Mast cell response to Leishmania mexicana and sand fly salivary proteins is modulated by androgens

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Abstract: Mast cells (MCs) play a crucial role during infections with Leishmania, that is transmitted through the bite of an infected sand fly that injects saliva together with the parasite. Sand fly saliva is a complex fluid that modulates the host immune response. In addition, hormonal factors modulate the host immune response, impacting the susceptibility to infections. Thus, to assess the impact of androgens and salivary proteins of sand fly vectors on the mast cell (MC) response to Leishmania infections, we infected orchiectomized male mice with the parasite in the presence or absence of sand fly salivary proteins and analyzed the inflammatory response of MCs. Our results showed a differential MC response to the parasite and to vector salivary proteins in mice deprived of gonadal hormones, as compared to sham-operated mice. Orchidectomy induced a different pattern of activation in MC of animals infected with Leishmania and vector-salivary proteins. Our results show that during Leishmania infection, androgens modulate the innate immunity response against the parasite and salivary proteins of the sand fly vector.
1. Introduction

Mast cells (MCs) are considered sentinels of the innate host defense due to their ability to detect pathogens and degranulate immediately upon activation. They are strategically distributed throughout most tissues of the body and possess the capacity to differentiate into life-long sessile tissue homing cells [1,2,3], making them unique. Mast cells mediated their physiological and pathophysiological roles through the selective release of various preformed and newly synthesized inflammatory mediators [4,6]. These include different proteases, histamine, heparin proteoglycan, chondroitin sulfate E, acidic hydrolases, and various cytokines and growth factors [4,6]. However, the role of activated MC in response to infections remains controversial. It is considered a double-edged sword: on the one hand, specific mediators can contribute to contain or eliminate the pathogen, yet they can also favor the progression of infections by diminishing or augmenting the intensity of inflammation, depending on the pathogen, age, gender, and the genetic background of the host [4, 5]. Leishmania infections (both in vitro and in vivo) have shown that MC degranulation influences the early inflammatory response, thereby contributing to the outcome of the infection, which also depends on the genetic background of the host (7,8,9). Thus, in BALB/c mice, MCs seem to facilitate disease progression, while in C57BL/6 mice, MCs promote disease control [7].

Leishmania transmission occurs through the bite of an infected female sand fly during blood feeding, which injects the parasite together with salivary components [10,11]. After the infection, both the parasite and proteins of the sand fly saliva affect the immune response of the host [12].

Furthermore, the immune system is also strongly influenced by the endocrine system since sex hormones modulate and regulate the differentiation, proliferation, and activation of immune cells [13,14].

Gender also modulates Leishmania infections (15,16,17,18,19). Thus, estrogens upregulate IL-4 and IL-10 production, whereas testosterone downregulates IL-2, IL-6, TNF-a, and nitric oxide production in macrophages.
During L. donovani infections, testosterone down-regulates p38 MAPK activation, inhibiting the macrophage inflammatory and microbicide response [20,21,22]. Additionally, dihydrotestosterone (DHT) increases Leishmania mexicana (L. mexicana) infectivity, both in vivo and in vitro [23]. Yet, the regulation exerted on MC by endocrine factors, Leishmania parasites and vector salivary proteins during acute and chronic infections, remain to be analyzed. Therefore, the aim of the current study was to analyze MC activation in male BALB/c mice deprived of gonadal hormones and infected with L. mexicana, in the presence and absence of sand fly-salivary proteins.

2. Results

2.1. Ear tissue histology after inoculation of L. mexicana and vector salivary proteins.

To assess MC activation in the presence or absence of male sexual hormones after L. mexicana and salivary gland lysate (SGL) inoculation, the ears of intact, sham, and orchiectomized BALB/c male mice were analyzed by histology. After the inoculation of L. mexicana and vector salivary lysate, intact BALB/c male mice showed a dermal inflammatory response, started at 30 minutes after the challenge and remained from 8 to 72 hours, characterized by neutrophilia (Figure A-E black arrows). Orchiectomized mice also showed edema, vascular congestion, and vasodilatation but a less neutrophilia and dermal neutrophil infiltration (Figures 1F-H, arrows) than intact mice between 30 min and 24 hours after inoculation. Interestingly, a delay of 48 hours was observed in the recruitment of neutrophils in orchiectomized mice (Figure 1I). In addition, as compared to sham mice (Figure 1I) the intense neutrophil infiltration in the orchiectomized mice occurred between 48 to 72 hours (Figure 1 I-J).

![Figure 1. Comparative histological images of kinetics of the inflammatory cell infiltration of sham and orchiectomized BALB/c mice, in response to L. mexicana together with salivary gland lysates (SGL). Hematoxylin and eosin-stained ear sections of sham-operated (A-E) and orchiectomized mice (F-J), at 30 min. (A & F), 8 h (B & G), 24 h (C & H), 48 h (D & I), and 72 h (E & J), following inoculation with L. mexicana and SGL. In sham mice, accumulation of inflammatory cells, principally neutrophils, is evident at 30 minutes after challenge (A), and remains intense up to 48 h (D), weakening thereafter at 72h (E). In contrast, in orchiectomized mice, the neutrophil infiltration is delayed, beginning after 24 h after challenge (H) and remaining intense throughout 48 h (I) and 72 h (J). Original magnification ×40 (A-I) and ×10 (J). Scale bar = 50 um Photomicrographs are representative of three independent experiments with five replicates for each group. Black arrows show neutrophils within venules and capillaries.
Furthermore, MC number recruitment and degranulation also showed slight differences between orchiectomized and sham mice. A slightly higher increase in MC infiltration was observed in gonadectomized mice, as compared to sham mice, at all time points (Figure 2).

**Figure 2.** MCs degranulation in sham and orchiectomized mice throughout 72 h. Total mast cell numbers were counted in toluidine blue-stained ear sections from sham-operated (A) and orchiectomized mice (B), at 30 min, 8 h, 24 h, 48 h, and 72 h, following inoculation with *L. mexicana* together with SGL. Counting was done by analyzing the photomicrographs of the total number of mast cell in 20mm². Three independent experiments with five replicates for each group were done. A* significant differences were found between BSH, CSH, 30SH and 8SH vs 24SH and 48SH. B* significant differences were found between BOr, COr, 30Or vs 8Or, 24Or and 48Or. Nonparametric ANOVA ordinary multiple comparison was employed (Significance p < 0.0001). (Abbreviations: BSH: basal sham; CSH: control sham; 30SH: 30 min sham; 8SH: 8 h sham; 24SH: 24 h sham, 48SH: 48 h sham; 72SH: 72 h sham. BOr (basal orchiectomized; COr control orchiectomized; 30r: 30 min orchiectomized, 8-72 Or: 8-72 h orchiectomized).

After *L. mexicana* and salivary lysate inoculations, MC recruitment started at 30 min until 48 hours, showing a similar pattern between orchiectomized and sham mice (Figure 2). However, sham mice showed slightly lower numbers of MC throughout all the time points, as compared to orchiectomized mice. In addition, cell counts in orchiectomized mice showed a higher standard deviation compared with sham mice. At 72 hours, no MC increase was observed in either group of mice (Figure 2).

In sham mice, MC numbers increased after 30 min and throughout 48 hours compared to basal values (BSH) (p<0.001). However, after 72 hours, a reduction in mast cell numbers was observed (p<0.0001).

In orchiectomized mice, a significant increase in MC numbers was registered from 8 to 48 hours (p<0.001), as compared to basal values, control, and 30 min (BOr, COr, 30Or). This significant increase was registered after 8 to 48 hours (p<0.001). No significant differences were observed when comparing sham and orchiectomized mice.

It is noteworthy that in orchiectomized animals, MC degranulation revealed a discrete group of granules at 30 min and 48 hours. However, at 72 hours, MC degranulation in orchiectomized animals was similar to that observed in sham mice at 30 min and 8 hours (Figure 3 A-B, J). Whereas in orchiectomized mice, MC degranulation resembled a "piecemeal process," in which the contents of the released granules were surrounded by plasma membranes (Figure 3G-H), in sham mice, the MC degranulation occurs more diffusely ("systematic mode") resembling activation after FceRI high-affinity receptor binding (Figure 3A-E).
Figure 3. Photomicrographs of mast cells degranulation in ear skin from sham and orchiectomized mice after inoculation with *L. mexicana* together with salivary gland lysates (SGL). Toluidine blue-stained ear sections from sham-operated (A-E) and orchiectomized mice (F-J), at 24 h (C & H), 48 h (D & I), and 72 h (E & J) respectively, following inoculation with *L. mexicana* together with SGL. Note the intact dark purple mast cells (black arrows) and degranulated mast cells, and mast cell-released dark purple insoluble granular particles (red arrowheads). In sham mice, a mild and minimal mast cell degranulation is detected at 24 h (C & H) and 48 h (D & I) after challenge, whereas at 72 h (E & J) some mast cells with replenished granules are observed. In orchiectomized mice, mast cell degranulation also initiates at 24 h (H) but remains intense at 48h (I) and it is maintained 72 h (J). Images are representative of three independent experiments with five replicates for each group. Scale bar= 50 µm.

2.2. Immunohistochemistry of ears after inoculation of *L. mexicana* and vector salivary gland lysates: inflammatory mediators released after MC activation.

Histamine, TNF-α, and tryptase were assessed after MC activation induced by injection of *L. mexicana* and vector SGL at 30 min, 8, 24, 48, and 72 hours. Inflammatory mediators released by orchiectomized mice showed differences compared to sham mice. Histamine, TNF-α, and tryptase released in orchiectomized mice at 30 min showed a weak mark compared with sham mice (Figure 4), and their mark increases throughout the infection time. At 48 hours, the inflammatory mediator mark increased in intensity but continued to be less intense than in sham mice (Figure 5). However, at 72 hours, the histamine, TNF-α, and tryptase, mark in orchiectomized mice increased in intensity, similar to that of sham mice (Figure 6). Therefore, the lack of male sexual hormones seems to reduce and retard MC activation, their release of granules, and their numbers in the tissues (Figures 4-6).
Figure 4. Comparative immunohistochemical assessment of mast cell release of histamine, tryptase and TNF-α in sham and orchiectomized BALB/c mice, at 30 min in response to the injection of *L. mexicana* combined with salivary gland lysate. Photomicrographs show dark brown mast cells (black arrows) in sham and orchiectomized BALB/c mice. In dermis from sham mice degranulation and diffuse immunoreactivity are evident at 30 minutes after challenge. Scale bar= 20 µm.
Figure 5. Comparative immunohistochemical assessment of mast cell release histamine, tryptase and TNF-α in sham and orchietomized BALB/c mice, at 48 hour after *L. mexicana* and salivary gland lysate injections. Photomicrographs show intact dark brown mast cells (black arrows). In dermis from sham mice degranulation and diffuse immunoreactivity are evident at 48 hours after challenge. Images are representative of three independent experiments with five replicates for each group. Scale bar = 20 µm.
Figure 6. Comparative immunohistochemical assessment of mast cell release histamine, tryptase and TNF-α in sham and orchiectomized BALB/c mice, at 72 hour after *L. mexicana* and salivary gland lysate injections. Photomicrographs show intact dark purple mast cells (black arrows). In dermis from sham mice degranulation and diffuse immunoreactivity are evident at 72 hours after challenge. Images are representative of three independent experiments with five replicates for each group. Scale bar= 20 µm.

2.3. Chronic infection of *L. mexicana* together with vector salivary proteins in orchiectomized BALB/c mice.

Chronic *L. mexicana* infections (4 and 8 weeks) in orchiectomized mice showed smaller ulcers than sham mice (Figure 7). Furthermore, a significant reduction in the number of amastigotes infecting macrophages was observed in gonadectomized mice compared to sham mice (Figure 7). The significant reduction of infected macrophages observed at eight weeks (50.4%) indicates that gonadectomized mice have better control of the parasite infection.
Figure 7. Comparative ulcer size at 4 and 8 weeks after infection and the counting of infected macrophages. *L. mexicana* promastigotes were inoculated and the chronic infection was analyzed. Orchiectomized mice produced smaller lesions, as compared to sham-operated mice. Low numbers of infected macrophages were evidenced in orchiectomized mice (42.8±.2 and 21.6±.4) after 4 or 8 weeks, respectively, as compared to sham-operated mice (71.2±.3 and 79.8±.2) at the same time points. Orchiectomized mice showed a strict control of the parasite infection at 8 weeks. Images are representative of three independent experiments with five replicates for each group. Significant differences between sham-operated and gonadectomized mice \( P \leq .05 \). (*,†). Abbreviations: W4Or and W8Or (week 4 and 8 in orchiectomized mice); W4SH and W8SH (week 4 and 8 in sham mice).

3. Discussion

The innate and adaptive immune response orchestrated by the endocrine system results in an intricate crosstalk between the different actors involved. This study demonstrated the absence of male hormones produced a delay in the degranulation of mast cells. Gonadectomized male immunological response to *L. mexicana* and salivary gland proteins shows a tight control of male hormones in developing the parasite infection and the mast cell activation. The chronic *L. mexicana* infection + salivary glands proteins and delayed mast cell degranulation during acute inflammation result in small cutaneous ulcers and lower parasite load.

It is well accepted that MCs orchestrate the development of the inflammatory process after an infection with *Leishmania*. Experiments done in vitro have shown that MCs release TNF-a, histamine, tryptase, and IL-1b after contact with the parasite [26,27,28,29]. Furthermore, a cause-effect variation in MCs numbers and in the percentage of degranulation has been reported in inoculation sites after *L. major* infections [24]. Thus, the release of inflammatory mediators, such as TNF-a, tryptase, and histamine is possibly involved in the inflammatory process that ensues after a natural sand fly bite [30]. Furthermore, a pharmacologically induced MC degranulation in susceptible mice was shown to make them more resistant to an *L. major* infection, with lower parasite loads and decreased lesion sizes [8].

During *Leishmania* infections in mice, a differential response has been observed according to gender and mouse strain. Previous studies in our laboratory showed a differential MC activation in male mice infected with *L. mexicana*, which was associated with their resistance to the parasite infection. Susceptibility to *L. major* and *L. mexicana* infections is higher in males than females [31,30,32,33]. Fundamental differences between susceptible and resistant mice include MC degranulation and the presence of inflammatory cells. In C57BL/6 males, which are slightly resistant to *L. mexicana*, a delayed MC degranulation was shown after parasite inoculation, as compared...
to highly susceptible BALB/c male mice [6]. These data seemed to indicate that the delay of inflammatory mediators in C57BL/6 mice favor their control of the infection.

Considering the evidence on the differential response of males to L. mexicana infections, we now analyzed whether the early activation of MC and their release of inflammatory mediators are influenced by male sexual hormones, thereby facilitating the L. mexicana infection. In our infection model we also included sand fly SGL, aiming to simulate natural sand fly infections. Therefore, two groups of mice, orchiectomized and sham, were inoculated with L. mexicana in combination with sand fly SGL. Our results show that in sham BALB/c mice, an early MC degranulation, accompanied by a massive neutrophil infiltration, was evidenced after the inoculation of L. mexicana and vector SGL (Figures 2 and 3). The MC degranulation released inflammatory mediators such as TNF-α, histamine, and tryptase (Figure 4-6), which can contribute to neutrophil recruitment, giving an advantage to the parasites. The non-protective effect of neutrophils has been proposed to resemble that of a “Trojan horse”, which shields phagocytosed parasites from extracellular immune mechanisms, permitting their replication within neutrophils. This early evasion strategy seems to facilitate the infection [34,35]. Our data now show that in contrast to sham operated mice, orchiectomized mice showed a discrete and piecemeal mode of MC degranulation and discrete neutrophil infiltration (Figure 3), accompanied by a lower release of inflammatory mediators (Figure 4-6). This notorious difference in MC activation seems to be related to disease evolution in L. mexicana infections, where in chronic infections of orchietomized led to smaller lesions, as compared to sham mice (Figure 7).

Early neutrophil recruitment after L. mexicana infections have been associated with an increased parasite load and reduction of an efficient, protective immune response [35], indicating that L. mexicana exploits neutrophils to block the protective immune response by impairing the recruitment of dendritic cells to the infection site [36]. The results of our study now support the observation that neutrophils facilitate the L. mexicana infection and strongly suggests that MCs play an essential role during the early inflammation produced after L. mexicana and vector salivary gland proteins enter the host. Furthermore, inflammatory mediators (TNF-α, tryptase, and histamine) also favor recruiting an early wave of neutrophils, facilitating the infection. Besides, TNF-α, histamine, and tryptase can contribute to creating an immune-microenvironment that facilitates the establishment of Leishmania infection, priming MC to adopt an alternative activation phenotype (MC 2), or priming DCs to produce cytokines that favor a Th2-type immune response [37,38,39,40,41,42]. Furthermore, the evidence of an extensive release of tryptase and histamine by MC after a natural infection of BALB/c mice with L. major and Ph. dubossci was observed by Sanchez-Garcia, 2018 [30]. Since the neutrophil-MC interaction facilitates L. mexicana infections, it is also essential to consider the gender in mouse models to describe the hormonal control of MC activation.

Throughout evolution, parasites have developed strategies to evade the host immune system and exploit it for the parasite benefit [43]. L. mexicana has developed a trans-regulation control of the host immune system, as evidenced by the fact that L. mexicana promastigotes show increased virulence and infection capacity in the presence of the male hormone dihydrotestosterone [23]. Furthermore, histamine release by MC is dose and gender-dependent [44]. Our results now demonstrate that the severity of L. mexicana infection is influenced by the male sex hormones, which apparently regulate MC activation, promoting rapid neutrophil infiltration and the early release of inflammatory mediators, such as TNF-α, tryptase, and histamine. Thus, hormones are crucial during the development of the L. mexicana infection in a male host, where the infection is more severe than in females [7]. The cytokine CXCL8 or IL-8) is one of the most potent chemoattractant for guiding neutrophils through the tissue matrix until they reach sites of injury in mice. Some pieces of evidence indicated a hormonal regulation of several inflammatory mediators such as CXCL8. There is a possibility that the absence of male hormones delayed the arrival of neutrophils because of the control of the inflammatory mediators [45,46].

Finally, the slight mast cell increase in the ear of the orchietomized mice seems to be related to the absence of male sexual hormones. This observation is in accordance with the significant enhancement of MC numbers reported in orchietomized male mice, reaching similar numbers as those reported for females, which have been reported to have higher MC numbers [47,48]. Furthermore, the transient mastocytosis in both sham and gonadectomized mice after the stimuli with L. mexicana and vector SGL may be related to the antigenic challenge, since this transient mastocytosis was also observed in sham male BALB/c mice after a natural sand fly bite [30].

4. Materials and Methods

Ethics statement
The protocol used in this study was approved by The Committee on Ethics and Use in Animal Experimentation of the Unidad de Medicina Experimental UNAM. The study was done following the guidelines of Mexican regulation for laboratory animals (NOM-062-ZOO-1999).

4.1. Animals and experimental groups

This study used four-week-old male BALB/c mice. The animals were singly housed in a controlled temperature (22-24°C) and 12:12 light-dark conditions, receiving sterilized rodent diet and water ad libitum. The animals were organized into six groups of 5 animals each: 1) intact control, 2) infected control, 3) sham intact, 4) sham infected, 5) orchiectomized intact, and 6) orchiectomized infected. Orchiectomized male mice acquired the following characteristics: smooth hair, gained weight and became less aggressive. At necropsy, animals that presented reminiscence of testes were discarded.

4.2. Surgical procedure

Orchiectomy was performed using a mix of ketamine (0.25%) and xylazine (0.4%) for anesthesia. A small incision was made in the scrotum, the underlying muscle was cut, and the testes were extruded to the lower abdomen, ligated, and removed. For the sham-operated group, the testes were reinserted and non-ligated. Mice were operated under the day-light lamp to keep them warm and were monitored daily until the staples had self-removed.

4.3. Parasite culture

L. mexicana amastigotes were isolated from footpad lesions of infected BALB/c mice, as previously described [23-24]. Promastigotes were obtained by culturing isolated amastigotes at 26°C in culture medium 199, pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, 100 µg/ml streptomycin, and 2 mM L-glutamine (all from Gibco-Life Technologies, Grand Island, NY, USA) and sub-passaged during the logarithmic growth phase (days 3–4 of culture). For in vitro and in vivo infections, promastigotes at the stationary growth phase (day 5 of culture) were used. In this phase, most promastigotes have transformed into highly infectious metacyclic promastigotes. All promastigotes were cultured for no more than four in vitro sub-passages.

4.4. Salivary Gland Collection

Wild female sand flies were caught in Cunduacan, Tabasco, located in the southeast of Mexico, near the Grijalva river and Chontalpa subregion. The salivary glands of the sand fly species Bichromomyia olmeca olmeca (females) were obtained between 4 and 8 hours after collection. The flies were immobilized in cold PBS for about 2 min. The dissected salivary glands were collected in phosphate-buffered saline (PBS) pH 7.2 and stored at -80°C in batches per collecting day (5 pairs per batch). Lysates of the salivary glands were obtained by sonication and freeze-thaw cycles and centrifuged at 9900 x g for 1 min. The supernatant was collected and used immediately. Protein concentration was assessed by DC (DC protein Assay BIORAD 5000002). The average vector salivary gland protein concentration was 0.66 ± 0.09 µg/µl, corresponding to the pair of salivary glands of each sand fly.

4.5. Infection procedure

The infection procedure was performed as previously described by Dantas y col., 2009 [23]. Briefly, 0.06µg/µl salivary proteins combined with 100 purified viable metacyclic L. mexicana promastigotes were injected into the dermis of both ears of each mouse. The evolution of the infection was monitored at 8, 24, 48, and 72 hours, and then at 4 and 8 weeks.

4.6. Histology and immunohistochemistry

The ears were gently flattened onto a piece of thick paper to avoid curling and cut by scissors into three equally sized fragments (0.7 x 1.6 cm). Ears pieces were fixed in 4% paraformaldehyde in 0.1 M Tris-HCl buffer (pH 7.2) for 24 hours. After carried out a conventional paraffin-embedded technique, the tissue sections were stained with hematoxylin-eosin (H&E) or 2% toluidine blue (198161, Sigma Aldrich) for histopathological analysis and MC identification, respectively. MC identification was made following the metachromatic principle [25]. TNF-a, histamine, and tryptase were assessed by immunohistochemistry in paraffin-embedded ear tissue sections. The immunohistochemistry procedure was as follows: tissue sections were de-waxed with xylene, rehydrated with 0.1 M Tris-HCl buffer (pH 7.2), and transferred to plastic Coplin staining jars containing 0.1 M citrate buffer (pH 6.0) for antigen retrieval. Slides in the Coplin jar were then heated in a pressure cooker for 20 min. at 200°C followed by 10 min. at 100°C. The slides were cooled in the jar at room temperature (RT) for 15 min. and then transferred to 0.1 M Tris-HCl buffer (pH 7.2) until needed. After antigen retrieval, endogenous peroxidase was...
inhibited by incubation for 30 min. at RT with 3% hydrogen peroxide diluted in methanol. To reduce nonspecific background staining, slides were then incubated for 1 hour at RT in a solution containing 0.1 M Tris- HCl buffer (pH 7.2), 2% BSA, and 0.01% Triton X-100. Slides were incubated overnight at 4°C with specific primary antibodies anti-TNF-a (1:50 anti-mouse N-19 sc 1350 Santa Cruz), anti-tryptase (1:100 Mast Cell Tryptase; anti-rabbit FL-275-Santa Cruz Biotech), or antihistamine (1:100 anti-rabbit ab78335 Abcam). After three washes, slides were incubated for 30 min. with biotinylated secondary antibodies, either with anti-mouse IgG (diluted 1:50) (Jackson Immuno Research Laboratories cat # 115035003) or with anti-rabbit IgG (diluted 1:50) (Sigma, USA) for 1 hour at RT. The avidin-biotin-HRP complex and 3,3'-diaminobenzidine were used, according to the manufacturer's instructions (Biocare Medical 901-DB801-01061). Finally, tissue sections were counterstained with hematoxylin (Sigma HHS16) for 1 min. Only dark-brown colored cells with a visible nucleus and cytological features of MC were identified as positively stained mast cells. Control tissue sections were processed in the same manner, but primary antibodies were omitted.

4.7. Statistics

Nonparametric ANOVA ordinary one-way and multiple comparisons were used to test the statistical significance between groups. Significance was considered as p < 0.001. Tests were run by using GRAPH PAD PRISM 8 software.

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**Institutional Review Board Statement:** The protocol used in this study was approved by The Committee on Ethics and Use in Animal Experimentation of the Unidad de Investigación en Medicina Experimental UNAM number. Furthermore, the experimental study followed the guidelines of Mexican regulation for laboratory animals (NOM-062-ZOO-1999) and the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, 8th Edition to ensure compliance with the established international regulations and guidelines.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All of the results were generated and included in this study.

**Conflicts of Interest:** The authors declare no conflict of interest.

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