</td>
<td>3.1 ± 0.8</td>
<td>6.4 ± 3.1</td>
</tr>
</tbody>
</table>

* 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.

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 × 400), (B) epididymal tubes filled with sperm (H & E × 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. However, information is scarce and controversial regarding *T. cruzi* infections (that affect the reproductive system) and the direct transmission of such infections through coitus. The latest report by Carvalho and others showed that *T. cruzi* can colonize different cells, including myod 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.

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 others 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 England and Cortez and others, whose results showed low correlations between the parameters.

There are no available reports that mention if the pH is affected by the trypansomatid infection. The pH values reported for the semen of healthy dogs vary from 6.3 to 6.7. In this study, the pH values of the semen in healthy and chronically infected dogs, respectively. Both values were slightly lower than those reported by others (who obtained averages between 4.0 and 4.6).

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.

In conclusion, 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 $5 \times 10^4$ to $1 \times 10^6$ metacyclic trypomastigotes per animal (intraperitoneal inoculated).

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