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The effect of sex hormones on progression of *Leishmania donovani* infection in liver and spleen of BALB/c mice

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Abstract--*Leishmania donovani* invades and resides within tissue macrophages in the spleen, liver, and bone marrow leading to a histological change such as hepatosplenomegaly. In the present study we experimented the effect of gender and sex hormones on the progression of the visceral leishmaniasis disease in the liver and the spleen of BALB/c mice. The mice were divided into two groups, males and females, then male group was divided into three sub groups, first injected normal saline (control), the second injected with 107 promastigotes intraperitoneally and the third injected with 107 promastigotes intraperitoneally plus estradiol subcutaneously. Female group was divided into three groups, the first injected with normal saline, the second injected with 107 promastigotes and the third injected with 107 promastigotes intraperitoneally plus testosterone subcutaneously. After six weeks, all animals were sacrificed and dissected then liver and spleen were removed, weighted and histological sections were made from them. The result revealed several changes in liver sections of infected males and infected females treated with testosterone like disarrangement of the hepatocytes around central vein, inflammatory cells infiltration, granuloma formation with amastigotes inside macrophages and congestion while the spleen shows disorganization of white pulp and red pulp and appearing of parasite amastigotes, while infected females and infected males treated with estradiol revealed mild changes like low numbers of inflammatory cells infiltration and spleen have minor congestion.

Keywords--sex hormones, estradiol, testosterone, visceral leishmaniasis, *Leishmania donovani*, hepatosplenomegaly.

Introduction

Among both humans and animals, the spread and severity of parasitic infections are higher in males than in females [1,2]. Sex differences in exposure as well as susceptibility to parasites may contribute to sex-based differences in the intensity and spread of parasites. Several studies illustrated that immunological differences exist between the sexes that may underlie increased parasitism in males. Females typically have higher immune responses than males [3]. Elevated immunity among females creates a strong factor against infectious diseases [4]. *Leishmania donovani* invades and resides within tissue macrophages in the spleen, liver, and bone marrow [5,6,7]. During this initial phase, which related to the 2-3 weeks post-infection in visceral leishmaniasis of murine models, most parasites are eliminated from the liver parenchyma by the hepatic immunologic response [8,9,10]. In other mammalian models, like dogs, the presence of infected reticuloendothelial liver cells (Kupffer cells) can be appeared as early as 2 days post infection and can expand to months or years [11]. The spleen can rapidly produce nonspecific, polyreactive antibodies, which efficiently contain infectious agents until the antigen-specific immune reaction occurs in the germinal center. This essential survival trait, which is afforded by short-lived plasma cells residing in its marginal zone, is hijacked by visceralising *Leishmania spp.*, leading to complete splenic anatomical and functional disorganization [12,13]

Methodology

Sixty males of *Mus musculus Balb/c* mice aged (10-12) weeks and weighted (30-38) Gms were brought from university of Kufa / College of Science / Animals' house. The animals were breed in animal house of Pharmacy college / University of Karbala in plastic cages at (25±4) C° and provided with water and fed pellet.

The sample of the parasite

Fresh sample of *Leishmania donovani*, strain (DUAA/ IQ/ 2005/MRU15) in NNN media brought from Department of Biology/ College of Science/ University of Baghdad then, new subcultures in NNN were made every week to keep the vitality of the parasites. The culture was examined every twice day to count the number of promastigotes by using Neubauer chamber. The dosage of the parasite was prepared by centrifuging the lock solution of number of cultured NNN tubes with speed 1500r/min. Then the precipitant was diluted with lock solution then, Neubar Hemocytometer chamber slide was used to count the number of parasites in order to prepare a dosage of 10^7 /mouse / 0.2ml. All groups, except negative control were injected with 10^7 parasites/mouse intraperitoneally.

Designing of the study

In this study 60 mice were used (30 males and 30 females). Both males and females were divided into three groups as the following: -

Male groups

- Negative control group: Injected with 0.2 ml normal saline only.

- Positive control group: Injected once with 1×10^7 parasites intraperitoneally only.
- Estrogen + Parasites group: Injected once with 1×10^7 parasites intraperitoneally and 545 $\mu\text{g}/\text{kg}$ 17β -estradiol subcutaneously twice a week for 6 weeks.

Female groups

- Negative control group: Injected with 0.2 ml normal saline only.
- Positive control group: Injected once with 1×10^7 parasites intraperitoneally.
- Testosterone+parasite group: Injected once with 1×10^7 parasites intraperitoneally time and 12 mg/kg testosterone subcutaneously twice a week for six weeks.

Histological preparation

For histological studies, the mice were jerked then, the animals were sacrificed directly. The liver and spleen were isolated from the abdominal cavity, weighted and measured then, histological sections were made and stained according to [14].

Results

Table (1) shows the changes in length, width and weight of liver and spleen of the animals, the table revealed that the size and weight of liver increased significantly in female +parasite +testosterone (length 3.6cm and width 3cm weight 2gm) and in male+parasite (length 3.5cm and width 2.4cm weight 1.8gm) compared to other groups while decreased in infected females and males treated with estradiol.

Table 1
The changes in length, width and weight of liver and spleen of the animals

parameters Groups	Liver			Spleen		
	Length \pm SE	Width \pm SE	Weight \pm SE	Length \pm SE	Width \pm SE	Weight \pm SE
Male control	2.7 \pm 0.19 ^b	2.2 \pm 0.33	1.54 \pm 0.19 ^b	1.7 \pm 0.22 ^b	0.42 \pm 0.05 ^b	23.5 \pm 3.2 ^b
Male+parasite	3.5 \pm 0.22 ^a	2.4 \pm 0.41	2.1 \pm 0.15 ^a	2.4 \pm 0.29 ^a	0.61 \pm 0.08 ^a	31.1 \pm 4.1 ^a
Male+parasite +E2	3.1 \pm 0.36 ^a	2.4 \pm 0.37	1.6 \pm 0.20 ^b	2.0 \pm 0.17 ^b	0.51 \pm 0.07 ^a	25.3 \pm 2.6 ^b
Control Female	2.4 \pm 0.35 ^b	2.1 \pm 0.19	1.33 \pm 0.15 ^b	1.7 \pm 0.25 ^b	0.44 \pm 0.05 ^b	22.1 \pm 3.3 ^b
Female+parasite	2.7 \pm 0.1.8 ^b	2.6 \pm 0.22	1.61 \pm 0.17 ^b	1.9 \pm 0.21 ^b	0.52 \pm 0.04 ^a	26.6 \pm 2.3 ^b
Female+parasite +testosterone	3.6 \pm 0.43 ^a	3 \pm 0.42 ^a	2.3 \pm 0.29 ^a	2.5 \pm 0.3.2 ^a	0.63 \pm 0.05 ^a	33.2 \pm 5.1 ^a

Histopathology

- Control normal mice: the liver sections of mice revealed normal cells and tissue in addition, the sections revealed normal radial arrangement of the

hepatocytes around the central vein. The sinuses also appeared normal in shape and size and have no congestion or bleeding (Figure 1, a)

- The infected males: - Show noticeable abnormality in tissue section of the liver. there were enormous inflammatory cells infiltration and the liver lost the radial arrangement of the cells around the veins (figure 1, b). Granuloma formed with amastigotes inside macrophages (Figure 1, c).
- Infected males treated with estradiol: - Revealed mild inflammatory cells infiltration, no parasite seen and no congestion. The radial arrangement around the central vein appeared normal (Figure 1, d).
- Infected females: The liver section of infected females shows mild inflammatory cells infiltration and the radial arrangement of cells around the central vein appeared normal (Figure 1, f).
- Infected females injected with testosterone: - The liver section revealed losing of the radial arrangement of the liver cells around the central veins, vein dilation, congestion, formation of granuloma with amastigotes inside macrophages in the center of granuloma and sinusoids dilation. (Figure 1, g-h).

Spleen

- Control: shows the normal organization of white and red pulp (figure 2-a)
- Infected males : show reducing in white pulp and disorganization of white and red pulp (figure 2-b) as well as sever congestion and hemorrhage in red pulp also the show amastigotes of the parasite inside the macrophage (figure 2-c). proliferation of megakaryocyte (figure 2-d)
- Infected males treated with estradiol: (figure 2-e) shows mild reducing in white pulp and normal organization of white and red pulp and mild congestion.
- Infected females: (figure 2-f) shows mild disorganization of white pulp and red pulp with mild congestion.
- Infected females treated with testosterone: Shows reducing in white pulp, disorganization of white and red pulp and proliferation of megakaryocyte (figure 2-g) while (figure 2-h) shows congestion, hemorrhage and found of amastigotes of the parasite inside the macrophages.

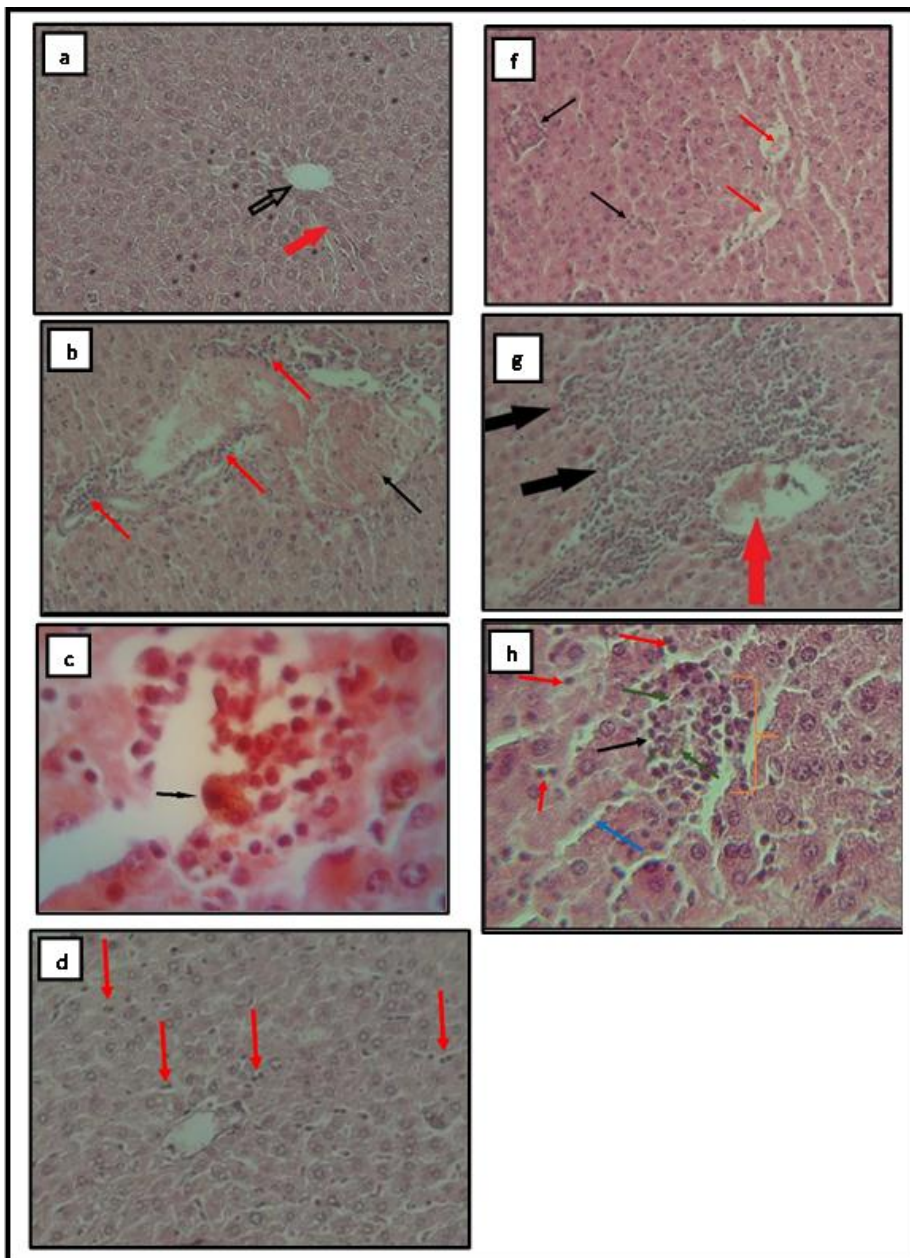


Figure 1. Liver sections: a-Normal liver shows hepatocytes → around central vein →100X. H&E.. b- Infected males shows vasodilation with congestion → and inflammatory cells infiltration →100X. H&E.. c- Infected male shows amastigotes inside macrophage → 400X. H&E stain.. d-Infected male treated with estradiol shows mild inflammatory cells infiltration →100X. H&E stain.. e- Infected female without treatment shows mild inflammatory cells infiltration → and mild vein congestion 100X. H&E stain. f-Infected females treated with testosterone shows enormous inflammatory cells infiltration around central vein → and central vein congestion→ 100X. H&E stain. g- granuloma formation] , sinusoidal dilation→ and macrophages contain amastigotes → 400X. H&E stain

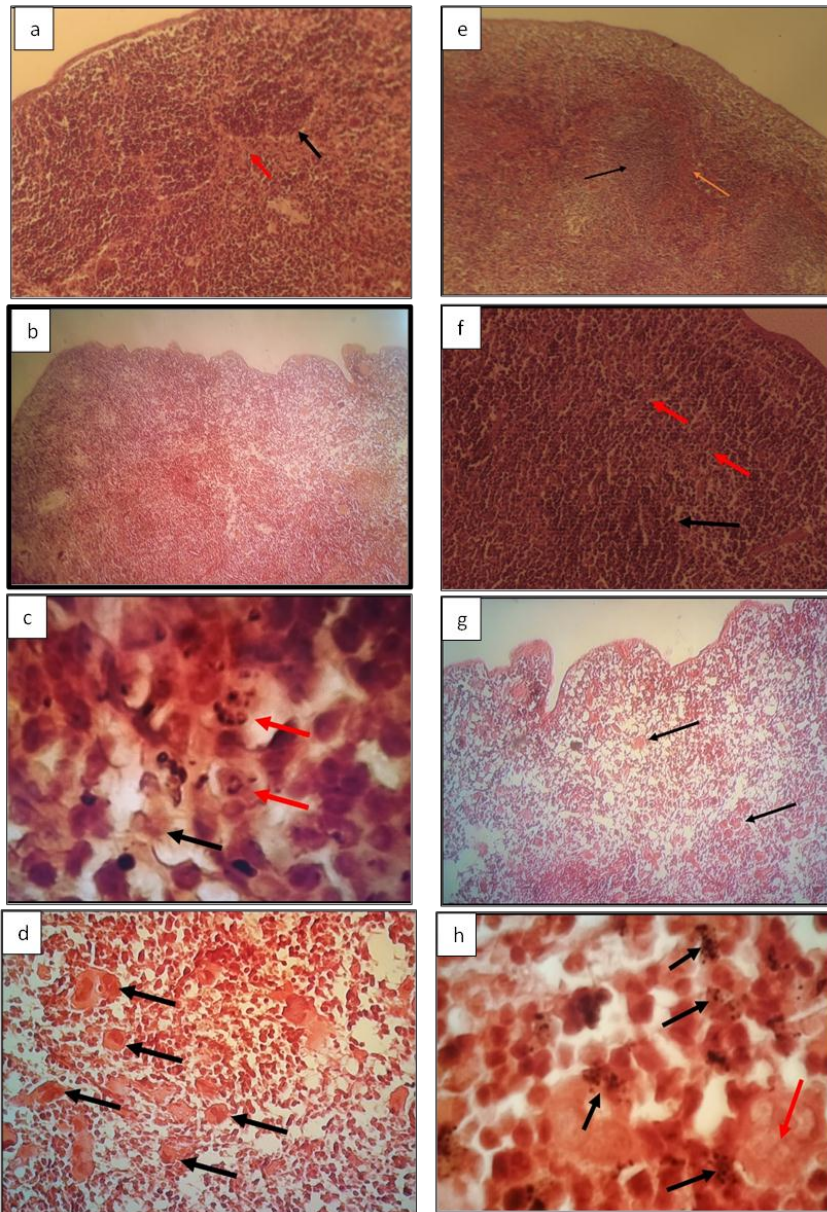


Figure 2. Spleen sections: a- Control normal mice shows: white pulp → and red pulp →.(40x) H&E stain b- Infected male shows: disorganization of white and red pulp (40x) H&E stain. c- Infected male shows: amastigotes → (500x) H&E stain. d- Infected male shows: proliferation of megakaryocyte → (100x) H&E stain. e- infected male treated with estradiol shows: white pulp (→) mild disorganization, with mild congestion of red pulp (→) (40x) H&E stain. f- Infected female shows: white pulp→ and red pulp→ with minor congestion (40x) H&E stain. g- infected female treated with testosterone shows: components disorganization and proliferation of megakaryocyte→ (40x) H&E stain. h- Infected female treated with testosterone shows: congestion (→) and amastigotes of the parasites (→) (400x) H&E stain

Discussion

The effect of *Leishmania donovani* on liver and spleen size

The present study revealed that *Leishmania donovani* infection caused a significant hepatomegally and splenomegally in males and females this agree with [9,15] they found that *Leishmania donovani* infection caused liver and spleen enlargement due to increasing in macrophage production by immune system as a response to parasite infection as well as hyperplasia of endothelia in liver and spleen. It was proved that hepatosplenomegally occurs in patients infected with *Leishmania donovani* as a result of accumulation of macrophages in liver and spleen [16,17]. Ramzi and Zghair found by an experiment on mice that the liver and spleen size increased gradually with progression of infection as a result of aggregation of inflammatory cells [18]. On the other hand Maeda *et al* demonstrated that the increasing in the liver weight was predominantly due to an increase in water weight in the liver as a result of non-obstructive hepatic sinusoidal dilation that lead to edema which associated with sinusoidal dilation [19].

Histopathological studies

The main organs that affected by the infection of visceral leishmaniasis are liver, spleen and bone marrow [18, 7]. Kennedy and Deleo explained that the severity of the disease is that the infected macrophages in the skin are the gateway to infect other organs, such as the liver, spleen, bone marrow and lymph nodes [20]. These infections can lead to focal lymphoid hyperplasia and as a result of parasitism of splenic macrophages and kupffer cells leading to hepatosplenomegally [21].

Control group

The control group of the liver revealed normal tissue structure that has normal radial arrangement of liver hepatocyte around the central vein and normal sinusoids and no findings of granulocytes. The control group of spleen sections show normal tissue organization of spleen components which consist of two compartments, white pulp and red pulp.

Infected males group

The study revealed several changes in liver section of infected males. The changes included disappearing of the ray arrangement of hepatocytes, granular infiltration, granuloma formation, vascular dilation, sinusoidal dilation and congestion. The section also revealed parasite burden inside macrophage and these findings matching results obtained by [22]. Ramzy and Zghair found that hydropic degeneration of liver cells leads to disorganization of hepatic cords [18]. Disorganization is resulted from decreasing of ATP which lead to K⁺, N and Ca overload and activation of endogenous proteases or phospholipases that damage the cytoskeletal supports. [23]. Furthermore, necrosis can happen due to blocking of blood vessels as a result of *Leishmania donovani* amastigotes and reaction of inflammation leading to ischemic effect on liver cells which leads to loss oxidative phosphorylation by mitochondria and decreasing ATP generation which is responsible for acute cell degeneration and necrosis [25].

Formation of granuloma in the liver section of the infected males was explained by Beatie *et al* who mentioned that granuloma is resulted as immune reponse for containing parasite dissemination and providing main site responsible for parasite removal [25]. The spleen section of the infected male shows disorganization of white and red pulp, proliferation of megakaryocyte, and congestion of red pulp this in line with Ramzi and Zghair [18]. As well as, the boundary between white and red pulp gradually disappeared. These findings also matching the results obtained by Veress *et al.*, [26]. It was found also that sever infection of leishmaniasis leads to disorganization of the spleen compartments[9].

Infected male treated with estradiol (E2) group

The section of the liver of the infected males treated with E2 revealed mild inflammatory cells infiltration but exhibit normal hepatocyte arrangement around central veins as well as disappearing congestion and parasite burden comparing to non treated infected males group mentioned previously. The healing of infected female may belong to the effect of estradiol as immune enhancer to reduce the effect of the parasite infection. It was considered that sex hormones androgens and oestrogens are involved in innate and adaptive immune response, so that determining pathogenicity, virulence and evolution of the disease depend on these hormones. As well as they found that estradiol is associated with immune function regulation leading to control the infectious process that involve proinflammatory mediator [27,28]. It was found that several cells of immune system of the mice oestrogenic receptors and androgens receptors in T and B cells, so that discovering these receptors may be a guide for the role of sex hormones in regulating immune system [30,31]. *Leishmania donovani* is considered organ-restricted immunity, so that a self healing infection can be observed in the liver which depend on the development of T-cell mediated immunity and granuloma formation.

Infected female treated with testosterone

The present study explained that treating the infected females with testosterone leads to huge changes in liver tissue, included disappearing hepatocyte arrangement, sinusoid dillation, congestion, bleeding and formation of granuloma in addition to vasodilation, inflammatory cells infiltration around veins and granuloma formation. This may belong to the effect of testosterone as immunosuppressor, this in line with Gubbeles and Jorgensen they found that testosterone has immunosuppressive effect on different components of the immune system as well as in different phases of responses [33]. immunosuppressor effect on infection belong to the effect of testosterone in elevating IL-10 and lowering IFN- γ [34]. It was domenstrated that IFN- γ promotes monocyte activation while IL-10 limits monocyte activation against *Leishmana donovani* infection [35,36]. In addition testosterone has effect on suppressing antibody response to vaccination and infection, suppressing inflammatory immune cells like dendretic cells and macrophages and inhibit the development and functioning of T and B cells.

Conclusions

- Infection with *Leishmania donovani* caused major changes in liver and spleen of males and females treated with testosterone such as hepatosplenomegaly, inflammatory cells infiltration, congestion, and parasite burden in macrophages but caused minor changes in females and males treated with estradiol.
- Estradiol has a powerful effect on reducing the strength of infection on the liver and spleen while testosterone has power effect on elevating the strength of infection in liver and spleen.

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