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Immunological assay and histopathological study of *Toxoplasma gondii* in Experimental male rats

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Abstract

Toxoplasma gondii is an intracellular entity that can infect nearly all cells of vertebrates with a mild blood temperature. The main objective of the study is to investigate the host immune aspects in *Toxoplasma gondii*-infected experimental animals by monitoring some cytokines production profiles and analyzing immune cell phenotypes. Therefore, the toxoplasma cysts were extracted and injected into 40 male rats compared to 10 rats as a control group. Then, leukocytes from peripheral blood were stained with anti-surface antigens antibodies and analyzed using flow cytometry. Pro and anti-inflammatory cytokines levels were evaluated 12 and 30 days after infection using a quantitative ELISA assay. Rats infected with *Toxoplasma* in placental suspension had significantly higher levels of IL-12, IFN-γ, IL-10, and IL-6 than control rats. Infected rats also had higher levels of $CD14^+$ and $CD19^+$ leukocytes. A difference between the stages tachyzoite and bradyzoite was also observed. Histopathology of *T. gondii*-infected rat tissues confirmed bradyzoite stage in liver, testis, brain, and kidney, further studies are needed to unravel the mechanisms behind stage switching. In conclusion: There are significant differences in the concentrations of cytokines associated with some cellular and humoral immune cells of rats infected experimentally with toxoplasmosis.

Keywords: *Toxoplasma gondii*, flow cytometry analysis, immune response, In vivo model, cytokine production; bradyzoite, tachyzoite, cyst, differentiation

Introduction

Toxoplasma gondii is an intracellular parasite from the apicomplexan phylum that infects 30-50% of the world's human population (1). Infection with this parasite is frequently asymptomatic (2). Contracting this infection during pregnancy can result in miscarriage, fetal death, and congenital malformation. This parasite can be transmitted by drinking water and eating infected raw or half-cooked meats of the intermediate hosts, or by eating vegetables contaminated with cat oocytes (3), cycle of parasite reproduction lead to the formation of a huge number of tissue cysts (bradyzoite), in all organs and viscera, which stimulate the immune system (4).Evaluation of cytokine production, especially interleukin 10 (IL-10), IL-12 and interferon-gamma (IFN-γ) is an important tool in investigating

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immune responses against stimuli such as intracellular parasites (5). Most investigations to study the immunity to toxoplasmosis depend on interleukins (6). Another method for investigating the immunological processes is to check leukocyte surface phenotypes by flow cytometry. Immunophenotyping monoclonal antibodies directed against surface CD (cluster of differentiation) molecules are used, these molecules are present on the surface of a wide variety of cell types and can serve as indicators of the functional capacities of leukocytes and several other cells (7). Our study is the first in Iraq and the Middle East on experimental infections of *Toxoplasma gondii* in laboratory animals and persistent abortions in Iraqi pregnant women, While, most studies in Iraq and Middle East used ready-made strains in experimental infection, we used local strains of *Toxoplasma gondii* to explore the immunological response in rats (8). The aim of the study is to explore the host immune response in experimental rats that have been infected with *Toxoplasma gondii*. This will be accomplished by tracking the production profile of various cytokines and doing phenotypic analyses of immune cells.

Materials and Methods

Parasite isolation

T. gondii was isolated from the placenta of 41 aborted women hospitalized in Al-Diwaniyah maternity and teaching hospital, Iraq. (8)

The presence of the parasite was confirmed by the impression method. The smear was prepared by cutting part of the placenta and impress on a glass slide, which was then dried in the air and fixed using ethyl alcohol at a concentration of 90% for ten minutes. Finally, slides were stained with Giemsa and examined under the microscope. (9). The placenta tissues were cut down into smaller pieces, combined with an equal volume of normal saline and ground in a mortar. The mixture solution was filtered through gauze and centrifuged at 3000 rpm for 10 minutes three times. 1000 I.U. of penicillin and 100 mg of streptomycin were added to the 1 ml of the suspension to prevent contamination. The preparation was then examined under a light microscope with (100X) immersion oil. The total number of tissue cysts was evaluated by Haemocytometer (10). 100 tissue cysts were used for inoculation. The inoculum was directly injected into the rats or kept in the refrigerator at a temperature of 4^{o}C for 24 hours.

Animals and experimental infection:

Animals: The study was conducted on 50-60 days aged Wister albino male rats obtained from the laboratory field of the College of Veterinary Medicine, University of Karbala. Their body weight ranged from 250 to 300 g. The rats were caged in clean plastic cages, under standard laboratory conditions. They were provided with adequate ventilation and 12 hours lighting, water bottles, and commercial food ad libitum. Parasite infection of rats: The fifty rats were divided into two groups: an experimental group (40 rats intraperitoneally (IP) inoculated with 0.3 ml of filtrated suspension) and a control group (10 rats: inoculated by 0.3 ml of distilled water). After 7 days of inoculation, the infection of the experimental rat was confirmed using the Latex Agglutination test according to Manufacture Company (Taytec®/ Germany). 1-2 ml of blood was drawn from the heart directly; serum samples were then prepared by a centrifugation at 3000 rpm for 5 minutes. Samples were considered positive when visible agglutination occurred.

Cytokine level analysis

The levels of rats IL-10, IL-6 (Bioassay Technology Laboratory/China) IFN-γ, and IL-12 cytokines (Elabscience®/China) were measured at 12- and 30-days post-parasite inoculation using quantitative ELISA kits following the manufacturer's guidelines.

Flow cytometry analysis

Blood samples were collected via cardiac puncture into EDTA tubes 12- and 30-days post inoculation. All antibodies used in Immunophenotyping were purchased from Bio-Rad Company and used according to the manufacturer's instructions (Biorad®, CA, USA). Leukocytes were stained using anti-

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mouse CD19 RPE, anti- mouse CD4 RPE, anti- mouse CD8 RPE, anti- mouse CD3 RPE and anti -mouse CD14 RPE monoclonal antibodies to detect cell surface antigens (all antibodies were purchased from BIO-RAD, California, USA). After 30 min incubation, erythrocytes were removed using ammonium chloride lysing solution. Then, data were analyzed by Epics XL flow cytometer (Beckman Coulter®, FL, USA).

Histopathology analysis

Rats were sacrificed after day 30. Organs from all animals (brain, testis, liver and Kidney) were collected and dissected. All tissues were processed and embedded in paraffin as per routine histopathology protocol, then, they were sliced into 5 µm thickness sections and stained with haematoxylin and eosin. The histopathological evaluation was performed using compound microscopy (Biobase®/China), 10 X, 20X and 40X magnifications.

Ethical approval

The protocols were confirmed by the local ethical committee for animal experimentation in Karbala University, Iraq. Written consent was acquired from each patient after explained nature & purpose of study and the rats were maintained and infected in accordance with institutional and national guidelines

Statistical analysis

Results were statistically analyzed using SPSS20.0 software (IBM SPSSR Inc., IL, USA). Statistical significance was assessed using Student's tests when comparing two conditions/groups. P value of ≤0.05 was set as the level of significance.

Results

1-Macroscopic and microscopic examination of toxoplasmosis in placenta:

We have confirmed the presence of the parasite from the aborted infected placenta. (Figure 1A). The microscopic examination by impression smear after Giemsa staining confirmed the bradyzoite phase of the parasite (Figure 1B).

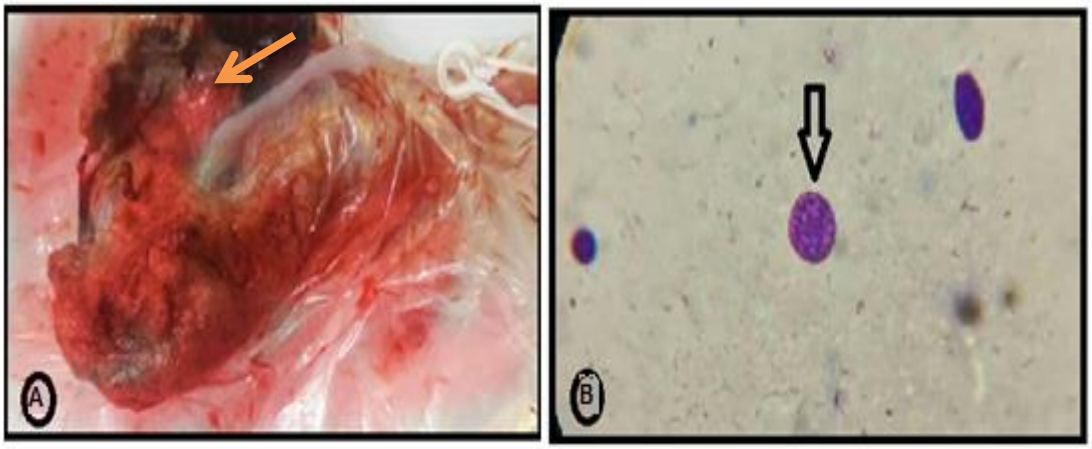

Figure 1: Phenotypical section of placental of aborted women A: fixed umbilical cord (arrow) in the placental tissues of *Toxoplasma* aborted women B: bradyzoite phase (arrow) in slide by 40X magnification field

2-Parasite detection in experimental rats' post-inoculation

Based on the latex agglutination test (figure 2), the infection rate of Toxoplasma parasite in rats that were experimentally injected with placenta suspension was recorded 100%

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Figure (2): Latex agglutination test results: Toxo-slide test *Toxoplasma gondii* positive agglutination test : 1, 2, 4 and 5 negative agglutination test : 3 and 6 (two control rats).

3-Histopathological examination of internal organs in male rats infected with Toxoplasma gondii

In our study, we have found bradyzoites in different tissues: brain; liver; testis and kidney.

- Brain section:

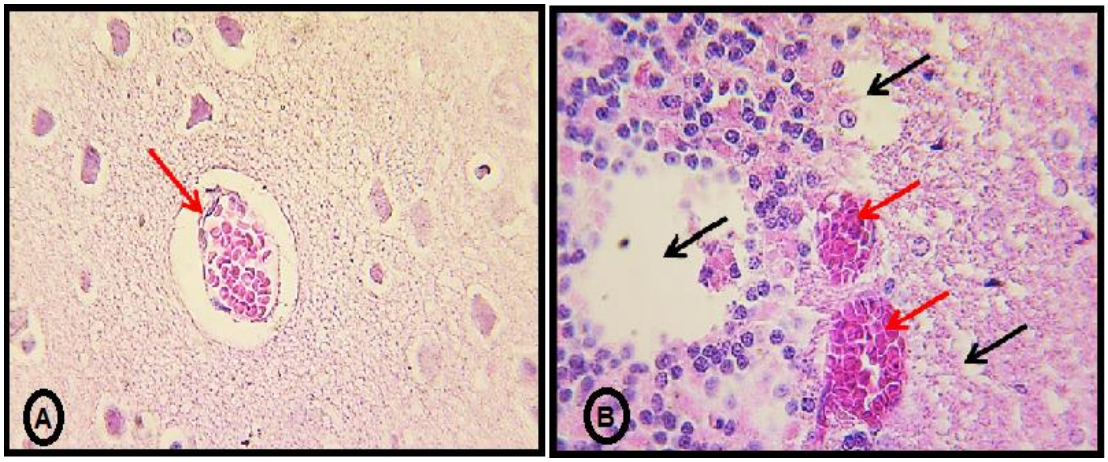

Figure (3): Histopathological examination of brain section: The tissue is stained with H&E stain, and the section is captured with a digital camera and 20X magnification optical microscope.

A: A clear infiltration of bradyzoites (Red arrows).

B: A clear damage and absence of neurons (Black arrows) and Bradyzoites can be seen infiltrated in the section (Red arrows).

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- Testis section:

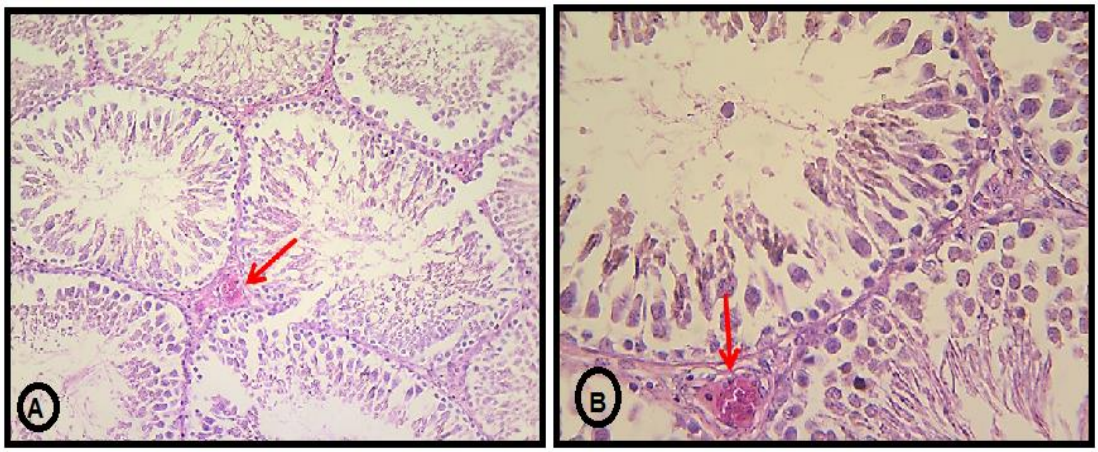

Figure (4) A and B: histopathological examination of testis: clear infiltration of bradyzoites (Red arrows). The tissue is stained by H&E stain and section is captured using digital camera and light microscope at 10X magnifier scale.

- Liver section

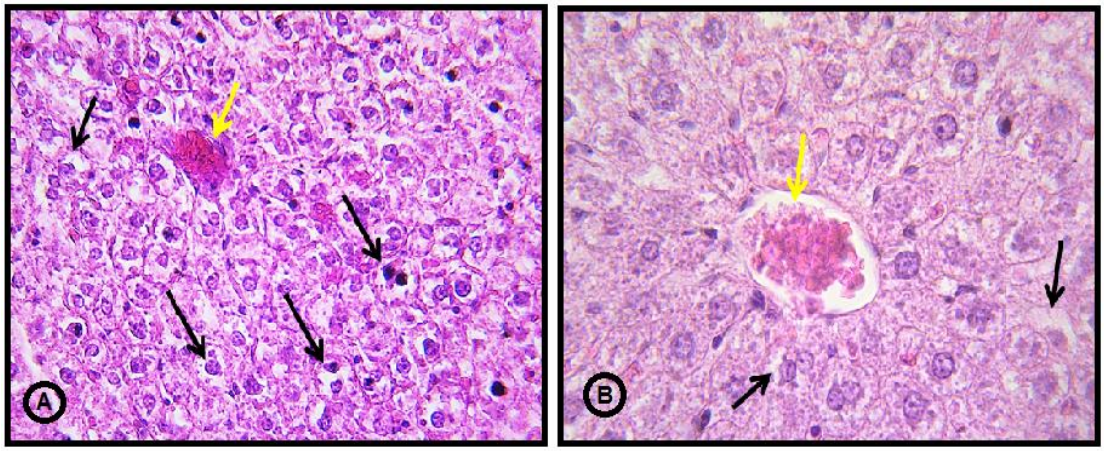

Figure (5): histopathological examination of the rats Liver section A and B: represented histopathological section in the rat's liver which shows clear infiltration of Bradyzoites (yellow arrow). Fatty degeneration can be seen in the hepatocytes as clear space giving theme ring shape appearance (Black arrows). The tissue is stained by H&E stain and section is captured using digital camera and light microscope at 20X (A) and 40 (B) magnifier scale.

- Kidney section

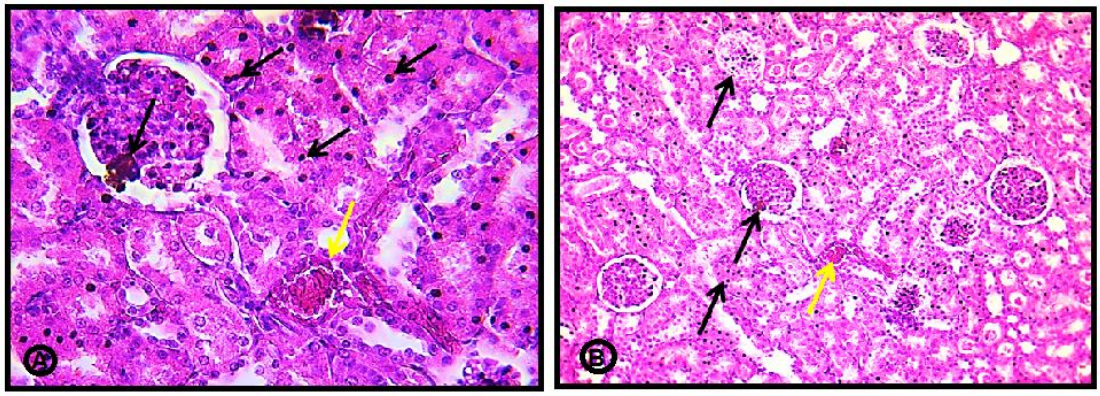

Figure 6: Histopathological examination of Kidney section A and B: The histopathological section in the Kidney shows clear infiltration of Bradyzoites that can be seen in the section (yellow arrow). The nucleus of the epithelial cells of proximal renal tubules and glomeruli s

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how hyperchromatic changes which appear dark staining (Black arrows). The tissue is stained by H&E stain and section is captured using digital camera and light microscope at 10X(A) 4X (B) magnifier scale and .

Cytokines level analysis

We quantified the cytokines in serum at two different times (Table 1). At 12 days post-inoculation, an experimental group of rats showed notably increased levels of IL-12, IL-6 and IFN-γ in contrast to the control groups (p=0.044, p< 0.037 & p< 0.038). On the other hand, for IL-10, there was no statistically significant difference between the studied groups. At day 30 post-inoculation, highly significant level of IL-12 and IL-10 were revealed in the experimental groups of rats when contrast to the control group (p< 0.037 & p< 0.041 respectively).

Table (1): Mean and standard deviation of different cytokine profiles among infected and control rats groups.

Cytokine	Infection stage	Concentration (Mean ±SD) (Pg/ml)		P value
		Infected group n= 40	Control group n=10	
IL-12	12 days Tachyzoite Stage	349.5 ± 23.6	158.4± 11.45	P= 0.044
	30 days Bradyzoite Stage	267.8± 18.56	142.5± 8.35	P= 0.037
IFN-γ	12 days Tachyzoite Stage	422.27 ±29.3	336.5 ±32.7	P= 0.037
	30 days Bradyzoite Stage	367.9± 31.1	361.6±37.2	P=0.167
IL-6	12 days Tachyzoite Stage	543.8 ±23.6	391.18±21.7	P=0.038
	30 days Bradyzoite Stage	362.7± 26.6	344.6±21.8	P=0.232
IL-10	12 days Tachyzoite Stage	425.7 ±13.6	492± 10.25	P=0.695
	30 days Bradyzoite Stage	301.2± 16.4	425.7±27.5	P= 0.041

Cytometric analysis of CD19, CD4, CD8, CD14 and CD3

A cytometric analysis of CD19, CD4, CD8, CD14 and CD3 surface antigens was performed in peripheral blood for both control and infected groups. All results were statistically analyzed. We have quantified the cytometric lymphocyte analysis in blood two different times in both 12-day and 30-day post-inoculation. Experimental group of rats showed notable increased levels of CD19, CD4, CD8, CD14 and CD3 compared to the control group-(P= 0.035, P= 0.026, P=0.027, P=0.042 and P= 0.025 respectively) , (P= 0.046, P= 0.044, P=0.039, P= 0.033 and P=0.048 respectively) (Table 2).

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Table (2): Statistical analysis regarding changes in percentage of łymphocyte surface antigens of whole blood lymphocytes of rats, infected with *T. gondii*, in the period from 12 to 30 days after infection, and in non-infected rats (control):

Lymphocytes Surface antigen	Infection stage	Concentration (Mean ±SD)		P value
		Infected group n= 40	Control group n=10	
CD19	12 days Tachyzoite Stage	15.7 ± 5.21	4.8± 1.98	P= 0.035
	30 days Bradyzoite Stage	16.8± 6.39	6.1± 2.24	P= 0.046
CD4	12 days Tachyzoite Stage	19.6 ±7.45	15.8±5.46	P= 0.026
	30 days Bradyzoite Stage	22.1± 5.72	10.34±6.61	P= 0.044
CD8	12 days Tachyzoite Stage	15.7±2.1	11.72 ±4.3	P=0.027
	30 days Bradyzoite Stage	16.9± 2.9	13.8±5.2	P=0.039
CD14	12 days Tachyzoite Stage	2.8±0.64	0.95± 0.35	P=0.042
	30 days Bradyzoite Stage	3.5 ± 1.01	0.78±0.21	P= 0.033
CD3	12 days Tachyzoite Stage	1.4±0.21	0.83± 0.26	P=0.025
	30 days Bradyzoite Stage	1.1± 0.4	0.77±0.1	P= 0.048

Study of the stage conversion:

We have analyzed the difference between the two stages tachyzoite and bradyzoite for the different parameters studied, a significant difference in IL12; IL-6 and IL10 was found.

Table (3): Cytokine profile between 2 Stages (tachyzoite and bradyzoite)

Interleukin	Tachyzoite stage	Bradyzoite stage	P value
IL-12	349.5 ± 23.6	267.8± 18.56	P=0.036
IFN-γ	422.27 ±29.3	367.9± 31.1	P=0.057
IL-6	543.8 ±23.6	362.7± 26.6	P=0.022
IL-10	425.7 ±13.6	301.2± 16.4	P=0.048

Table(4): Comparison of lymphocyte antigen surface expression between the 2 Stages (tachyzoite and bradyzoite)

Lymphocytes Surface antigen	Tachyzoite stage	Bradyzoite stage	P value
CD19	15.7 ± 5.21	16.8± 6.39	P=0.074
CD4	19.6 ±7.45	22.1± 5.72	P=0.055
CD8	15.7±2.1	16.9± 2.9	P=0.157

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CD14	2.8±0.64	3.5 ± 1.01	P=0.044
CD3	1.4±0.21	1.1± 0.4	P=0.063

Discussion

Toxoplasma gondii is an apicomplexa parasite able to invade any nucleated cells in warm-blood animal. It is estimated that approximately one-third of the world's population is infected with toxoplasma because it is omnipresent throughout the globe. *T. gondii* tissue nodules can form in various organs, including the liver and kidneys (11). In our study, we have found bradyzoites in different tissues: brain; liver; testis and kidney.Despite this ability to infect any nucleated cell of intermediate hosts, tissue cysts during chronic toxoplasmosis are not randomly distributed. Bradyzoite differentiation and tissue cyst formation are predominantly triggered in neural and muscular cells thus explaining the predilection for these tissues (12). The manifestations of *T. gondii* infections depends on genetics of the human host, genetics of the parasite, immune status of the host and probably inoculum size and parasite stage acquired. These factors influencing pathogenesis are only partially characterized (13).In this study, serum samples were collected from a total of 50 male Wistar albino rats, with 40 rats being infected with Toxoplasma through placental suspension. After 12 days of placental suspension, the infected groups exhibited a significantly higher average concentration of IL-12 in their serum compared to the control groups. (14) demonstrated the importance of IL-12 in regulating resistance to Toxoplasma gondii in rats with severe combined immunodeficiency (SCID). Their research revealed that IL-12 plays a protective role in SCID mice by activating natural killer (NK) cells to produce interferon-gamma (IFN-gamma).Indeed, IL-12 is a heterodimeric cytokine known for support cell-mediated immunity and type 1 T helper cell (Th1) responses (15). Numerous studies utilizing anti-IL-12 antibody neutralizing and IL-12 deficient rats proposed that endogenous IL-12 play a crucial role in the host defense against multiple intracellular pathogens (16). The Th1 cytokine response is responsible for regulating infection. There is evidence that dendritic cells, neutrophils, and macrophages respond directly to parasite antigens by releasing IL-12 and TNF-alpha, indicating that the innate immune response is implicated in the early generation of pro-inflammatory cytokines during infection in naive animals (17). Since resistance to Toxoplasma infection relies heavily on Th1-type cell-mediated immune responses (18), IFN-γ, released by CD4+ and CD8+ T lymphocytes, plays a critical role in immunity against *T. gondii* (19). In this study, the concentration of interferon-gamma (IFN-γ) was highly significant in the infected groups in contrast to the control groups after 12 days post infection. Actually, early in infection, natural killer (NK) cells are the primary producers of IFN-γ, contributing to innate immunity. IFN-γ activates both hematopoietic and non-hematopoietic effector cells to exert anti-Toxoplasma activity (20). However, in our 30-day experimental study, we observed a clear decrease in IFN-γ concentration, which was not significantly different from the control concentration. This decrease is attributed to the parasite's ability to evade the immune system. IFN-γ response, mediate by the STA T1 transcription factors, was important for host defending against intracellular pathogens. Recent findings indicate that Toxoplasma infection inhibit the assertion of type II interferon with induced secondary response genes, including Class II transactivator (CIITA), a nuclear protein that regulates the transcription of class II major histocompatibility complex genes and is known as the "master control factor" for their expression (21).The study examined also the concentration of interleukin 6 (IL-6) and interleukin 10 (IL-10) in two groups. After 12 days, IL-6 levels were found to be significantly higher in the infected group

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compared to the control group. IL-6 plays a critical role in the development of protective immunity against *Toxoplasma gondii*, as indicated by studies showing that mice deficient in IL-6 experience high cyst burdens and severe encephalitis due to a failure to control parasite replication (22). On the other hand, IL-10 levels did not show significant differences between the infected and control groups after 12 days. IL-10 is important for the survival of the host during *T. gondii* infection, as excessive expression of IFN-γ can be detrimental. The parasite employs antigen shedding, releasing excretory/secretory products (ESP) into the host's body, which triggers the immune system (23). Consequently, some findings have indicated a decrease in IL-10 expression in mice inoculated with T. gondii (24).Flow cytometric analysis of immune cell surface molecules revealed significant findings for various cell types. The percentage of CD19 lymphocytes showed a significant increase in both Tachyzoite and Bradyzoite stages, indicating the stimulation of the humoral immune response connected to CD19 B lymphocytes. This increase was observed at the twelfth day following infection and continued to rise gradually until the thirtieth day, coinciding with the presence of chronic T. gondii IgG antibodies (25). The analysis of CD3 lymphocytes display a significantly increased in the percentage of T lymphocytes in rats infected with T. gondii from 12 to 30 days post-infection compared to the control group. This finding is consistent with previous research demonstrating elevated CD3 levels in patients with T. gondii infection (26). CD3, which is part of the T-cell receptor (TCR) complex, plays a crucial role in immune signal response. It consists of four distinct chains (CD3-γ, CD3-ε, CD3-δ, and the zeta ζ chain) that associate with the TCR to form the TCR complex (27).Moreover, the analysis of lymphocytes containing CD4 and CD8 in rats infected with *T. gondii* revealed significant differences compared to the control group at both the twelfth and thirtieth day. CD4 lymphocytes, which are associated with the specific cellular immune response (Th1), likely, play a significant role during *T. gondii* invasion. This response may result from the release of *T. gondii* peptide AS15 antigens, indicating a potential form of immune evasion. Additionally, CD4 T cells produce interleukin-10 (IL-10) and can contribute to immune-mediated pathology (28,29). On the other hand, CD8 lymphocytes, known for their role in immune protection against intracellular pathogens like T. gondii, exhibited a gradual and significant increase throughout the study period. CD8 T cells produce cytokines such as IFNγ and TNFα, exert cytotoxic effects, and play a crucial role in the control of acute and chronic T. gondii infection (30). On the other hand, CD14 leukocytes exhibited higher mean values in the infected group compared to the control group throughout the study period, indicating their activation during T. gondii infection. Monocytes/macrophages, as phagocytic cells, play a role in the effector phase of the immune response (31). Cytokine production and cell death and in the infected tissue are typical host responses to Toxoplasma infection; nevertheless, some parasites escape the site of primary infection and travel to other parts of the body. So, tachyzoites are not completely eliminated by the immune system, they differentiate into dormant bradyzoite stage contained in tissue cysts. The factors that control *T. gondii* stage conversion in an intermediate host are diverse and complex. In our study, we found a difference between the two stages in the production of IL12, IL6, and IL10. The pathogenesis, transmission, and establishment of chronic infection are critically impacted by this stage conversion (from tachyzoite to bradyzoite), Moreover, disorders brought on by the reversal of this process (from tachyzoites to bradyzoites) are not uncommon. Therefore, it is essential that researchers continue to elucidate the molecular mediators and routes that regulate stage conversion, both by the parasite and its host cell environment (30)

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Conclusion

Acute toxoplasmosis can be effectively treated with medicines that target enzymes in T. gondii's metabolic pathways. However, there is currently no clinical treatment available to completely eliminate the infection in its chronic state (32). As a result, manipulating the process through which bradyzoites differentiate can be a useful therapeutic target and shedding light on these processes is crucial.

Conflict of Interest: The author declares that there is no conflict of interest

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