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Experimental visceral leishmaniasis: immunopathology and histology

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Abstract

This study aimed to assess the immunological and histopathological effects of Leishmania donovani infection in mice, and the impact of pentostam treatment. L. donovani promastigotes were cultured in Nicolle-Novy-McNeal (NNN) medium. Thirty mice were divided into three groups of ten: a negative control group given saline, a positive control group infected with promastigotes, and a treatment group infected with promastigotes and treated with pentostam. The mice were treated daily for 21 days. Blood samples were collected after 7, 14, and 21 days to measure serum levels of IL-1. After 21 days, the mice were euthanized, and their livers and spleens were collected for histopathological analysis. The results showed a significant decrease in IL-1 levels in the infected group compared to the control group, while IL-1 levels increased slightly in the treated group. Histopathological analysis revealed pathological changes in the liver and spleen of infected mice, which were reduced in the treated group. The study concluded that L. donovani infection leads to a decrease in IL-1 production and causes pathological damage to the liver and spleen, and that pentostam treatment is effective in mitigating these effects.

Keywords: *Leishmania donovani,* NNN media, *Interleukin* 1, Parasitic disease, histopathological

1. Introduction

Leishmaniasis is one of the main predominant parasitic health issues in the world. It is a vector-borne disease caused by obligate intracellular protozoa it is an endemic disease in a wide area of the tropic, subtropic and Mediterranean basin [1]. With the exception of Antarctica and Australia, visceral leishmaniasis (VL) is prevalent in more than 70 nations, putting an estimated 200 million people at risk [2]. Different types of *Leishmania* parasites are endemic in Iraq and cause various diseases as mentioned by Al-Bashir *et al.* [3] and Younis and AL-Thawni [4]. According to the World Health Organization (WHO), almost 13,000 cases of VL occurred in 2020 [5].

The parasite undergoes a digenic life cycle, a flagellated motile promastigote stage and a non-motile phagocytic macrophage stage (amastigote) in the midgut of vector [6]. Various diseases caused by genus *Leishmania* in human and animals including cutaneous (CL) mucocutaneous (MCL) and visceral leishmaniasis (VL) with different symptoms from self-healing cutaneous leishmaniasis to visceral fatal disease. In children, a systemic visceral infection typically starts abruptly with cough, diarrhea, fever, and vomiting. In adults, the medical condition typically lasts two weeks to two months and is accompanied by symptoms including fatigue, weakness, and lack of appetite that worsen as the disease progresses [6].

The diagnosis of human VL is difficult which was established by serological test as indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) to bone marrow examination and liver biopsy [7]. Visualization of the amastigote in splenic or bone marrow aspirate is the gold standard of diagnosis; however, this is a technically difficult procedure that is often unavailable in regions of the world where visceral leishmaniasis is endemic; in these regions, and serological testing is much more common [8]. Interleukin 1(IL-1) is a pleiotropic, nonspecific hormone-like cytokine that is of considerable concern to immunologists. Since IL-1 is a potent pro-inflammatory cytokine that is produced by inflammasomal action and secreted by a range of cell types, including the innate immune system cells, such monocytes and macrophages, research on inflammasome activation in leishmaniasis has recently focused more on it [9-10]. This study was done to measure the concentration of IL 1 in the serum of infected mice with promastigote of *L. donovani* and demonstrated the histopathological changes in liver and spleen of infected mice with infected stage and after treatment by pentostome.

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2. Material and methods

Leishmania donovani (LD) promastigote was obtained from the Department of Biology, College of Science, University of Babylon and was cultured in 21C° Nicolle-Novy McNeal (NNN) medium and incubated at 27C° for maintenance.

The NNN media consists of two stages, blood agar base (solid stage) and lock solution (liquid stage), which was prepared for cultivation and maintenance of parasite growth.

2.1. NNN media

2.1.1. Solid phase

According to the method prepared by Kagan and Norman [11], the medium from the following ingredients was prepared with modified agar(20 gm), blood (200 ml), brain heart infusion (37gm), gentamicin (250 ml), dextrose (10 gm), distilled water (1000 ml), all ingredients added to distill water except blood and antibiotic, after being heated and dissolved blood was added, well mixed and the pH was adjusted to 7.4, then autoclaved at 121C° and cool to about (50-55)C°, antibiotic added, then dispensed (4ml) into sterile screwed-cup cultured tube, and incubate at 37C° for 24h to make sure the media free of contamination then stored at 4C° in refrigerator till used.

2.1.2. Liquid phase (lock solution)

Oral rehydrating solution has been used in the preparation of the medium, which contains the following components, sodium chloride (2.6gm), potassium chloride (1.5gm), glucose (13.5gm), sodium citrate (2.6gm) all ingredients added and dissolved in 1000 ml of DW, autoclaved at 121C° for 15min., cool then antibiotic added to avoid contamination and placed in sterile bottles and then refrigerated at 4C° until use.

2.2. Animal Groups

Thirty albino mice, aged 6-8 weeks and weighing 20-25 grams, were housed in a laboratory animal facility for one week to ensure they were disease-free prior to the experiment. Subsequently, the mice were randomly divided into three groups of ten animals each. These groups were treated daily for 21 days according to the following protocols[12]:

1. First group: administrated orally with 0.1ml /day normal saline and considered as negative control (non-infected group).
2. Second group: injected with *L. donovani promastigoate* intraperitoneally (I/p) 1x107 cell/ml of LD promastigote and left without treatment which was considered as positive control (infected group).
3. Third group: infected with 1 x 107 promastigotes (I.P.) and treated daily with pentostam (0.041 mg, I.M.). [12].

After 7, 14, and 21 days blood samples were collected to obtain sera for immunological study. Then all animals were killed and the liver and spleen were removed and saved in formalin 10% for histopathological study.

The immunological study was carried out by measuring the level of IL 1 using sandwich Enzyme Linked Immune Sorbent Assay (ELISA).

Histopathological study of liver and spleen was done through processed and stained tissue samples as mentioned by Luna [13].

2.3. Statistical analysis

Data from the immunological study, specifically serum IL-1 levels, were analyzed using appropriate statistical methods. Data were analyzed for each time point (7, 14, and 21 days) separately. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences in IL-1 levels between the three groups (negative control, infected, and infected treatment). When the ANOVA indicated a significant difference ($p < 0.05$), Tukey's Honestly Significant Difference (HSD) test was then performed to determine which comparisons between groups were significantly different from each other. Results are presented as means ± standard deviation (SD) and all statistical analyses were performed using SPSS version 20.0. The significance level was set at $p < 0.05$ for all analyses. Histopathological findings were evaluated qualitatively. Images of liver and spleen sections were taken and compared across the three groups, with particular emphasis on cell types, inflammatory foci, and structural integrity of the organs. The severity of changes was determined using a descriptive approach.

3. Results

The present study aimed to investigate the effect of *Leishmania* infection on IL1 serum level as immunological marker and histopathological changes in experimentally infected mice in comparison with treated and negative control animals. The immunological study shed light on detection of IL1 in sera of animals in three studied groups, the results revealed that the mean interleukin levels were 165.5 ± 22.30, 164.5± 22.20, after 7, 14 and (164.8± 22.10) after 21day with significant difference ($P \leq 0.05$).

While 226.6± 5.40, 299.5± 5.40)in infected none treated animal after 7, 14 with highly significant difference at ($P \leq 0.001$) and 352.7± 5.40 in infected none treated with significant difference ($P < 0.05$) in comparison with negative control, in addition to that it was found that the level of IL1 in infected and treated animals group with penostam (175.4 ± 42.82, 223.2 ± 17.48) in infected and treated animals after 7, 14 days with highly significant difference at ($P \leq 0.001$) and 226.1± 12.27 after 21day with significant difference ($P \leq 0.05$) compared control group values (Table 1 and Figure 1).

Table 1. Serum level of IL1 (pg/mL) in the study groups of mice after three weeks.

Groups	7 days Mean±SD	14 days Mean ±SD	21 days Mean ±SD
Control +ve	226.6± 5.40	299.5± 5.40	352.7± 5.40
Control-ve	165.5 ± 22.30**	164.5± 22.20**	164.8± 22.10*
Pentostam	175.4 ± 42.82**	223.2 ± 17.48**	226.1± 12.27*

* significant difference at ($P \leq 0.05$)
** highly significant difference at ($P \leq 0.001$)

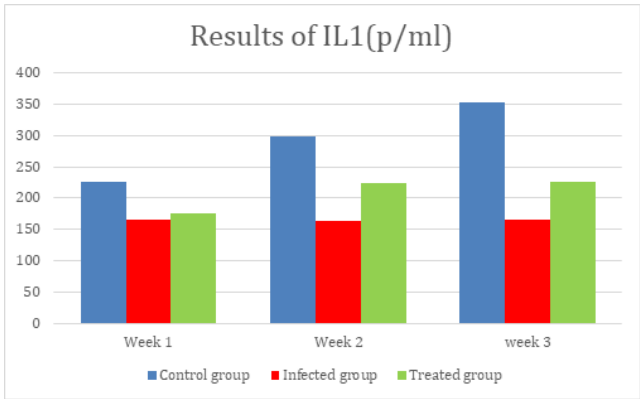

Fig. 1. IL-1 concentration level in the serum of different animal groups according to the experimental study period (7,14,21 days).

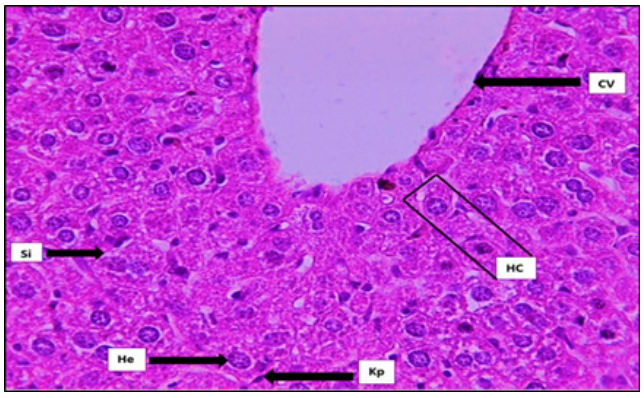

Fig. 2. A cross-section of Liver tissue showed normal histological which consists of hepatic cord. (HC), sinusoid (Si), Kupffer cell(Kp). the central vein with thread arrangement of hepatocyte (He), (Control group) (H&E) (X20).

The histopathological changes in the liver and spleen of mice infected with Leishmania promastigotes, with and without treatment, were also examined. Figure 2 depicts a normal liver histological section from the control group, showing a central vein and hepatocytes arranged in cords.

The histopathological changes of the second group showed degenerative hepatocyte cells in addition to necrosis in some cells, beside congestion and dilatation in the central veins and multiple areas of granuloma, also there was vacuolation of hepatocytes with congestion of sinusoid, some sections showed hydropic degeneration of hepatocyte which lead to and narrowing of the sinusoid in addition to disorganization of hepatic cord and infiltration of inflammatory cells as shown in Figure 3 in comparison with negative control (Figure 2). Whereas liver section of treated animals (Third group) showed look – like normal histological structure appearance with mild inflammatory cells (Figure 4).

The Splenic Section of the first animal group showed the normal histological structure of white and red pulp (Negative Control) as shown in Figure 5. The Section of splenic tissue of the second group showed disturbance of white pulp, aggregation of numerous plasma cells and widening of white pulp besides reduction of red pulp with severe congestion and inflammatory cells which gave a clear indicator for high-grade inflammation as appeared in Figure 6, while Spleen Section of treated animals with pentostam (Third group) showed look-like normal appearance of white pulp of splenic tissue structure with low inflammatory cells Figure 7.

4. Discussion

Leishmaniasis is a dangerous endemic disease in Iraq, transmitted to humans by sandflies, affecting individuals of all ages, particularly children [3,14]. Numerous clinical symptoms can arise from leishmania infections, and the intricate host-parasite relationship determines the outcome of disease.

When promastigotes of different Leishmania species transform into amastigotes within macrophages and dendritic cells, the innate immune system initiates a primary defense against the parasite. Simultaneously, the presence of the parasite within macrophages (Kupffer cells) triggers

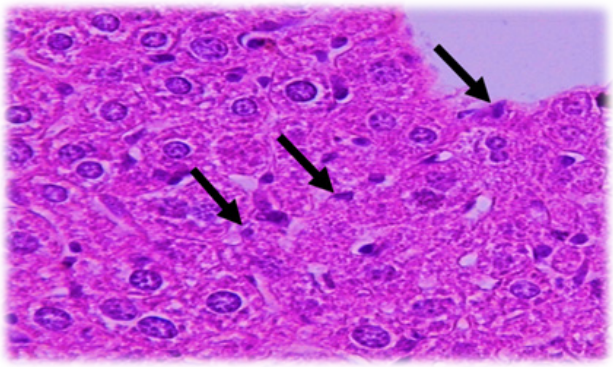

Fig. 3. Section of the liver from mice infected with *L. donovani* and treated with pentostam drug after three weeks showed like normal histological appearance with mild dilatation of sinusoid (Si) and (IN). Necrosis and inflammatory cell infiltration (H& E) (X40).

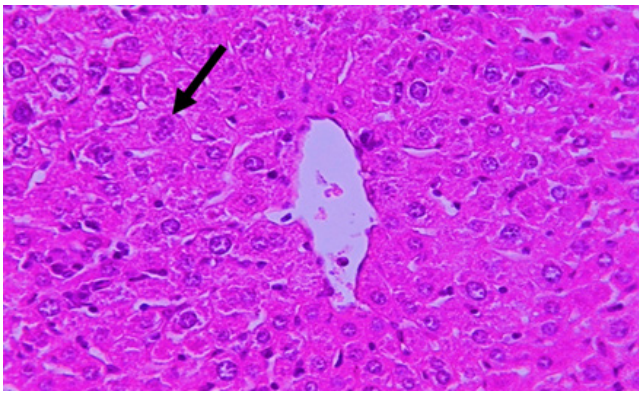

Fig. 4. Cross-liver section of mice showed mild inflammatory cells (Third group animals after three weeks treated with pentostam) (H & E) (X40).

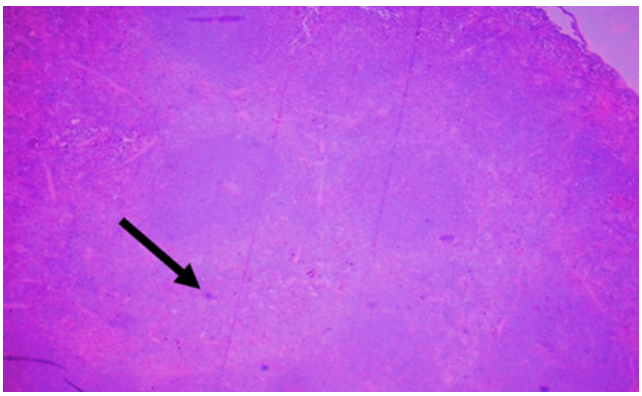

Fig. 5. Cross Spleen Section showed the normal histological structure of white and red pulp in negative Control (Forth group) (H&E) (X10).

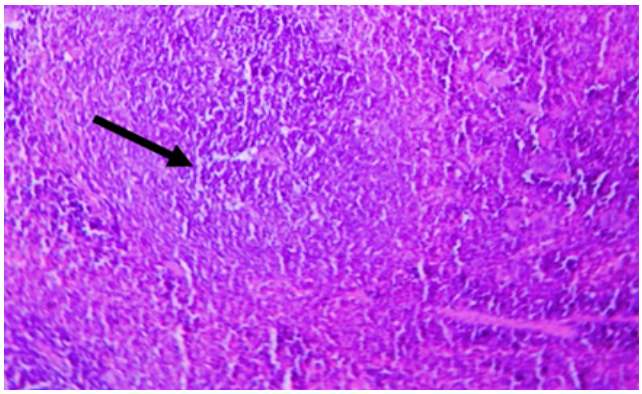

Fig. 6. Cross section of splenic tissue of infected animals (Fifth group) showed widening white pulp and reduction of red pulp with filtration of inflammatory cells (H & E) (X40).

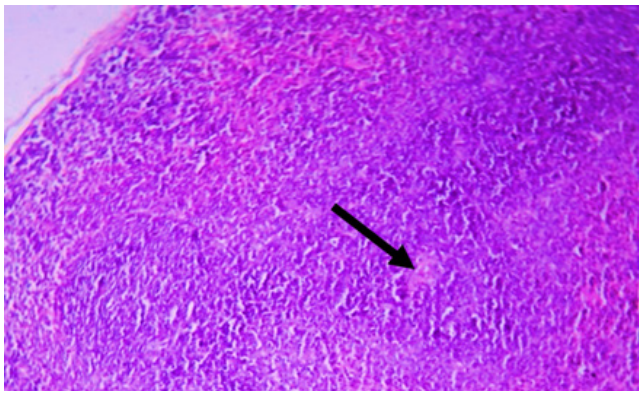

Fig. 7. Cross spleen section of treated animals with pentostam (Sixth group) showed a normal appearance of white pulp of splenic tissue structure with low inflammatory cells (H & E) (X40).

the release of chemokines, which in turn affect granulocytes and monocytes [15].

It is now widely agreed that the outcome of microbial infections is significantly influenced by a number of cytokines, including proinflammatory interleukin, which is released by immunocompetent cells such as dendritic cells and macrophages, and as known the IL 1 is an important proinflammatory cytokine, so its serum level increase with different infection [16]. Cytokines are crucial for maintaining a balanced immune system, but when their regulation goes awry, it can contribute to different diseases. Excessive production of pro-inflammatory cytokines referred to as a cytokine storm, can occur in conditions like sepsis, autoimmune diseases, and specific viral and parasitic infection [17].

It was found the level of IL1 in parasitized peripheral blood of mice infected with *L. donovani promastigoate* intraperitoneally (1x107 cell/ml) showed a decline in IL1 level in comparison with that come from control, then the level slightly elevated after treatment with pentecostal and this result coincides with the explanation of Jiaxiang et al, 2018 [18] who mentioned that the infection by protozoa which belonging to the genus *Leishmania* occurred inside the immune cell especially macrophage and that responsible for impaired production of this interleukin. Nevertheless, the function of interleukin 1 (IL-1) in the host's reaction to leishmaniasis remains unclear, and it was discovered that L. donovani did not cause IL-1α to be produced in macrophages from either mouse strain [19]. Also, Parmar *et al*., [20] discovered that the IL-1R receptor is degraded following a phosphorylation process that the pathogen initiates during the early phases of macrophage infection with *L. donovani*. Furthermore, *L. donovani*-induced mTOR activation in macrophages led to an increase in IL-10 and TGF-β levels and a decrease in IL-1α, IL-12, and TNF-α production, resulting in upregulation of the parasitic survival and multiplication [21]. These findings suggest that *L. donovani* has developed a capacity to infect mononuclear phagocytes without inducing the production of potentially host-protective cytokine IL1.

The presence of mild vacuolation in the hepatocyte cytoplasm, along with congestion of the sinusoids, suggests a resistance response against the invading parasite. While Kupffer cells exhibit endocytic and phagocytic capabilities as macrophages, they do not produce toxic oxygen intermediates, which are a key antimicrobial mechanism [22].

This study assessed the impact of visceral leishmaniasis (VL) on the liver and spleen, specifically examining the histopathological changes induced by *Leishmania* promastigotes. The results showed variable changes and an accumulation of inflammatory cells in these organs due to the infection. As promastigotes transform into amastigotes within immune cells, the immune system releases various factors to combat the parasite, leading to structural disruption of the infected organs and the recruitment of inflammatory cells. The liver sections from infected mice showed hepatocyte degeneration and necrosis, in contrast to the control group. However, these changes were reduced, and the liver tissue appeared more normal after treatment with pentostam, indicating the drug's efficacy against the parasitic infection and the host's immune response (Fig.3) which is evidence of the effect of the drug on progression of parasitic infection beside the immune response. Oxidative damage may be the cause of a decrease in lymphocytes in spleen tissue. According to other studies, lymphocytes are thought to be the most sensitive type of blood cell: Lymphopenia is the first blood alteration that occurs after whole-body exposure [23]. Carlos *et al*. [24] reported that hepatosplenomegaly is caused by hyperplasia, which is brought on by the growth of the parasite *L. donovani*, and that cytokine-mediated systemic inflammation develops, triggering acute phase reactants from the liver. Vomiting, fever, and cachexia can all be brought on by these cytokines getting to the brain. Numerous systemic infections brought on by bacteria, viruses, and parasites result in broad alterations in the spleen's structure [25].

Histopathological changes in spleen are widespread in many systemic infections as bacteria, viruses and parasites [25] so in this study, the splenic changes were investigated in infected mice which revealed disorganization of its structure and aggregation of inflammatory cells in white pulp in addition to reduction of red pulp and that come in agreement with [26-27] results which demonstrated that the experimentally infected mice with *L. donovani* promastigote show the hepatosplenomegaly sign of the pathological effect. These pathological lesions depend upon the virulence of the strain and the resistance of the individuals [28].

5. Conclusion

This study provides valuable insights into the immunopathological effects of Leishmania donovani infection and the therapeutic efficacy of pentostam in a murine model.

The findings demonstrate that L. donovani infection leads to a significant decrease in serum IL-1 levels, suggesting a suppression of the host's pro-inflammatory response. Furthermore, the infection induced notable histopathological changes, including hepatocyte degeneration, necrosis, and inflammatory cell infiltration in the liver, as well as disruption of splenic architecture with increased inflammation and reduced red pulp. Importantly, pentostam treatment was shown to be effective in mitigating these effects. The treated group exhibited a slight increase in IL-1 levels compared to infected mice, and histopathological analysis revealed a marked improvement in liver and spleen tissue structure, with reduced inflammation. These results confirm that L. donovani infection causes substantial damage to the liver and spleen, alongside suppression of IL-1 production, and that pentostam treatment is effective in reducing the pathological burden of the infection and modulating immune response. This suggests that pentostam is a viable therapeutic option for visceral leishmaniasis. Future research is warranted to explore the precise mechanisms underlying the interaction between *L. donovani* and the host's immune system.

To better understand the impact of *Leishmania donovani* on the host, both histologically and immunologically, further future studies are needed. Therefore, we can recommend the following:

1. Investigating the histological tissue effect of drugs on other organs such as kidney and bone marrow.
2. Studying the effect of pentostam on the parasite by using an electron microscope and lymph node.
3. Study the effect of pentostam on the parasite at the molecular level and gene expression.

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