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Original Research

Sexual Dimorphism in the Physiopathology and Immune Response during Acute *Toxocara canis* Infection

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Abstract

Background: *Toxocara canis* (*T. canis*) is a helminth parasite of zoonotic and veterinary health significance that causes the disease known as Toxocariasis. This disease has been associated with conditions of poverty, especially in tropical climate zones throughout the world. Although it rarely causes important clinical manifestations, *T. canis* can lead to blindness, meningoencephalitis, or other nervous manifestations in humans. Moreover, some studies show its importance in the development of tumor growth, which have been associated with the parasite's ability to modulate the host's immune response. While different studies have evaluated the immune response during this disease, currently, there are no studies where the infection is analyzed from the perspective of sexual dimorphism. **Methods**: To evaluate sex differences in susceptibility, we analyzed lesions and parasite loads in lung and liver at 7 days post-infection. In addition, immune cell subpopulations were analyzed in spleen, mesenteric and peripheral lymph nodes. Finally, the production of cytokines and specific antibodies were determined in the serum. Statical analyses were performed using a Two-way ANOVA and a post-hoc Bonferroni multiple comparison test. **Results**: Female rats had a higher number of larvae in the liver, while male rats had them in the lungs. The percentages of immune cells were evaluated, and in most cases, no significant differences were observed. Regarding the cytokines production, infection can generate a decrease in Th1 such as IL-1β in both sexes and IL-6 only in females. In the case of Th2, IL-4 increases only in infected males and IL-5 increases in males while decreasing in females due to the effect of infection. IL-10 also decreases in both sexes as a consequence of the infection, and TGF-β only in females. Finally, the infection generates the production of antibodies against the parasite, however, their quantity is lower in females. **Conclusions**: This study demonstrates that *T. canis* infection is dimorphic and affects females more than males. This is due to a polarization of the inadequate immune response, which is reflected as a higher parasite load in this sex.

Keywords: *Toxocara canis*; zoonoses; sex differences; paratenic host; larval migration; dimorphic cytokine production

1. Introduction

Sex-associated differences in the immune response can be defined as the variations in cellular and molecular events in the immune system that exist when the organism is exposed to an antigenic challenge caused by a virus, bacteria, or a parasite. These differences have been associated with the role of hormones such as sex steroids that play an important role in the regulation of the immune system [1–5]. During different studies where the immune response against helminths has been evaluated, previous studies have evaluated the influence of hormones on the immune function. It has been observed that sex (male or female) plays an important role in the regulation of the immune response. Therefore, it has generally been demonstrated that one sex is more susceptible than the other to different parasite infections [6–9]. In some parasitic infections, hormonal regulation has been associated with larval reactivation that is vertically transmitted (transplacental, lactogenic) from mother to offspring, as is the case of Toxocariasis [10,11]. However, so far, it has not been described whether there is a dimorphic effect on the immune response that could influence the migration of the larvae towards organs such as the uterus and the mammary gland, thereby modifying susceptibility in males and females.

Toxocara species are parasitic nematodes whose definitive hosts are dogs and cats. *Toxocara canis* (*T. canis*) infects dogs as the definitive host, whereas *Toxocara cati* (*T. cati*) is found in cats. Additionally, this parasite can affect humans; therefore, it is considered a zoonotic disease; as well as other mammals, birds, and invertebrates among

others, causing the disease known as Toxocariasis [11–13]. In humans, the infection can go from asymptomatic to produce different clinical manifestations caused by the migration of *T. canis* larvae through the bloodstream and other organs. This disease can be classified as Visceral Larva Migrans (VLM), Neurotoxocariasis (NT), Ocular Larva Migrans (OLM), and Covert Toxocariasis (CT) [14–16].

During *T. canis* infection, the predominant immune response is a Th2 type, although a dichotomy has also been mentioned in terms of the polarization of the immune response. It is possible to find molecules of both the Th1 and/or Th2 type. This response may be influenced by aspects such as the host species (dog, cat, mouse, rat, human), time of infection (acute or chronic), and target organ (lung, liver, brain), among others. However, it is worth mentioning that, currently, the immune response during *T. canis* infection has not been analyzed from a sex perspective. In general, the immune response is assembled against somatic antigens and excretory-secretory antigens (TcES-Ag) [17–19]. This response is characterized by a strong adaptive immune response, where a Th2-type response predominates during the chronic infection. The Th2 response is characterized by the production of cytokines such as interleukin (IL) IL-4, IL-5, IL-6, IL-13, IL-33, and regulatory cytokines such as IL-10, among others [17–21]. However, during the acute phase of infection, a strong pro-inflammatory response takes part in the immunological profile, where a mixed response with inflammatory cytokines of innate origin, together with Th17 and Th2 type cytokines has been reported [22].

Th2 cytokines are involved in the activation of other immune cell subpopulations such as mast cells, macrophages, eosinophils, and the secretion of Immunoglobulins (Ig) IgE and IgG1 [19,20]. These cytokines and Ig are associated with leukocytosis, peripheral blood eosinophilia, eosinophilic infiltration around larval sites of migration, as well as specific antibody production [22].

Macrophages also play an important role during infection, and macrophages obtained from mice within ten days of infection with *T. canis*, and cultured *in vitro* with lipopolysaccharide, have been reported to produce higher amounts of IL-10 and tumor growth factor-beta (TGF-β), and lower amounts of Th1 cytokines such as IL-12 and tumor necrosis factor-alpha (TNF-α) [23]. Later, macrophages that display a different phenotype from the classical ones, namely, alternatively activated macrophages (AAMs), are involved in the initiation of immune responses against helminths, as well as in tissue repair [24–26]. In addition, some helminths can modulate the immune response by inducing the formation of T and B regulatory cells, which also produce IL-10 and TGF-β [27], the former being one of the strategies that help parasites evade the host immune response. In the case of *T. canis* infection in paratenic hosts, it has been determined that regulatory T cells participate in immunopathological events at the hepatic level from 5 weeks post-infection [28], while in definitive hosts it was shown that the secreted and excreted antigens of *T. canis* increase the frequency of regulatory T cells and the production of IL-10 [29].

In addition, different studies have shown that the immune system can be regulated by different hormones, such as sex steroids [30–34]. On this basis, differences in susceptibility and the associated immune response have also been reported in some other parasitic diseases [6,8,9,35–39]. Nonetheless, as far as we are concerned, there is a lack of information that demonstrates the immune modulation caused by sex steroids during Toxocariasis. Within this framework, this study aims to evaluate susceptibility, immune response, and immune effector mechanisms during acute *T. canis* infection from the point of view of immune sexual dimorphism, to describe an immunomodulatory effect that could increase susceptibility to infection to this parasite in male or females.

2. Materials and Methods

2.1 Ethics Steatment

The protocol for the use and care of the animals was endorsed by both Institute's Animal Care and Use Committee (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio, CICUAL, permit number 201-2016) following the official Mexican regulations (NOM-062-ZOO-1999). These regulations are in accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health of the USA, ensuring compliance with established international regulations and guidelines.

2.2 Experimental Animals

Twenty-eight male and Twenty-eight female Wistar rats of sixty days old, were obtained from the experimental animal facility of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. The rats were divided into four groups and allocated in polycarbonate boxes (50 cm L $\times$ 23 cm W $\times$ 21 cm H). The groups were set as follows: (I) Control males (♂ Ctrl $n = 14$), (II) Infected males (♂ Infx $n = 14$), (III) Control females (♀ Ctrl $n = 14$), and (IV) Infected females (♀ Infx $n = 14$). The animals were kept in cycles of 12 hours light/darkness. Water and food were provided *ad libitum* in sterile conditions.

2.3 Collection and Processing of T. canis Eggs

T. canis eggs were obtained from adult parasites donated by Centro de Control Canino de Cuautitlán, Estado de México. Female worms were separated, rinsed in tap water, and placed in phosphate buffer solution (PBS) with a pH around 7.2–7.4. Subsequently, uteri were collected through an incision in the first third of the body and placed in a physiological saline solution. Eggs were obtained using a fine pore filter. The ova were washed several times in PBS and centrifuged at 3250 $\times$g for 5 minutes. Finally,

the eggs from the pellet were resuspended in a PBS 1×/2% formaldehyde solution and incubated at 27 °C for 28 days to obtain 80–90% of the infective form (larvated eggs) of the parasite.

2.4 Rats Infection

Male and female rats were infected with 1000 *T. canis* larvated eggs at sixty days old. The inoculum for the infection was washed three times to eliminate the PBS/formaldehyde solution. Eggs were then resuspended in PBS and concentrated to 1000 larvated eggs per ml. The infection was performed by intragastric administration using a type of Foley metallic probe. Seven days later, the rats were humanely euthanized using sevoflurane (Sevorane®) anesthetic overdose.

2.5 Serum Obtention

Right after the rats were euthanized, blood was collected in Serum Separation Tube (SST)-Vacutainer tubes BD (BD Biosciences, San Diego, CA, USA). After collection, the blood was allowed to clot by leaving it undisturbed at room temperature for 3 minutes and the clot was removed by centrifuging at 2000 ×g for 10 minutes in a refrigerated centrifuge. Following centrifugation, the serum was immediately transferred into a clean glass tube using a Pasteur pipette. Serum samples were then stored at –20 °C until further analysis.

2.6 Larvae Recovery from Lung and Liver

The lungs and liver of infected rats were weighed, and small pieces of these organs were obtained by cutting them off. The tissue was then macerated in PBS 1×. For digestion, artificial gastric juice [1% pepsin (250 units/mg, SIGMA- ALDRICH®, St Louis, MO, USA) and 1% HCl 37%. pH: 2.0 (10 mL of artificial gastric juice/1 gr of tissue)] was used, and the process lasted for 24 hours. After digestion, samples were centrifuged at 791 ×g for 5 minutes, and the pellet was resuspended in 10 mL of 4% paraformaldehyde. Parasite counting involved 10 counts per 20 µL sample, and the total number of larvae was multiplied by 50 to calculate the number of larvae per ml and, consequently, the number of larvae per gram of tissue.

2.7 Flow Cytometry Assay

For the assay, spleen, mesenteric lymph nodes (MLN), inguinal, and axillar peripheral lymph nodes (PLN) were collected from rats immediately after euthanasia. The samples were disaggregated using a sterile nylon mesh (50 µm) and a syringe plunge in PBS at 4 °C. The splenic cell suspension was centrifuged at 182 ×g for 3 min, decanted, and resuspended in 500 µL of ACK buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.3, JT Baker, CDMX, Mexico) for 10 min at room temperature to lyse erythrocytes present in the sample. Afterward, 700 µL of Fluorescent Activated Cell Sorting (FACS) buffer was added, followed by centrifugation at 182 ×g for 3 min. All samples (Spleen and lymph nodes cells) were washed with PBS 1×, fixed with 4% paraformaldehyde (SIGMA- ALDRICH®, St Louis, MO, USA) for 10 min/37 °C; and then resuspended in 500 µL FACS buffer. Finally, cells were transferred to 96-well plates (Costar®, Coppell, TX, USA).

For extracellular staining, cells were incubated with a primary antibody solution for 10 min at 4 °C. The following selected primary antibodies were used: AF 488 anti-rat CD3 (Biolegend, Clone 1F4, San Diego, CA, USA) (1:100), PE/Cy5 Mouse anti-rat CD4 (BD bioscience. Clone Ox-35) (1:300), PE Mouse anti-rat CD8α (BD bioscience. Clone Ox-8) (1:200), PE anti-rat CD45RA (Biolegend. Clone Ox-33) (1:200), PE anti-rat TCR$\gamma\delta$ (Biolegend. Clone V65) (1:200), AF647 anti-rat CD161 (Biolegend. Clone 10/78) (1:200) and anti-rat CD11b/c-biotin (Biolegend Clone Ox-42) (1:200) + PE/Cy5 Streptavidin (Biolegend) (1:200). Primary antibodies were set in 3 different groups: T1; AF 488 anti-rat CD3 + PE/Cy5 Mouse anti-rat CD4 + PE Mouse anti-rat CD8α. T2; AF 488 anti-rat CD3 + PE anti-rat CD45RA + anti-rat CD11b/c-biotin. T3; AF647 anti-rat CD161 + PE anti-rat TCR$\gamma\delta$. Cells were washed twice with 150 µL FACS buffer after incubation, resuspended in 200 µL of FACS buffer, and stored at 4 °C in the dark. Cell analysis was performed with an Attune NEXT (Applied Bioscience®, Beverly Hills, CA, USA) flow cytometer in the National Laboratory of Flow Cytometry. Data analysis was performed using FlowJo software v10.0 (Treestar Inc.®, Ashland, OR, USA). Isotype controls were used for each of the organs analyzed to prepare the reading gates in the cytometer.

2.8 Multiplex Assay

Eight different cytokines: IL-1β, IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10 and IL-13, were measured using the MILLIPLEX®MAP Rat Cytokine/Chemokine Magnetic Bead Panel (RECYTMAG-65K) (Merck KGaA, Darmstadt, Germany). TGF-β1 was determined with MILLIPLEX®MAP TGF-β1 Single Plex Magnetic Bead Kit (TGFBMAG-64K-01) (Merck KGaA, Darmstadt, Germany). Frozen serum samples were thawed and prepared according to manufacturer's instructions. Median fluorescence intensity was obtained from duplicates of serum samples using a MAGPIX from LUMINEX (Seattle, WA, USA) by Millipore (San Luis, MX, USA), facilitated at the Unidad de Investigación from Facultad de Medicina Veterinaria y Zootecnia, UNAM. The obtained data was further analyzed with Myassays software package (version 10.2.75.625) using the Four Parameter Logistic Curve fitting method to calculate the sample cytokine concentration (MyAssays.com).

2.9 T. canis Antigens Purification and Quantification

TcES-Ag was obtained by culturing the larvae. *T. canis* embryonated eggs were centrifuged at 3250 ×g for 5 min. To disaggregate the outer egg layer, 1 ml of sodium

hypochlorite was added. After 10 min of continuous agitation, eggs were washed with 10 ml of Milli-Q water, centrifuged at 3250 ×g for 5 min, and washed 3 times with PBS 1×. Eggs were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, GrandIsland, NY, USA) with 1% antibiotic-antimycotic (GIBCO, GrandIsland, NY, USA). Larval hatching was stimulated with magnetic stirrer for 20 minutes, and the egg suspension was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 overnight. Larvae were separated from eggshells through a Baermann technique. The supernatant was collected weekly filtered with a 0.2 µm syringe filter (Millex-GV, Merck Millipore Tulagreen, Carritwohill, Ireland), and the medium was replaced. Protein was precipitated with acetone at –20 °C, resuspended in PBS and stored at –20 °C until use.

The integrity and purity of antigens were determined by SDS-PAGE and Coomassie staining. Protein content was quantified using the Bradford method. Different TcES-Ag dilutions were performed in PBS and mixed with the Bradford reagent (Bio-Rad, Hercules,CA, USA). The protein concentration was determined by comparing the optical density (O.D.) of the sample to a standard curve (10, 20, 40, 60, 80, and 100 µg/mL) made with bovine serum albumin (BSA, SIGMA-ALDRICH, St Louis, MO, USA). The concentration of total antigens obtained in the test was 235.14 µg/mL.

2.10 Specific anti-T. canis IgG Determination

To determinate specific antibodies, 96 wells flat-bottom polystyrene plates (Maxisorp, NUNC Labs, Norristown, PA, USA) were sensitized with 1 µg/mL TcES-Ag in bicarbonate buffer (pH 9.6) for 24 hours at 4 °C. Subsequently, the plates were washed three times with 0.01% PBS-Tween 20 (SIGMA-ALDRICH), blocked with 3% bovine serum albumin (BSA), and stored at 4 °C for 24 hours. After the blocking time finished, the plates were washed three times with PBS-Tween 20 at 0.01% and stored at 4 °C. For each sample, 50 µL of serum/well was used in duplicate, diluted 1:200 with PBS, and incubated at 37 °C for 2 hours. Subsequently, the plates were washed three times with PBS-Tween 20 at 0.01%, and 50 µL/well of secondary antibody [1:1000 peroxidase-conjugated AffiniPure Goat Anti-Rat IgG (H+L), Jackson Immunoresearch, West Grove, PA, USA] was added and incubated at 37 °C for one hour. The plates were washed five times with the same solution (PBS-Tween 20 at 0.01%), and color development was achieved using 0.05% o-phenylenediamine (OPD) and 0.001% hydrogen peroxide in citrate buffer (SIGMA-ALDRICH). The plates were incubated for 15 min in total darkness at 37 °C, and then, 50 µL/well of 0.06% orthophosphoric acid was added. Plates were read at 492 nm with 15 seconds of agitation in an ELISA reader (Stat Facs 4200, Awareness Technology, Palm City, FL, USA).

2.11 Statical Analysis

Data are presented as mean ± Standard Deviation (SD). Results are depicted in bar graphs describing the mean and standard deviation. Analyses were performed using Prism V 8.0 software (GraphPad Software Inc®. Boston, MA, USA). The experimental design considers 2 independent variables: sex [male (♂) or female (♀)] and infection [Control (Ctrl) or *T. canis* infection (Infx)]. Data regarding parasite charges (larval recovery) only consider the infection variable. These values were evaluated by a Two-way ANOVA and a *post-hoc* Bonferroni multiple comparison test between all groups. A significant difference was considered when $p < 0.05$.

3. Results

3.1 Macroscopic Lesions and Larvae Number during Acute T. canis Infection

The number of lesions and larvae number was significantly different depending on sex. In males, lesions and larvae counts were higher in the lungs seven days post-infection. In contrast, in females, the extent of the lesions and the number of recovered larvae were higher in the liver (Fig. 1a,b). Combining the mean number of larvae obtained in both organs we observed statistically significant differences ($p < 0.001$). In males, a total of 94.6 larvae were recovered (28.7 in the liver + 65.9 in the lung), while in females, 119 larvae were collected (95.5 in liver + 23.5 in lungs) (Fig. 1b).

3.2 Innate Immune Cells Subpopulations

Because sex steroids can modulate the immune response in infections, we decided to evaluate the immune response during a *T. canis* infection as an antigenic challenge. Fig. 2 shows Macrophages (Mϕ), Natural Killer cells (NK) and T gamma-delta lymphocytes (T$\gamma\delta$L) in the spleen, PLN and MLN. In the spleen, differences were only seen in Mϕ percentage between males and females, as the percentage of these cells was higher in the former, regardless of the infection status ($p < 0.05$) (Fig. 2A). In the PLN, there were no statistically significant differences in the subpopulations analyzed that could be attributed to sex or to an infection condition ($p > 0.05$). In male-collected MLN, infection generated an increase in the percentage of Mϕ ($p < 0.01$) (Fig. 2G).

3.3 Adaptative Immune Cells Subpopulations

Fig. 3 shows the percentage of total T cells, T helper lymphocytes (ThL) and T cytotoxic lymphocytes (TcL) in the spleen, PLN and MLN. In the spleen, the infection generated an increase in the percentage of ThL only in males ($p < 0.05$) (Fig. 3B). In the PLN, the percentage of ThL was higher in control females in contrast to control males ($p < 0.05$). Yet, when the infection was present in females, this percentage decreased ($p < 0.0001$) (Fig. 3E). Finally, total T cell percentages in MLN were higher in females than in males. Statistically significant differences were ob-

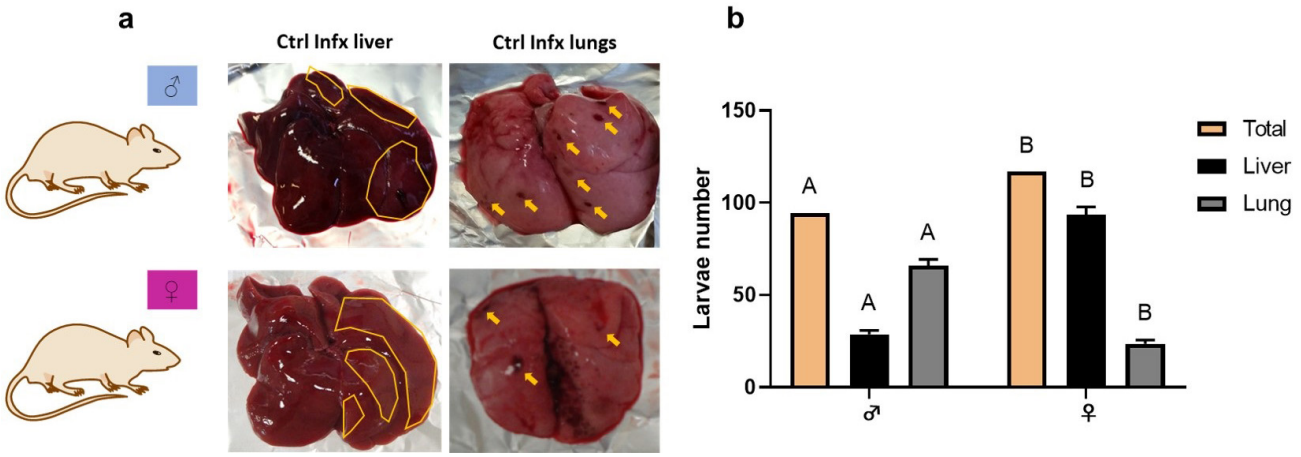

Fig. 1. Macroscopic lesions and larvae number of *T. canis* in the lungs and liver. (a) Liver and lungs injuries. In the liver, the areas of damage are outlined in yellow. In the lungs, yellow arrows point to areas of damage. (b) Total larval number in both organs and larval number in liver and lungs. Bars represent the mean ± Standard Deviation (SD) of larvae number. Letters (A, B) indicate the significant differences between sexes ($p < 0.001$).

served in control ($p < 0.001$) and infected ($p < 0.01$) animals (Fig. 3G). Regarding ThL, the infection causes a decrease in the percentage of these cells in both sexes ($p < 0.05$) (Fig. 3H).

Finally, in B lymphocytes (BL), the infection generates an increase in the number of these cells ($p < 0.01$) in females in PLN (Fig. 4B). In the spleen and MLN, there were no significant differences due to sex or infection ($p > 0.05$) (Fig. 4A,C).

3.4 Cytokine Production

To evaluate cell response in the immune system, we analyzed the production of cytokines during *T. canis* infection in both sexes. For Th1 cytokines, the infection reduces the concentration of IL-1β in both sexes ($p < 0.0001$). Additionally, the levels of this cytokine are lower in females compared to males in both the control ($p < 0.05$) and the infected group ($p < 0.01$) (Fig. 5A). IFN-γ concentration also shows a dimorphic pattern where males have higher levels than females ($p < 0.01$) (Fig. 5B). TNF-α was not detected in any of the experimental groups (Fig. 5C). IL-6 levels are higher in control females ($p < 0.05$), but during an infection, the cytokine levels decrease ($p < 0.001$) (Fig. 5D). Regarding Th2 cytokines, infection causes an increase of IL-4 ($p < 0.01$), IL-5 ($p < 0.01$), and IL-13 ($p < 0.05$) levels, but only in males (Fig. 5E–G). Sex-related differences in IL-5 levels were observed, as the generation of this cytokine was higher in males than in females during infection ($p < 0.01$) (Fig. 5E). In contrast, IL-5 levels are higher in control females than in males; interestingly, infection did not increase theses IL-5 levels ($p < 0.01$) (Fig. 5F). IL-13 shows no significant differences due to sex ($p > 0.05$) (Fig. 5G). Likewise, regarding regulatory cytokines, there are no differences related to sex ($p > 0.05$), but the infection causes a decrease in the expression of IL-10 in both sexes ($p < 0.0001$) (Fig. 5H), and TGF-β decreases only in females ($p < 0.05$) (Fig. 5I).

3.5 Specific anti-T. canis IgG Production

Since the production of specific antibodies is an important strategy for the control of helminths, we decided to determine the presence of these antibodies in the serum of the animals. The infection caused an increase in the production of specific Ig-G anti-*T canis* in both sexes ($p < 0.0001$), but in females, this increase is lower than in males ($p < 0.01$) (Fig. 6).

4. Discussion

In this study, we estimated the effect of sex on susceptibility to *T. canis* infection by evaluating the immune response during the acute phase of the infection in a rat model. The initial assessment of susceptibility focused on the analyzing parasitic loads in organs such as lungs and liver. We chose to evaluate these organs because, during the time of infection, migrating somatic larvae are present throughout them [13–40] (Fig. 1B). Our data align with findings reported by Lescano *et al.* [40], who infected male Wistar rats with 500 larvated eggs, observing that the number of larvae recovered at eight days post-infection in the liver and lung was 12.3 (2.46% of total larvated eggs) and 35.7 (7.14% of total larvated eggs), respectively. In studies with other species of *Toxocara* (*T. cati*), by Santos *et al.* [41], (2009) infected adult male Wistar rats with 300 larval eggs, reporting a recovery of 4 larvae (1.33% of total larvated eggs) from the liver and 13.6 larvae (4.53% of total larval eggs) from the lung. Additional experiments by dos Santos *et al.* [42], (2017) evaluated the pattern of somatic larvae migration in male and female Wistar rats infected with 300 larvated eggs. They found that seven days post-infection,

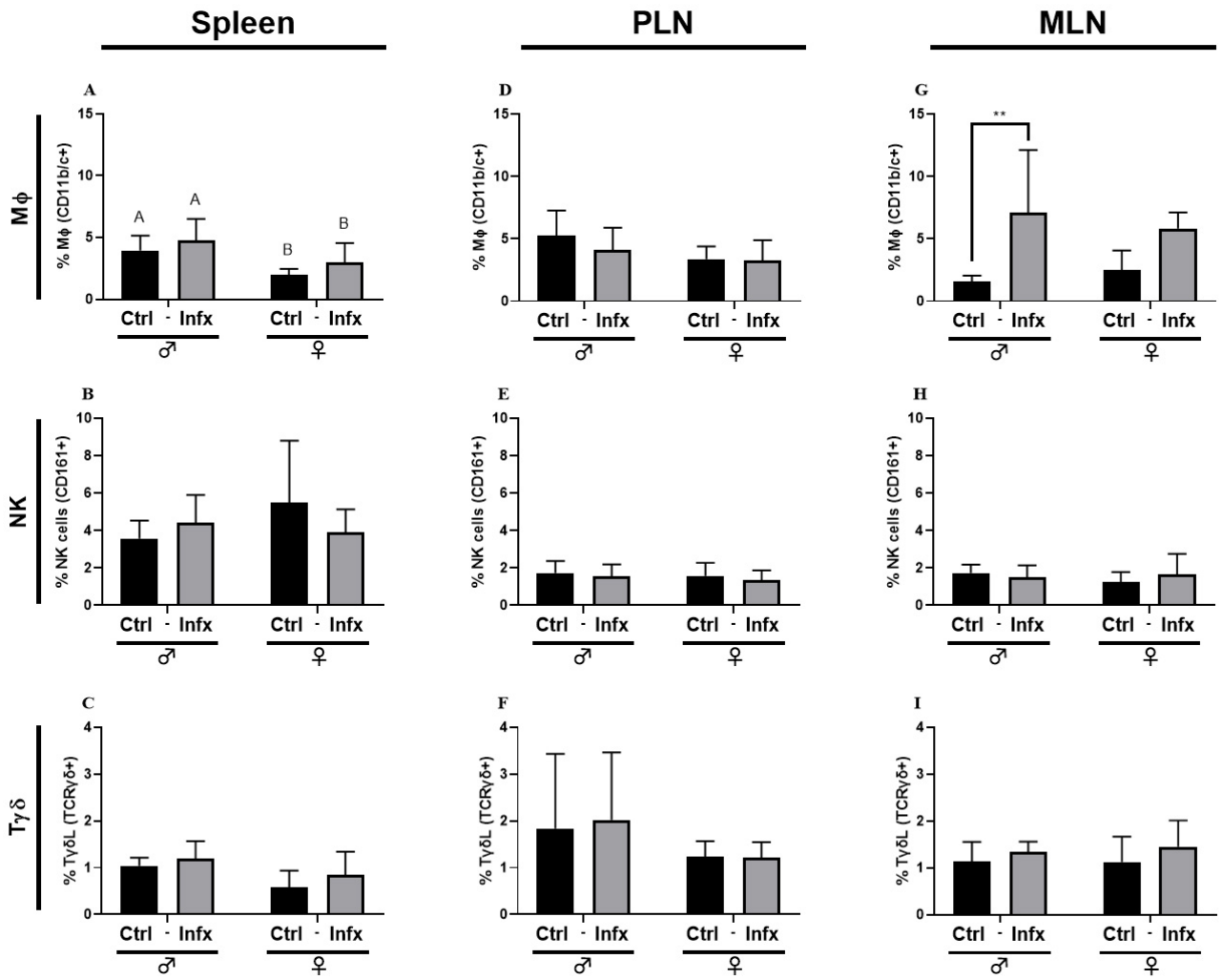

Fig. 2. Percentage of the innate immune cells subpopulations in male and female rats control or infected with *T. canis*. (A) Mϕ (CD11b/c$^+$) in the spleen. (B) NK cells (CD161$^+$) in the spleen. (C) T$\gamma\delta$ L (TCR$\gamma\delta^+$) in the spleen. (D) Mϕ (CD11b/c$^+$) in Peripheral Lymph Nodes (PLN). (E) NK cells (CD161$^+$) in PLN. (F) T$\gamma\delta$ L (TCR$\gamma\delta^+$) in PLN. (G) Mϕ (CD11b/c$^+$) in Mesenteric Lymph Nodes (MLN). (H) NK cells (CD161$^+$) in MLN. (I) T$\gamma\delta$ L (TCR$\gamma\delta^+$) in MLN. Bars represent the mean $\pm$ SD of the percentage of immune cells. Asterisks indicate the significant differences between Ctrl and Infx animals (**$p < 0.01$) and the letters (A, B) between sexes ($p < 0.05$). Ctrl = Control; Infx = Infected

the recovery of larvae in males was 13 in the liver (4.33% of total larvated eggs) and 2 in the lungs (0.66% of total larvated eggs). In females, the number of larvae recovered from liver was 27.5 (9.16% of total larvated eggs) and 1.5 in the lungs (0.5% of total larvated eggs). The increase in the number of larvae at the hepatic level in females was maintained from seven to 60 days post-infection. Moreover, the total number of larvae recovered in these organs was higher in females than in males, indicating that females are more susceptible than males to *T. canis* infection [42]. These results align with those reported by our research group, where we observed a dimorphic distribution of larvae in the liver and lungs, with females having a higher number of larvae in the liver than males. Although these differences in parasitic loads at the pulmonary level shows statistically significant differences, with males having a greater number of larvae than females. Additionally, the total number of larvae in both organs was higher in females than in males (Fig. 1B). Even though it remains unclear why these differences occur in certain larvae located in these organs, it has been previously reported that differences in enterohepatic circulation exists in rats between both sexes. For instance, a higher recirculation and consequently, a reduced excretion of some molecules have been demonstrated [43]. This fact could allow the larvae to remain in the liver of females for a longer period compared to males. The present results differ from the prevalence reported in definitive hosts, where it is mentioned that males are more susceptible to infection than females. This difference may be attributed to the fact that in males, transmission is completed only through in-

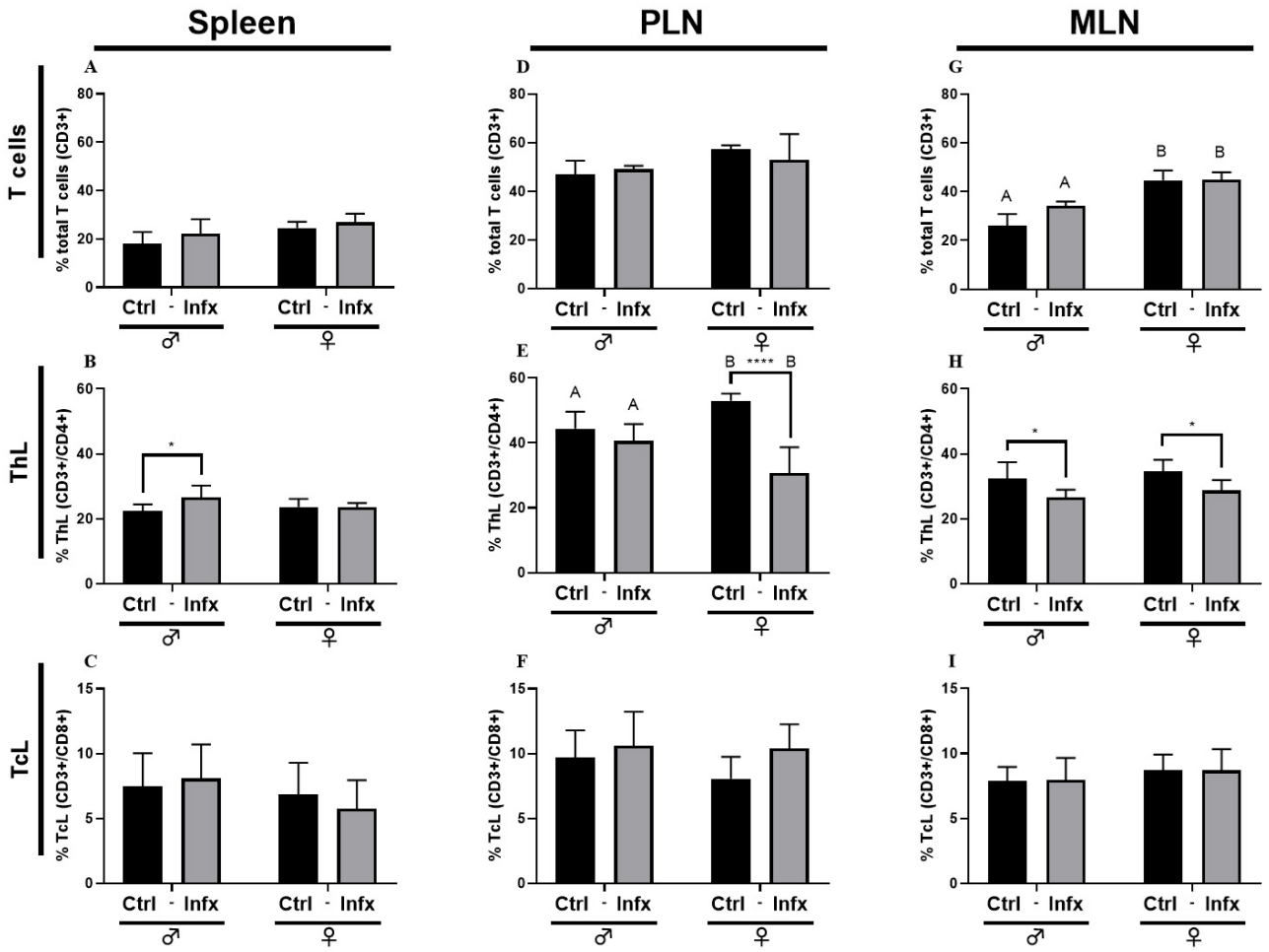

Fig. 3. Percentage of T lymphocytes in male and female rats control or infected with *T. canis*. (A) Total T cells ($CD3^+$) in the spleen. (B) ThL ($CD3^+/CD4^+$) in the spleen. (C) TcL ($CD3^+/CD8^+$) in the spleen. (D) Total T cells ($CD3^+$) in PLN. (E) ThL ($CD3^+/CD4^+$) in PLN. (F) TcL ($CD3^+/CD8^+$) in PLN. (G) Total T cells ($CD3^+$) in MLN. (H) ThL ($CD3^+/CD4^+$) in MLN. (I) TcL ($CD3^+/CD8^+$) in MLN. Bars represent the mean ± SD of the percentage of immune cells. Asterisks indicate significant differences between Ctrl and Infx animals (*$p < 0.05$, ****$p < 0.0001$), and the letters (A, B) indicate significant differences between sexes ($p < 0.05$).

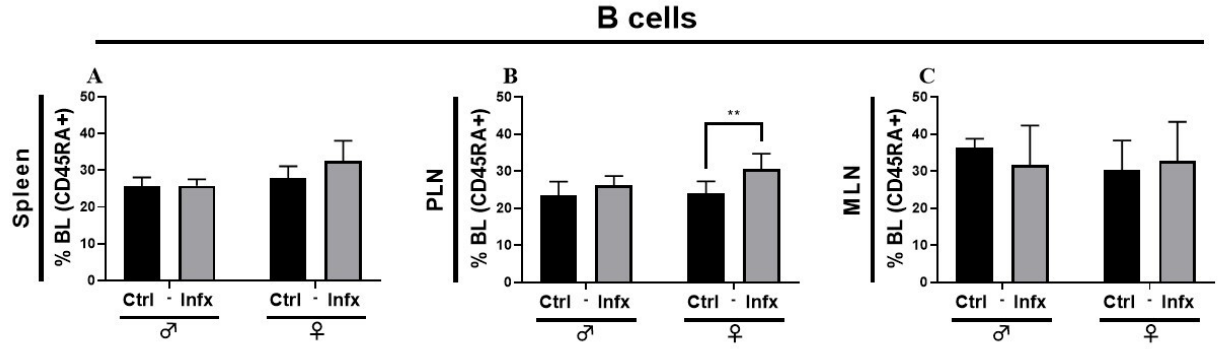

Fig. 4. Percentage of B Lymphocytes (BL) in male and female rats control or infected with *T. canis*. (A) BL (CD45RA+) in the spleen. (B) BL (CD45RA+) in PLN. (C) BL (CD45RA+) in MLN. Bars represent the mean ± SD of the percentage of immune cells. Asterisks indicate the significant differences between Ctrl and Infx animals (**$p < 0.01$).

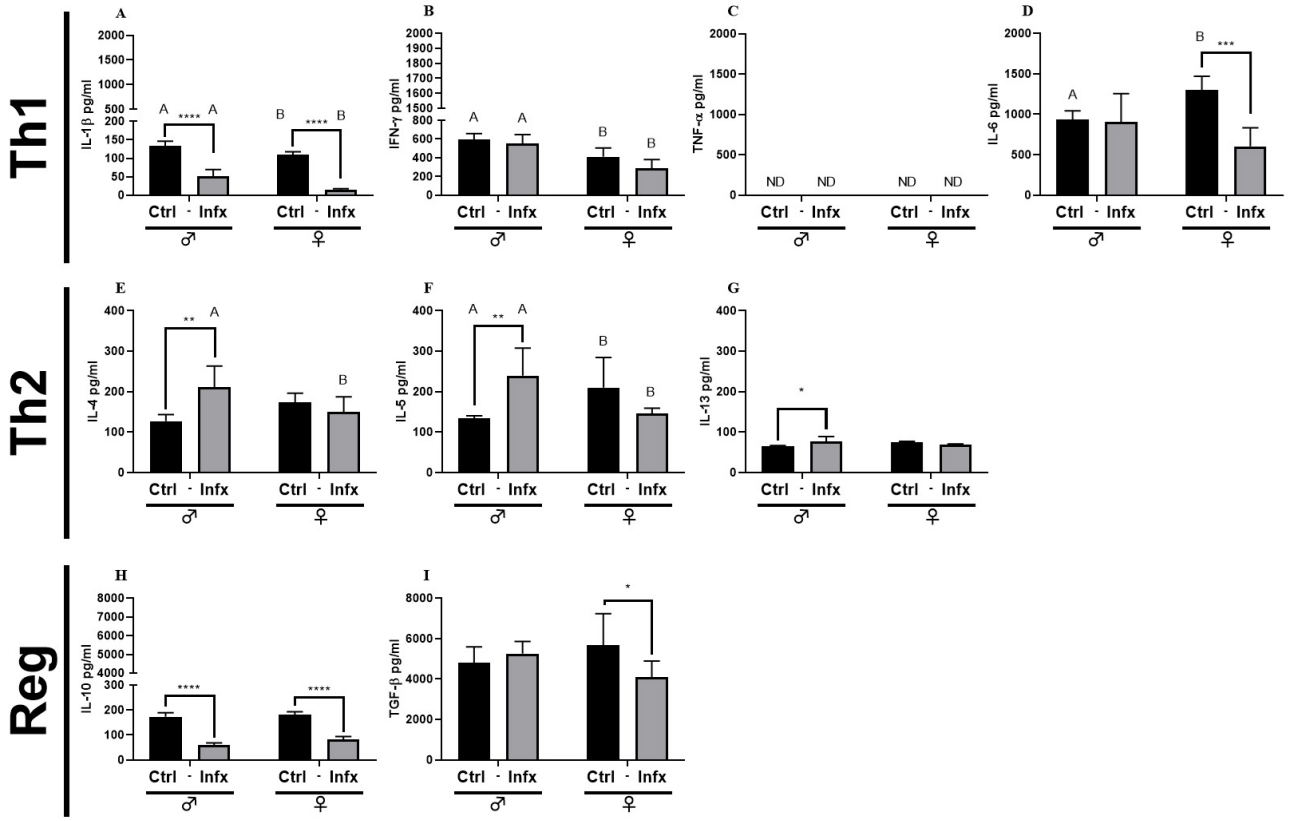

Fig. 5. Analysis of systemic soluble factor expression in serum of male and female rats, control, or infected with *T. canis*. (A) IL-1β. (B) IFN-γ. (C) TNF-α. (D) IL-6. (E) IL-4. (F) IL-5. (G) IL-13. (H) IL-10 and (I) TGF-β. Bars represent the mean $\pm$ SD of cytokines concentration. Asterisks indicate the significant differences between Ctrl and Infx animals (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) and the letters (A, B) indicate differences between sexes ($p < 0.05$). ND = No Detected.

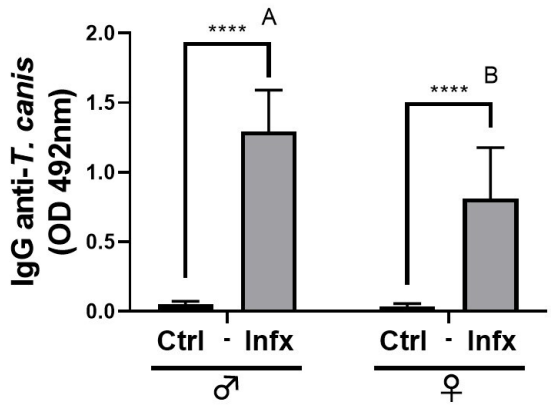

Fig. 6. Analysis of specific anti-*T. canis* antibody production in control and infected male and females. Bars represent the mean $\pm$ SD of IgG antibodies levels. Asterisks indicate the significant differences between Ctrl and Infx animals (****$p < 0.001$) and the letters (A, B) indicate differences between sexes ($p < 0.05$).

testinal patent infection, whereas in females, a larval reactivation phenomenon occurs with consequent transplacental and lactogenic transmission [44]. On the other hand, additional factors, such as the immunosuppressive effect of testosterone, may contribute to these differences [45]. However, it is worth highlighting that in these reports, the age of the animals is not mentioned or no statistically significant differences were found between both sexes. Additionally, in this study, paratenic hosts, namely rats, were used, and the parasite does not develop into its adult form in this rodent host; it can only be transmitted to the offspring by the lactogenic way [46] or by ingestion of prey infected with somatic larvae.

Since the immune response plays a key role in the development of resistance or susceptibility to various diseases, whether of non-infectious or infectious origin, we decided to evaluate the primary cells of the innate and adaptive immune response responsible for controlling helminth infections in the spleen, MLN, and PLN. Concerning cells from the innate response, only Mϕ showed differences in their percentage at the splenic level, where females presented a lower percentage of these cells than males, regardless the presence of an infection or not (Fig. 2A). In MLN, the infection causes an increase in the percentage of these cells in males but not in females (Fig. 2G). Mϕ have been proven to be important cells in the regulation of the

immune response against helminths, acting as competent antigen-presenting cells. Thus, these changes in percentages could influence the susceptibility to developing an infection [20,35,47]. Although in our experiments, we did not elucidate the polarization of Mϕ towards an AAM phenotype, different studies have shown that females have a higher number of Mϕ than males [4–33]. However, we did not confirm this finding in the current study. Regarding adaptive immune cells, infection causes a reduction in the number of ThL in PLN and MLN in females (Fig. 3E,H), whereas in males, these cells increase in the spleen but decrease in MLN (Fig. 3B,H). Similarly, Nava-Castro *et al.* [48], (2019) demonstrated that male and female BALB/c mice during acute infection (5 days post-infection) with *Trichinella spiralis*, present a decrease in the percentage of ThL in MLN, although a different antigenic challenge was used in the referred trial. Similar experiments by Ruiz-Manzano *et al.* [19], (2019), who used a model of chronic *T. canis* infection in females, showed no differences in the percentage of splenic ThL in infected animals compared to the control group. However, no differences in PLN were demonstrated, in contrast to our results. BL are crucial cells in the control of nematodes, as they mature into plasma cells and produce specific antibodies against these parasites. In BL, we observed an increase in the percentage due to the infection in PLN of females (Fig. 4B). In MLN, there were no significant changes in both sexes; this also agrees with reports published by Nava-Castro *et al.* [48], (2019), where no differences were seen in the percentage of these cells during the *Trichinella spiralis* acute phase of the infection. In contrast, during chronic *T. canis* infection, the percentage of these cells increases in the spleen and PLN of females [19]. When evaluating differences in cell subpopulations, it must be considered that there is a lack of information in the literature on the impact of sex differences on the immune response during Toxocariasis. Additionally, other factors must be considered, such as the time of infection, type of host, and the number of ingested larval eggs, among others, which makes the analysis even more complicated.

As mentioned earlier, the Th2 response plays a crucial role in orchestrating the immune response during helminthic infections. Here, we report a decrease of Th1 cytokines, IL-1β in both sexes and IL-6 only in females due to infection (Fig. 5A,D). Th2 cytokines IL-4, IL-5, and IL-13 are increased only in males due to this same effect (Fig. 5E–G). The regulatory cytokine IL-10 shows lower levels due to infection in both sexes (Fig. 5H), while TGF-β only decreases in females (Fig. 5I). Contrary to our findings, Pecinali *et al.* [49], (2005) observed increased levels of IL-6 and IFN-γ cytokines in plasma and bronchoalveolar fluid in male BALB/c mice infected with approximately 1000 larval eggs of *T. canis*. Th2 cytokines exhibit an increased production pattern similar to that reported when there is an infection by helminths only in males- the sex in which almost all studies evaluating the immune response to this parasite have been carried out [19,20,22]. Regarding IL-10 production, Torina *et al.* [50], (2005) demonstrated in pregnant bitches, that IL-10 levels increase while IFN-γ levels decrease during the first week of pregnancy. These differences in IL-10 levels may be attributed to the fact that, in the aforementioned study, dogs were naturally infected. Therefore, the exact phase of infection, namely the acute or chronic period of toxocariasis when IL-10 levels were determined, remained uncertain. In contrast, the current study was conducted in a paratenic host during the acute phase of the infection. Finally, the production of specific anti-*T. canis* antibodies is lower in females compared to males (Fig. 6). although different studies have shown that *T. canis* infection generates the production of specific IgG antibodies against the parasite [19,51,52], the production of antibodies anti-*T. canis* between males and females has not been analyzed until now. These differences underscore the importance of studying infections from the perspective of immune sexual dimorphism. Although some research groups have long been focused on studying parasitic diseases from a dimorphic standpoint, this aspect is lacking in many other parasitic diseases.

Finally, sexual dimorphism involves the biological differences between males and females of the same species, including developmental, physiological, and pathological dissimilarities as presented in here. The scientific significance of the present piece of research, it is of high priority to people interesting in an update on sex differences in parasitic infections, and biological factors of risk, and explains the latest sex-associated factors for *T cannis* Toxocariasis first, and ultimately for many helminth infections that are dimorphic in their presentations.

5. Conclusions

Our study demonstrates that *T. canis* infection is dimorphic and affects females more than males. This is attributed to a polarization of the inadequate immune response, which is reflected as a higher parasite load in this sex.

Availability of Data and Materials

The datasets generated and analyzed during the current study are included in the present manuscript. Furthermore, they are available from the corresponding author on request.

Author Contributions

VHDRA, KENC and JMM conceptualization. VHDRA, YAC and CAGC methodology and validation. VHDRA, YAC, CAGC and JMM formal analysis. VHDRA, YAC and JMM investigation and resources. VHDRA, YAC, CAGC, KENC, and JMM data curation. VHDRA writing—original draft preparation. VHDRA, YAC, CAGC, KENC, and JMM writing—review and editing.

VHDRA and JMM supervision. JMM project administration. VHDRA, YAC, KENC, and JMM funding adquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The protocol for the use and care of the animals was endorsed by both Institute's Animal Care and Use Committee, (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), permit number 201-2016) following the official Mexican regulations (NOM-062-ZOO-1999).

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Conflict of Interest

The authors declare no conflict of interest. Given his role as Guest Editor, Jorge Morales-Montor had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Giuseppe Murdaca.

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