



An analysis of the influence of sex hormones on Balb/c mice infected with *Plasmodium berghei*



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ABSTRACT

Sex steroids can determine several responses in the clinical evolution of malaria. Seventy Balb-c mice were randomly distributed into 7 groups (10 mice per group): G1 to G6 corresponding to castrated females, castrated females that received estradiol cypionate, uncastrated females, castrated males, castrated males that received intramuscular testosterone decanoate and uncastrated males infected with *Plasmodium berghei*, and G7, the control group. The mice were evaluated with regard to survival, parasitemia, temperature, body weight, hemoglobin level (anemia) and splenic index. Castrated infected females had lower rates of survival. In the castrated male, the administration of testosterone had a negative influence on survival. There was a progressive increase in parasitemia without repercussions for survival. Castration had a significant influence on weight gain in females. Weight loss was observed in all mice, except those in groups G2 and G5, although this bore no direct relation to parasitemia. A significant and progressive decline in temperature and hemoglobin levels occurred in mice over the course of their infection, which differed from the G7 group. The weight of the spleen in relation to total body weight did not differ among the groups of infected mice, but was significantly higher than it was for the control group.

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1. Introduction

Currently, it is incontestable that sex hormones directly influence the immune system and, thus, susceptibility to viral, bacterial and parasitic diseases. In this context, prominent diseases in the field of public health such as tuberculosis, human immune deficiency virus (HIV), influenza, hepatitis and malaria should be investigated. In human beings, not only do sex hormones vary between the sexes, they also change with age and interact with the innate immune system and T and B cells [1,2].

Plasmodium berghei causes serious infection in mice. While it

does not infect humans, this species constitutes a practical model for the study of malaria on account of its analogous physiological structures and life cycle with the plasmodia that provoke the disease in human and non-human primates [3].

In plasmodium-infected mice, a higher mortality rate is observed in males than in females, which may denote differences in the immune response between the sexes. In male mice infected with *Plasmodium chabaudi* or *P. berghei*, castration reduced mortality and the exogenous administration of testosterone increased mortality [4,5]. Understanding the different responses to infection with *P. berghei* in mice of both sexes, castrated and uncastrated, with the exogenous administration of sex hormones can aid in the comprehension of the physiological mechanisms of plasmodial infection in humans.

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2. Ethics

The animals were studied in accordance with Brazilian legislation regarding the raising and use of animals (Lei federal nº 11.794, de 2008) and the norms of the Brazilian College of Animal Experimentation. The research was approved by the Ethics in the Use of Animals Committee of the State University of Pará (registered 34/2012).

3. Methods

3.1. Study time and sample selection

This is an experimental study with 70 adult mice from the Balb/c lineage: 35 nulliparous females and 35 males, with a median weight of 24 g.

The animals were provided by the vivarium at the Evandro Chagas Institute/SVS/MS (Ananindeua, Pará) and were randomly distributed, with ten animals per group:

I – Group 1: Ten castrated Balb-c female mice infected with *P. berghei* NK65.

II – Group 2: Ten castrated Balb-c female mice infected with *P. berghei* NK65, which all received estrogen.

III – Group 3: Ten uncastrated Balb-c female mice infected with *P. berghei* NK65.

IV – Group 4: Ten castrated Balb-c male mice infected with *P. berghei* NK65.

V – Group 5: Ten castrated Balb-c male mice, infected with *P. berghei*, which all received testosterone.

VI – Group 6: Ten uncastrated Balb-c male mice infected with *P. berghei* NK65.

VII – Group 7: Five Balb-c male mice and five Balb-c female mice that were neither infected nor castrated (the control group).

3.2. Plasmid strain and experimental infection

For experimental infection, we used a strain of *P. berghei* NK65 that was cryopreserved in liquid nitrogen and provided by the Laboratory for Basic and Applied Research on Malaria at the Evandro Chagas Institute/SVS/MS.

After thawing the cryopreserved blood containing *P. berghei* NK65, five mice were inoculated with 0.2 ml of the sample (1st replication). The mouse who first achieved a parasitemia level of approximately 5% was designated as the donor mouse, from which blood was drawn for the inoculation of the other five mice (0.2 ml/mouse, 2nd replication). When these mice reached parasitemia levels above 1%, an inoculation with 10^6 parasites was prepared, containing infected and non-infected erythrocytes, which was then administered to the mice in groups G1 through G6 (0.2 ml/mouse, intraperitoneally).

Both the 1st and 2nd replication mice were submitted to euthanasia (cervical dislocation) prior to the collection of biological material.

3.3. Treatment with synthetic steroids

Animals in group 2 received veterinary estradiol cypionate (2 mg/ml) at a dose of 2.5 µg/100 g of body weight, using canola oil as a delivery vehicle. Animals in group 5 received testosterone decanoate at a dose of 15 mg/kg. The doses of both hormones were administered intramuscularly four days after the day of inoculation and were maintained until the animals' deaths.

3.4. Clinical and laboratory parameters

The animals were distributed according to their respective study groups (G1 to G6) in plastic cages lined with wood shavings (bedding), which was changed daily. They did not receive treatment for plasmodial infection. The control group was separated into separate cages according to sex. All the mice received water and rations *ad libitum*.

At the end of the afternoon each day (for the authors' convenience), drops of blood were drawn from the base of each mouse's tail to determine parasitemia levels (thin blood smear), which were calculated as the percentage of parasitized red blood cells in ten microscopic fields [6].

Hemoglobin levels were evaluated at the same time as the parasitemia levels by collecting a drop of blood from the tail base of each animal with disposable needles. Once collected, the blood drops were placed in *microcuvettes* and immediately analyzed in the Hemocue® (Transfusion Technology, USA) device.

In mice from all the groups, body temperature was taken rectally at the end of each day (from the date of inoculation onward) with a digital thermometer for veterinary use. The mean temperature presented by the control group was considered the normal temperature (taken on the same day the temperatures of mice in Groups G1 to G6 were taken).

3.5. Operating procedures

The animals were anesthetized intraperitoneally using a combination of Acepromazine (3 mg/kg), Ketamine (100 mg/kg) and Xylazine (10 mg/kg). After surgery, the animals received 0.05 ml of veterinary-pentabiotic for small animals, which was administered intramuscularly via the animal's hind paw to avoid post-operative infection.

The oophorectomies were performed by means of a para-vertebral bilateral incision in the animal's torso. The orchiectomies were performed starting from the median longitudinal incision, with access to the abdominal cavity, identification and ligation of the spermatic funiculus and testicular artery following the resection of the testicles and epididymis. Both procedures were in accordance with the techniques performed in the Experimental Surgery Laboratory of The State University of Pará.

3.6. The evaluation of the degree of splenomegaly

After the death of the mice, a precision electronic scale (Filizola brand) was used to gauge both the weight of the animals and the weight of the spleen. The degree of splenomegaly was determined by calculating the ratio of the spleen weight (g) to the total animal weight (g).

3.7. Statistical analysis

The categorical variables are presented in the form of absolute values or percentages. The quantitative variables were analyzed with measures of central tendency (mean) and measures of dispersion (coefficient of variance and standard deviation).

The survival analysis was obtained using Kaplan–Myer survival curves, and the difference between the groups' survival curves was obtained using the Log-Rank test.

The quantitative data were compared using a signal test in related samples and using the Kruskal–Wallis test on independent samples.

A significance level of $\alpha \leq 0.05$ was adopted in all applied tests that were performed using the Program BioStat 5.0 (2007) [7].

4. Results

The castrated females (G1) had a significantly lower survival rate in relation to that of the castrated females that received exogenously administered estradiol (G2) and uncastrated females (G3). Group G2 also had a significantly better survival rate than G3 ($p < 0.05$, Log-Rank test) (Fig. 1).

In the males, castration (G4) or the absence of castration (G6) had no significant influence on survival ($p > 0.05$; Log-Rank test). However, the administration of decanoate and testosterone to the mice in group 5 (previously castrated) was a determining factor for a lower survival rate of male mice ($p < 0.05$, Log-Rank test) (Fig. 1).

Castrated females lived significantly shorter lives than the castrated males, with lifespans of 13 and 20 days, respectively ($G1 \times G4$, $p < 0.0001$, Log-Rank test). The castrated males and females that received hormones exogenously did not have different survival rates compared with uncastrated males and females ($G2 \times G5$, $p > 0.05$; $G3 \times G6$, $p > 0.05$; Log-Rank test) (Fig. 1).

The castrated male mice that received testosterone (G5) had faster parasitemia growth from the time of their inoculation (D0) onward, and this growth persisted throughout the tracked period until their last day of survival (D14). The male mice (G4 and G6) had similar parasitemia and survival rates (Fig. 2).

Similar to the uncastrated males (G6) and the castrated males (G4), the uncastrated (G3) females initially had a slower evolution of parasitemia growth and greater survival and higher parasite loads at the time of death. The castrated females (G1) had greater parasitemia than the castrated females that received estradiol (G2) and the uncastrated females (G3), and the castrated females also had lower survival rates (Fig. 2).

4.1. Weight

From the post-castration period until the day of inoculation, there was significant weight gain in the females of G1 ($22.0 \text{ g} \pm 1.30 \text{ g}$ – $26.5 \text{ g} \pm 1.90 \text{ g}$) and G2 ($23.4 \text{ g} \pm 1.77 \text{ g}$ – $27.2 \text{ g} \pm 1.31 \text{ g}$) ($p < 0.001$ for G1 and G2, signal test), as well as in the males that received testosterone (G5) ($22.0 \text{ g} \pm 2.16 \text{ g}$ – $22.3 \text{ g} \pm 2.87 \text{ g}$) ($p = 0.0039$, signal test). However, the castrated males (G4) had significantly decreased weight ($25.8 \text{ g} \pm 2.18 \text{ g}$ – $25.1 \text{ g} \pm 2.80 \text{ g}$) ($p = 0.0039$, signal test).

With the exception of G5 (castrated males that received testosterone) and G2 (castrated females that were administered estradiol), all groups of infected mice had significant weight loss from inoculation until death ($p < 0.05$, signal test). Among those mice that were not infected (G7), weight ($24.5 \pm 1.67 \text{ g}$) remained

stable until euthanasia (Table 1).

Significant weight gain was observed only among the G1 (castrated females) \times G5 (castrated males that received testosterone); G2 \times G5 (females and males castrated with hormones); G2 (castrated females to which estradiol was administered) \times G3 (uncastrated females) groups ($p < 0.05$ for G1 \times G5, G2 \times G5, and G2 \times G3; Kruskal–Wallis test).

At death, G2 (castrated females that received estradiol) females weighed significantly more than G4 (castrated males) and G5 (castrated males that received testosterone) ($p < 0.05$, Kruskal–Wallis test). Castrated females (G2) also had significantly higher weights than non-castrated females (G3) ($p < 0.05$, Kruskal–Wallis test). There were no significant differences for this variable in all other intergroup comparisons.

At death, there was no correlation between body weight and parasitemia among any groups ($p > 0.05$; Spearman Correlation).

4.2. Hemoglobin values

Independent of their groups, all the mice presented with significant and progressive declines in hemoglobin levels through the course of their *P. berghei* infections. In this respect, the infected mice differed from the control group mice, which, upon death had hemoglobin levels similar to those they had at the onset of the experiment (Table 2).

On the day of inoculation, there was a significant decrease in the level of hemoglobin between the following groups: G5 (castrated males that received testosterone) \times G1 (castrated females); G5 \times G3 (uncastrated females); G5 \times G6 (uncastrated males); G4 (castrated males) \times G3; G2 (castrated females that received estradiol) \times G3 ($p < 0.05$ for all comparisons, Kruskal–Wallis test). The drop in hemoglobin among all the other groups was not significant ($p > 0.05$, Kruskal–Wallis test).

4.3. Rectal temperature

From the time of their inoculation onward, rectal temperature decreased significantly in each group of infected mice, a feature not observed in the control group (Table 3).

In the intergroup analysis, on the day of the application of the inoculant, there were significant differences in the lower temperature of the animals among the following groups: G1 (castrated females) \times G4 (castrated males), G5 (males that received testosterone) \times G2 (castrated females that received estradiol), and G5 \times G4. The G5 mice had a temperature significantly lower than either G3 (uncastrated females) or G6 (uncastrated males) mice

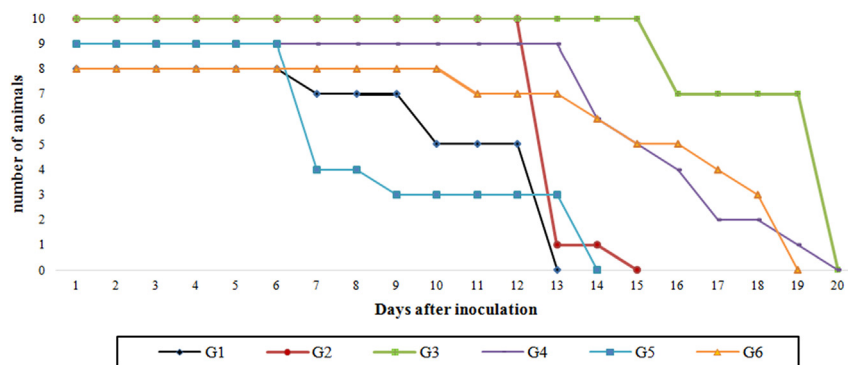


Fig. 1. Survival Curve, in days, of experimental groups after *P. berghei* inoculation ($G1 \times G2$, $p = 0.0316$; $G1 \times G3$, $p < 0.0001$; $G2 \times G3$, $p < 0.0001$; $G4 \times G5$, $p = 0.0004$, $G4 \times G6$, $p = 0.7846$, $G5 \times G6$, $p < 0.0001$; Log-Rank test).

Source: Research protocol.

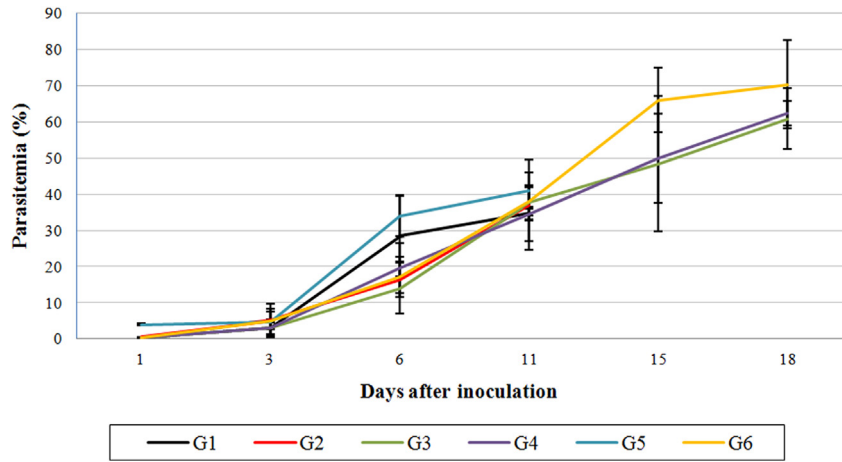


Fig. 2. Percentage of parasitemia (mean and standard deviation) in mice groups G1 to G6, from *P. berghei* inoculation to death (days).
Source: Research protocol.

($p < 0.05$, Kruskal–Wallis test).

Upon the death of the animals, the drop in rectal temperature was significant in two groups of females: G1 (castrated) and G2

(uncastrated that were administered estradiol) when compared with the mice in group G3 (uncastrated). A drop was also observed between the G3 × G4 (castrated males) mice and between the

Table 1
 Weight (mean and standard deviation) in mice groups G1 to G7.

Groups	Weight (in gram)	Weight (in gram) (on death)	p value (signal test)
Male control (G7)	25 ± 1.38	25.3 ± 1.79	p > 0.05
Female control (G7)	24 ± 1.40	24.6 ± 1.30	
<i>P. berghei</i> inoculation			
G1	26.5 ± 1.90	22.0 ± 2.60	p = 0.002
G2	27.2 ± 1.31	26.1 ± 1.22	p > 0.05
G3	23.7 ± 1.94	18.4 ± 1.49	p = 0.002
G4	25.1 ± 2.80	19.1 ± 2.16	p = 0.001
G5	22.3 ± 2.87	21.2 ± 2.61	p > 0.05
G6	26.5 ± 1.90	21.3 ± 2.11	p = 0.0039

Source: Research protocol.

Table 2
 Hemoglobin values (mean and standard deviation) in mice groups G1 to G7.

Groups	Hemoglobin (g) (Day of inoculation)	Hemoglobin (g) (on death)	p value (signal test)
Male control (G7)	17.5 ± 1.11	17.55 ± 1.25	p > 0.05
Female control (G7)	17.8 ± 0.88	16.80 ± 1.31	
<i>P. berghei</i> inoculation			
G1	18.94 ± 1.96	7.54 ± 2.63	p = 0.001
G2	16.99 ± 1.00	6.28 ± 1.04	p = 0.001
G3	18.99 ± 0.91	3.97 ± 1.06	p = 0.001
G4	16.88 ± 1.19	4.24 ± 1.24	p = 0.001
G5	16.11 ± 0.75	8.75 ± 4.56	p = 0.002
G6	18.8 ± 1.12	4.41 ± 1.33	p = 0.003

Source: Research protocol.

Table 3
 Rectal temperature (mean and standard deviation) in mice groups G1 to G7.

Groups	Temperature (°C) (Day of inoculation)	Temperature (°C) (on death)	p value (signal test)
Male control (G7)	34.80 ± 0.66	35.3 ± 0.76	p > 0.05
Female control (G7)	35.26 ± 0.61	35.1 ± 0.72	
<i>P. berghei</i> inoculation			
G1	34.13 ± 0.74	29.59 ± 0.84	p = 0.001
G2	35.12 ± 0.38	30.29 ± 0.70	p = 0.001
G3	34.92 ± 1.00	22.75 ± 1.22	p = 0.001
G4	35.63 ± 0.47	27.84 ± 5.14	p = 0.001
G5	32.68 ± 0.76	30.08 ± 1.64	p = 0.002
G6	35.11 ± 0.63	26.96 ± 2.71	p = 0.003

Source: Research protocol.

G3 × G5 (castrated males that received testosterone) mice ($p < 0.05$ for G1 × G3, G2 × G3, G3 × G4 and G3 × G5; Kruskal–Wallis Test). The decrease in temperature was similar in comparison between all other groups ($p > 0.05$, Kruskal–Wallis test).

4.4. Splenomegaly index

The splenomegaly index, the weight of the spleen in relation to body weight, was not different among the groups of infected mice, but was significantly higher than that of the control group ($p < 0.05$, Kruskal–Wallis test) (Fig. 3).

5. Discussion

In this study, male and female mice displayed different clinical and parasitological behaviors after infection with *P. berghei*, in concordance with the sexual dimorphism presented by this species of protozoan [4,8].

Castrated females had lower survival rates in relation to those that were castrated, but not compared to mice that received exogenous hormones and those that were not castrated. This protective female-hormone effect was evidenced by Klein et al. [9] when evaluating the effect of estrogen on malarial infection in female mice C57BL/6. This effect was also observed by Libonati et al. [10]; who studied cerebral malaria in female CBA mice infected with *P. berghei* ANKA. In this study, they found that females with estrogen replacement had significantly lower parasitemia (and as a consequence, higher survival) in relation to castrated mice without replacement, as well as major nitric oxide synthase (NOS) activity. It is possible that the estrogen stimulates the production of nitric oxide, and the subsequent plasmodicide activity would lower parasitemia in females with replacement of this steroid.

Castration did not have a significant influence on the survival of male mice. However, the administration of testosterone decanoate to the mice in group 5 (previously castrated) was a determining factor for the males that had lower survival rates. The exact mechanism of testosterone in malaria is still not completely understood. However, it is believed that this hormone has the capacity to provoke immunosuppression in animals, inducing a permanent vulnerability to infection caused by diminishing levels of circulating protective antibodies. It is theorized that the hormone produces this effect by altering the pattern of cytokines and reducing the expression of genes in the parasite's hepatic cycle [11].

Parasitemia evolved in a growth mode in all the groups (G1 up to G6). This growth, however, was not correlated with survival among

the groups and intergroups. In females, the initial increase in parasitemia was greater in those that were castrated (G1), with diminished survival in comparison with hormone-possessing females (endogenous or exogenous), which had a slower rhythm of parasitic development, denoting a protective effect of this feminine hormone, probably due to an increase in nitric oxide [10,12].

Similar parasitemia and survival among castrated males (G4) and uncastrated males (G6) demonstrated the absence of an association between the circulating level of endogenous hormones and a greater severity of infection. This finding is in opposition to what was observed by Mosqueda-Romo et al. [13]; who found that castration had a protective effect in males (increased plasmodial resistance) and higher parasitemia in those that possessed endogenous hormones compared with those that were castrated.

In the mice that received endogenously administered testosterone (G5), there was a precocious and faster increase in parasitemia. Consequently, there was a more rapid clinical evolution of infection in these mice, with significant reductions in survival, probably as a result of the immunosuppressive effect of this hormone [1,13].

In the period from post-castration until the day of inoculation, there was significant weight gain in castrated females (G1), castrated females that received estradiol (G2) and between G1 × G5, G2 × G5, and G2 × G3. By altering the serum levels of estrogen, castration determines metabolic alterations that influence body weight, consonant with the oophorectomy-related weight increase observed in rats in an experimental model [14].

Castrated male mice (G4) had significantly decreased weight, which contrasted the weight gain observed in those that received testosterone (G5). In this respect, our findings were similar to the results described by Velloso [15] in male Wistar rats with hypogonadism due to surgical castration. These data suggest a loss of weight due to the loss of muscle mass [16]. Except for the G5 (castrated males that received testosterone) and G2 (castrated females that were administered estradiol) mice, all groups of infected mice had significant weight loss from inoculation until death. This seems to denote that *P. berghei* infection *per se* cancels out the hormone-influenced weight gain, regardless of the lack of correlation between weight and intragroup parasitemia observed in this study. This could possibly be similar to what occurs in plasmodial infection in humans, in whom parasitemia is not the only factor that regulates the physiological events of malaria [17,18].

In the intergroup comparison, the weight of castrated females that received estradiol (G2) – while still low in relation to the weight acquired before inoculation was administered – was higher than that of castrated males (G4) and that of males that received testosterone (G5), underscoring the importance of sexual dimorphism in the relationship between the parasite and host [4,8].

In the course of their infection, all of the infected mice had a significant and progressive decline in hemoglobin levels. In this respect, they were unlike the group of uninfected mice (G7), whose hemoglobin levels stayed similar to those they had had at the onset of the experiment. This discrepancy can be justified by the fact that *P. berghei* infects mature erythrocytes and reticulocytes in an erythrocytic cycle similar to that of human plasmodia [19]. It is worth noting, however, that anemia in plasmodial infection is of multifactorial origin, with the participation of immunological and autoimmune mechanisms not contemplated in this study – mechanisms that certainly exerted an influence on the role of masculine and feminine sexual hormones in erythropoiesis [20].

Castration may justify a significantly lower hemoglobin level in G4 because the androgens stimulated erythropoiesis by increasing the production of erythropoietin by the kidneys and by acting directly on hematopoietic stem cells [21]. Group G5 had lower hemoglobin levels than group G6, probably because this group (G5)

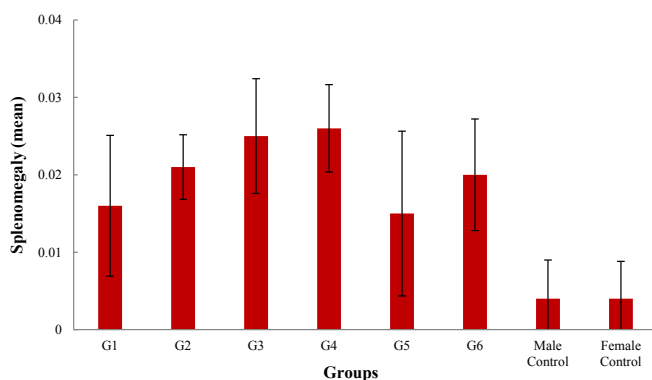


Fig. 3. Splenomegaly index (mean and standard deviation) in mice groups G1 to G7. Kruskal–Wallis test. Among infected groups ($p > 0.05$); Infected groups vs. control groups ($p < 0.05$).

Source: Research protocol.

had a higher parasitemic average despite testosterone replacement.

From inoculation onward, temperature decreased significantly in each group of infected mice, similar to what has been observed in other experimental models with malaria and different than fever with paroxysm observed in plasmodial infection in humans [18]. Considering that *P. berghei* determines the lethal infection in mice, similar to the severe forms of malaria caused by *Plasmodium falciparum*, the progressive decrease in temperature for all animals in this study (in contrast with the stability and normality of temperature in the control group) denotes that in all mice, the process evolved with severe infection [17,18].

After inoculation, castrated female mice (G1) and castrated female mice that received exogenous administration of estradiol (G2) had significantly lower temperatures than those of the castrated male mice (G4) and/or castrated male mice that received testosterone (G5). We therefore posit that in these cases gender contributed to lowering the temperature of female mice more lowering the temperature of males. So long as it is assumed that the degree of hypothermia indicates higher severity, this means that gender acts as a negative factor in clinical evolution. However, acceptance of this hypothesis is limited by the fact that there was no difference in hypothermia between uncastrated males and females (G3, G6), except that the absence of hormonal alterations (castration, hormone replacement) kept neuroimmune integration more harmonious.

The spleen plays a fundamental role in the clearance of *P. berghei* by the removal of infected red blood cells [22]. In this manner, infections from this protozoan induce a dramatic response, generally with hyperplasia and hypertrophy of the lymphoid follicles [23]. Postmortem analysis of the spleen weight demonstrated splenomegaly in all groups, though not between groups. This finding contrasts that of Libonati et al. [10]; who found that mice that received estrogen had smaller spleens than those of castrated mice.

In patients from endemic areas, spleen weight is a useful tool for determining the intensity of plasmodial infection [24]. Although Balb-c mice infected with *P. berghei* developed significant splenomegaly, extrapolation of this result in the comparison of the splenic immune response between mice and humans should be made with caution, considering mice as general experimental models for the study of malaria [23].

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