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The Effect of Contraceptive Pills on the Severity of Visceral Leishmaniasis by Measuring Some Physiological Parameters

Hanan F. Ghazi [a], Sabaa T. Mohammed [a], Areej A. Zabbon [a], and Mohamed Tawfiek Shaaban [b]

[a] Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
[b] Department of Botany and Microbiology, Faculty of Science, Menoufia University, Egypt

CORRESPONDANCE
Hanan F. Ghazi
Hanan.faisal.G@uomustansiriyah.edu.iq

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ABSTRACT: ***Background:*** *Leishmania donovani can cause a serious infection if it is left without treatment. The parasite is transmitted by the bite of an infected female phlebotomine sandfly. The disease is prevalent throughout the world and in at least 88 countries.* ***Objective:*** *This study aims to know the effect of contraceptive pills (OC) on the severity of infection of Leishmania donovani in infected mice, by measuring the levels of the hormones estrogen and progesterone, cholesterol, and total protein in the sera of infected mice. It is taken by dosage in the form of tablets dissolved in water at a dose of 0.012 ml/day and is compared with the drug pentostam, which is given by injection at 0.04 ml/day for a treatment of 21 days.* ***Methods:*** *The animals were divided into five groups, only the first group was uninfected, while the remaining four groups were infected, as follows: The second group was infected only. It was not dosed with pills and was not injected with pentostam treatment., the third group was dosed orally with contraceptive pills, the fourth group was considered a treatment group (pentostam), and the fifth group was dosed orally with pills and injected with treatment for comparison. Blood was collected from mice after three weeks on the 7, 14, and 21 days in special tubes containing a gelatinous substance for the purpose of conducting the required tests.* ***Results:*** *The results showed that the OC led to a significant increase in all indicators in the third week, which is compared with the drug group pentostam group, Data expressed as mean ± SD and values of ($P>0.05$) were considered statically non-significant, while ($P<0.05$) and ($P<0.01$) were considered significantly different, highly significantly different, respectively.* ***Conclusions:*** *It can be concluded that OC pills had an important role in increasing the severity of parasite infection in laboratory animals through the noticeable increase in the indicators that were measured.*

KEYWORDS: *Contraceptive pills; Estrogen; Progesterone; Cholesterol; Total protein*

INTRODUCTION

Visceral leishmaniasis (VL), especially the species *Leishmania donovani* can cause 100% fatality if left untreated [1]. In 2015, the World Health Organization classified this disease as a neglected tropical disease [2]. Infection with this parasite is characterized by high temperatures, weight begins to decrease significantly, spleen damage, and liver hepatospleenomegaly swelling [3]. The therapeutic supports for leishmaniasis are the pentavalent antimony compounds that were first introduced in the 1930s [4]. The VL cause originated from an animal infection transmitted by a disease vector [5]. Visceral leishmaniasis is affected by hormone levels in male blood [6]. Biological sex difference affects physiology and disease outcomes, males and females are known to have a similar number of genes, but the difference is only in the genes encoded by the sex chromosomes [7]. Sex-associated hormones, estrogen, and progesterone play a role in modulating immune responses and lead to different pathological outcomes [8]. Contraceptive pills (CPs) are taken daily and contain hormones. Thus, the way hormones work in the body changes how the body works [9], [10]. CPs work on the uterus

by changing its lining, which in turn becomes against the fertilization of eggs [11]. The two known types of tablets: Oral Contraceptive pills (OC) are non-combined and combined tablets. Most OCs in each cycle are active, which means they are hormones [12].

Progesterone and estrogen are steroid hormones, steroid hormones are lipid molecules derived from common cholesterol precursors [13]. Parasites can take cholesterol precursors from the host and exploit them through the hormonal microenvironment to enhance its formation, which ultimately would control the infection and associated damage. A host-parasite relationship is established in the context of which close biochemical coevolution and communication at all organization levels between two complex organisms developed. The ability of the parasite to establish in its host is associated with several evasion mechanisms to the immune response and its capacity for exploiting host-derived molecules [14]. Progesterone can modify the immune response during normal physiological processes during parasitic infections, it works directly on the parasite [15]. Thus, this study aims to determine the serum levels of sexual hormones (estrogen and progesterone), cholesterol and total protein in infected female mice inoculated with oral contraceptives and pentostam drug.

MATERIALS AND METHODS

Parasite Strain and Culture

Leishmania donovani was obtained from the College of Science/University of Baghdad, it was cultured and maintained by serial passage in NNN media every 8 days and incubated at 27 C° [16].

Preparation of Contraceptive Pill Concentrate

The pill 5mg containing 0.5 mg of hormones was dissolved in 20 ml of distilled water, and the concentration became 0.0025 mg per ml, then 0.012 ml was withdrawn, and the mouse was inoculated orally for 21 days.

Preparing the Pentostam Injection

First, 0.01 ml of Pentostam treatment was withdrawn and the volume was completed to 1 ml, then using an insulin needle. 0.04 ml was withdrawn and injected into the mouse, the injection was done subcutaneously.

Animal Grouping

A total of ninety female albino *BALB/c* mice aged between 8-12 weeks, were obtained from the National Center for Drug Control and Research/ Baghdad/ Iraq, Seventy-two mice were subcutaneously injected with 1×10^7 parasite/mL of promastigotes *Leishmania donovani* and the eighteen uninfected mice were considered the control group. The study groups were divided into five groups:

- The first group (G1): non-infected.
- The second group (G2): infected.
- The third group (G3):(infected), the OC was inoculated orally in the form of tablets dissolved in water with 0.012 mL each day for 21 days.
- The fourth group (G4): (infected) injected and treated with 0.04 mL daily by Pentostam drug intramuscularly each day for 21 days, it was considered a treatment group.
- The fifth group (G5): (infected) the OC inoculated orally in the form of tablets dissolved in water with 0.012 mL and it was injected with 0.04 ml daily by Pentostam drug.

Blood Collection

After the following days 7, 14, and 21, the blood samples were collected from the ophthalmic vein in special tubes containing gel to separate the serum to conduct the required tests, and the sera were stored at -20 C° until use.

Measurement of Estrogen and Progesterone Levels

The levels of estrogen were measured in serum, this was done according to the manufacturing instructions by the Backman coulter company in Brea, California, United States. Progesterone was determined, according to the instructions of manufacture by Boditech MED INC/ South Korea, The same company manufactures the kit used in this device and the method to work is the open system.

Measurement of Cholesterol and Total Protein Levels

The levels of cholesterol and total protein in sera were made according to Spin 200 manufacturing instructions/ Spinreact/Spain, The same company manufactures the kits used in this device and the method to work is the open system.

Statistical Analysis

Statistical analysis was performed by SPSS (version 20). ANOVA test was used for analyses repeated measuring between tests concentration and control. Data expressed as mean ± SD and values of *(P>0.05)* were considered statically non-significant while *(P<0.05)* and *(P<0.01)* were considered significantly count different, and highly significant different, respectively, for the purpose of calculating significant differences between test means, the LSD test was used.

RESULTS AND DISCUSSION

Measurements of Estrogen and Progesterone Levels

The results showed in Table 1 and Table 2 that there were significant changes in the levels of estrogen. There was a decrease in the G2 group compared to the G1 on the 7th day. Only in G3, the level continued to rise until the twenty-first day to 54.33 pg/ml and decreased in G4 and G5 at 25.87 pg/ml, and 37.67 pg/ml, respectively. These results showed that there were significant differences between all groups in the three weeks *P<0.05*, as shown in Table 1.

The progesterone levels also increased in G2 compared to the G1 on the 7th day, only G3 and G5 groups the level continue to increase on the 21st day to 3.00 and 1.48 ng/ml, respectively, while in G2 was 1.40 ng/ml and G4 was 1.48 ng/ml, the levels decreased. There were significant differences *(P<0.05)* between all groups on the 14th day and the 21st day, as shown in Table 2.

Table 1. The serum levels of estrogen pg/ml in the study groups

EST. **N.V:18-147 pg/ml**	**7 days** **Mean ± SE**		**14 days** **Mean ± SE**		**21 days** **Mean ± SE**		**P-value**
Negative control	31.00	0.89	31.00	0.89	31.00	0.89	NS
Positive control	A 27.70	0.70	A 20.32	0.29	A 19.85	0.21	NS
Contraceptives pills	C 68.33	1.31	C 103.50	3.06	C 154.33	2.74	0.01
Pentostam drug	A 34.00	1.71	A 22.72	0.25	A 25.87	0.93	NS
Contraceptives pills + pentostam	B 50.50	1.26	B 44.00	0.93	B 37.67	0.84	NS
P-value	0.05		0.01		0.01		
LSD	5.7		4.9		7.21		

LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C and D for column) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean.

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Table 2. The serum levels of progesterone ng/ml in the study groups

PROG. N.V:0.23-2 ng/ml	**7 days Mean ± SE**		**14 days Mean ± SE**		**21 days Mean ± SE**		**P-value**
Negative control	1.15	0.04	1.15	0.04	1.15	0.04	NS
Positive control	1.20	0.01	B 1.41	0.01	A 1.40	0.01	NS
Contraceptives pills	1.63	0.04	D 1.93	0.02	C 3.00	0.23	0.01
Pentostam drug	1.08	0.01	A 1.23	0.02	A 1.32	0.02	NS
Contraceptives pills + pentostam	1.82	0.07	C 1.60	0.01	B 1.48	0.02	0.05
P-value	NS		0.05		0.05		
LSD	NS		0.19		0.06		

LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C and D for column)represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean.

Measurements of Cholesterol Level

The results demonstrated that the cholesterol levels decreased in G2 compared to the G1 group on the 7th day. The cholesterol levels in G3 continued to increase till the 21st day to 187.67 mg/dl, while decreased in G2 to 60.17 mg/dl, G4 was 73.17 mg/dl and G5 was 60.83mg/dl. There were significant differences between all groups on the 14^{th} and 21^{st} days compared with the G2, as shown in Table 3.

Table 3. The serum levels of cholesterol mg/dl in the study groups

S. Chol. N.V:>200 mg/dl	**7 days Mean± SE**		**14 days Mean±SE**		**21 days Mean±SE**		**P-value**
Negative control	71.00	5.57	71.00	5.57	71.00	5.57	NS
Positive control	95.83	1.08	A 81.33	1.56	A 60.17	1.78	0.05
Contraceptives pills	77.83	1.85	C 111.17	4.33	C 187.67	8.66	0.01
Pentostam drug	126.67	7.61	B 97.17	1.08	B 73.17	3.21	0.01
Contraceptives pills + pentostam	112.33	2.08	B 92.33	1.45	A 60.83	3.79	0.01
P-value	NS		0.05		0.05		
LSD	NS		10.4		11.3		

LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C and D for column) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean.

Measurements of Total Protein Levels

It was recorded that the total protein levels increased in G2 compared to the G1 group after the seventh day, which were 6.83 and 8.18 g/dl consecutively, In the other groups (Group 3 was 6.80 g/dl, Group 4 was 8.43 g/dl, and Group 5 was 8.78 g/dl). There was a record d an increase in the level of total protein in the G2 was 9.98 g/dl, and the G3 was 9.33 g/dl; it was noted that the total protein levels continued to increase until the twenty-first day. A decrease in the levels of total protein was observed in the G4 and G5 groups as follows 7.14 and 6.31 g/dl, respectively, and these results showed significant differences between all groups, as shown in Table 4.

Table 4. The serum levels of total protein g/dl in the study groups

TP 6-8 g/dl	7 days Mean±SE		14 days Mean±SE		21 days Mean±SE		P-value
Negative control	6.83	0.23	6.83	0.23	6.83	0.23	NS
Positive control	8.18	0.23	C 9.25	0.23	C 9.98	0.16	NS
contraceptives pills	6.80	0.18	B 8.33	0.15	C 9.33	0.32	0.01
Pentostam drug	8.43	0.27	A 7.38	0.23	B 7.14	0.22	0.05
contraceptives pills + pentostam	8.78	0.19	A 7.73	0.11	A 6.31	0.48	0.01
P-value	NS		0.05		0.05		
LSD	NS		0.33		0.37		

LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C and D for column) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean.

These results are consistent with what has been observed by the researchers Ali *et al.* [17] showed that estrogen may be involved in the stimulation of antibodies and may have enhanced susceptibility to infection. Also, Navarro *et al.* [18] showed that estrogen increases the inflammatory response, stimulates estrogen and induces inflammatory cytokine production, including interleukin (IL 6). A study by Kumar *et al.* [19] reported that *Leishmania donovani* induces host T cell suppression, as they observed a significant enrichment of forkhead box protein 3 (FoxP3)+ interleukin (IL)-10+ FoxP3+ regulatory T cells (Treg) in the bone marrow of patients with high parasite load compared with low parasite load. Hasby Saad *et al.* [20] mentioned that mice infected and receiving the contraceptive pill showed a significant increase in the parasite *Trichinella spiralis* burden due to the gene encoding the receptor tyrosine kinase protein. This study contrasts with that reported by Abbas *et al.* who demonstrated that the innate defence against leishmaniasis infection during pregnancy increases as female hamsters with *Leishmania* treated with estrogen showed a higher proportion of nitrogen oxide (NO)-producing cells at the site of infection than controls that are associated with reduced parasite burdens at least in part as a result of estrogen-mediated up-regulation of iNOS expression and NO production [21].

These results are also in agreement with what was observed by Dardona *et al.* who showed that increased progesterone in pregnant women has been indicated to bias the maternal immune response towards a Th2 type and increases the susceptibility of female C57BL/6 mice to *L. major* depending on that female sex hormones influences the Th1-Th2 balance [22].

Gatto *et al.* [23] showed that patients with active VL exhibited lower levels of total cholesterol and, therefore reduced serum cholesterol concentrations as a function of their splenic parasite burden. Also, Diotallevi *et al.* [24] indicated that in VL cases, serum concentrations of cholesterol are inversely correlated with the density of amastigotes in the spleen and cellular concentrations of cholesterol levels are low in patients with high amastigote burdens in their spleens. Cellular cholesterol is required for the assembly of membrane lipid rafts, and *L. donovani* has affected the antigen presentation of macrophages by disrupting such rafts by decreasing membrane cholesterol [25]. Likewise, Satya *et al.* [26] reported membranous rafts are important pathogen portals required for *Leishmania* entry because cholesterol, maintains the membrane fluidity required for proper cell function to antigen-presenting. Leroux *et al.* [25] reported that cholesterol in the form of a liposomal formulation can stimulate the innate immune arm and reactivate macrophage function, increase levels of reactive oxygen species and reactive nitrogen intermediates, along with proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- alpha) and IL-6. Also, Gupta *et al.* [27] reported that the membrane fluidity of antigen-presenting cells has a significant effect on T cell stimulation capacity and is dependent on the cholesterol content in the membrane, splenic macrophages as a prototype for antigen-presenting cells in affected hamsters resulted in reduced apoptotic cholesterol and an inability to drive T cells, the effect was cholesterol-specific because liposomes made up of the analogue 4-cholesten-3-one provided no protection. The infection resulted in an increase in IL-10, shifting growth factor-beta, IL-4 signalling and a concomitant decrease in interferon-gamma (IFN-γ), tumor necrosis factor-alpha, and inducible NO synthase signalling. Messaoud *et al.* [28] reported that the parasite transformation from the vector promastigote stage to the intracellular amastigote host cell stage is based on four lipid classes: phospholipids, free fatty acids, triglycerides and sterols. Naz *et al.* [29] recorded that oral contraceptive pills increase the level of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol,

total cholesterol and triglycerides because they contain steroid hormones. The cholesterol level increased in this study reached 187.67 mg/dl in the group that inoculated with oral contraceptive pills only.

The present study agreed with that reported by Ali [30] who showed an increase in total protein in patients because it attacks the visceral organs when infected with *Leishmania donovani*. Also, these results agreed with that reported by Varma and Naseem [31] that *Leishmania donovani* targets the reticuloendothelial system, causing reduced bone marrow activity, cellular destruction in the spleen, and results in anaemia, leukopenia and thrombocytopenia, the various hematological manifestations of hepatosplenomegaly, weight loss, and hypergammaglobinemia appear. Mukerrama et al [32] reported that the decrease in hepatocellular mass and the progression of fibrosis to the point that there is architectural destruction with the formation of the regenerative nodule, the induction of fibrosis occurs with activation of the stellate cell, resulting in increased formation of collagen and other components of intracellular matrix leading to increases the total protein level. In addition, Sahni [33] reported that infection with *Leishmania donovani* is characterized by hypergammaglobulinemia, which increases the total protein level. Likewise, Paltrinieri *et al.* [34] showed that the total proteins and total globulin are frequently increased, especially in the acute phase of the disease, the increase of total protein can correlate with the severity of the clinical score.

From these studies, along with the current study, it was possible to discover the effect of taking oral contraceptive pills with Leishmaniasis infection by determining the levels of sex hormones (estrogen and progesterone) and showing the level of cholesterol and total protein in the blood of infected mice.

CONCLUSION

The current study concluded that contraceptive pills lead to increased severity of visceral leishmaniasis infection and also lead to an increase in the levels of the hormones estrogen and progesterone, cholesterol, and total protein *in vivo*. It had a clear role in increasing the severity of parasite infection in laboratory animals by observing an increase in some of these physiological indicators.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Hanan F. Ghazi: Mice inoculation, data curation, and writing-original draft preparation. Sabaa T. Mohammed: Supervised mice injection, conception, methodology, and design experiments. Areej A. Zabbon: Supervised mice dissection and preservation of samples, organizing and analyzing data. Mohamed Tawfiek Shaaban: Writing the review and editing the manuscript.

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None.

DATA AVAILABILITY STATEMENT

None.

ETHICAL APPROVAL

his study was approved by the ethics community of Biology Department/ Mustansiriyah University under reference number: BCSMU/1123/0040Z on 20/11/2023.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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