

ORIGINAL ARTICLE

Histological Effects, Immunological and Physiological Parameters in Women Infected with *Toxoplasma gondii* in Kirkuk Province

¹Lima T.Y. Lazar, ²Mohannad H. Jasim

¹Department of Basic Nursing Sciences, College of Nursing, Kirkuk University, Iraq

²Biology Department, College of Education, University of Fallujah, Iraq

ABSTRACT

Key words:

Abortions, Toxoplasmosis, cytokine IFN- γ , vitamin C activity

*Corresponding Author:

Mohannad Hamid Jasim
Biology Department, College of Education, University of Fallujah, Iraq.
muhaned.h.jassem@uofallujah.edu.iq

Background: *Toxoplasma gondii* is a widespread parasitic disease, upon infection, it forms a vacuole inside cells, evading immune system. When *Toxoplasma gondii* infects the body. T-helper cells, especially TH1, play a crucial role in immune response through interferon-gamma secretion, vitamin C helps reduce oxidative stress in infected individuals. **The objective** was therefore to determine the extent to which the infected tissue of the placenta is affected by the *T. gondii* parasite and what is the vital role of interferon gamma and vitamin C activities during infection. **Methodology:** One hundred women aged between 20-40 years were involved in the current study. These comprised changes in placenta and interferon gamma and vitamin C activities in women who had natural births, abortions with *Toxoplasma gondii*, and pregnant women without infection. **Results:** In the present study, the results showed that out of the total examined samples, 50 were infected and 50 were uninfected. Histopathological investigations showed distinct alterations in the placental tissue of infected cases compared to uninfected controls. The affected placentas showed severe histological changes such as tissue necrosis, degeneration, vacuolation, fibrosis, and widespread internal hemorrhage, which were absent in the uninfected tissues. Furthermore, a significant elevation in the cytokine interferon-gamma (IFN- γ) concentration was observed in the serum of women who experienced abortion (359.438 pg/ml) compared with both pregnant women without infection (24.447 pg/ml) and women who had normal deliveries (78.561 pg/ml). **Conclusions:** Placental tissues infected with *Toxoplasma gondii* exhibited distinct phenotypic abnormalities compared to those from uninfected women. Moreover, the concentration of interferon-gamma (IFN- γ) was significantly higher in infected women than in uninfected controls. Although a decrease in vit. C activity was observed in infected women; this reduction was not statistically significant at $P \leq 0.05$.

INTRODUCTION

Toxoplasma gondii was first described by Nicolle and Manceaux (1908) because of its crescent-shaped form. *T. gondii* is one of the most widely spread parasitic diseases globally, and infection rates vary in different countries depending on many factors, such as health, economic, social, and other factors (age and gender), and other environmental factors. Infection of the mother in the first three months of pregnancy leads to miscarriage or the occurrence of congenital malformations in the fetus ¹. However, if the mother's infection occurs in the middle months of pregnancy, it leads to congenital malformations and the appearance of symptoms of jaundice and enlargement of the liver and spleen (hepatosplenomegaly), while infection during the last three months of pregnancy is not accompanied by early clinical symptoms of the fetus, but develops as the child ages, leading to inflammation, chorioretinitis, and then infection with ocular toxoplasmosis or neurological disorders ^{2,3}.

When the parasite enters the human body, it will form what is known as a parasitic vacuole inside the cells. Therefore, it will be camouflaged from the body's immunity, allowing it to multiply, infect the body, and be transmitted to the fetus, leading to abortion or causing deformities, either congenital or post-natal. Upon infection, T-helper cells play an essential role in the immune response against *T. gondii*. It has become clear that (TH1) cells are responsible for the immune response in killing the parasite when it is inside the infected cell (intracellular killing) through their secretion of (IFN-gamma), which leads to the activation of phagocytic cells for the purpose of killing or inhibiting the parasite inside them. It has been observed that low secretion of IFN- γ is one of the causes of chronic toxoplasmosis, while T-Helper 2 cells are responsible for killing the parasite when it is outside the cell. Additionally, when the cells are infected, there will be a clear rupture of the tissue of the infected placenta or intestinal cells ^{4,5}.

Furthermore, ascorbic acid, also known as vitamin C, is regarded as a water-soluble chemical and is one of the most significant and potent non-enzymatic antioxidants in humans, through which the body can reduce the risk of free radicals being released during oxidative stress. This vitamin is involved in numerous non-enzymatic processes. As an electron donor or reducing agent, Vitamin C activity in *T. gondii* infected patients decreases due to the formation of free radicals and the occurrence of oxidative stress, and its presence helps those infected with the *T. gondii* to prevent oxidative damage ⁶. Its low level in plasma, amniotic fluid, and white blood cells is associated with miscarriage and premature rupture of the membrane. Research has shown that in addition to impairing placental implantation, low vitamin C levels cause oxidative stress to the developing foetus, potentially increasing the chance of placental abruption. As a result, vitamin C deficiency may also contribute to placental abruption. Preeclampsia and miscarriage ⁷.

The aim of this research, therefore, was to check the changes in the structure of placental tissue and to assess the immunological and biochemical responses in regard to *Toxoplasma gondii* infection among pregnant women. More precisely, the study focused on determining the concentrations of IFN- γ and the activity of vitamin C in infected and uninfected women, with the purpose of elucidating their participation in the pathogenesis of placental damage and pregnancy outcomes.

METHODOLOGY

This study was done in the Obstetrics and Gynecology Department of Kirkuk Teaching Hospital, March- July 2023. The sample had 100 women aged 20-40, among others, who had given birth regularly, those who had aborted due to being infected with *T. gondii*, and pregnant women who were not infected. The population of the study was split into three categories:

1. Women having *Toxoplasma gondii*-induced abortion (n = 50)
2. A pregnant woman who was free of *T. gondii* infection (n = 25).
3. Women who gave birth to normal non-infected births (n = 25).

In this study, demographic and clinical data such as name, age, occupation, blood group, and place of residence of each participant were collected. This information is recorded through the use of a structured questionnaire, ensuring the data is accurate and consistent.

Gathering and Processing Blood Samples

All the participants were collected with 5 mL of their venous blood under aseptic conditions using sterile

single-use syringes. The blood samples were then collected and put in empty vacutainer tubes and allowed to coagulate at room temperature. Then the samples were centrifuged at a rate of 5000 revolutions per minute (rpm) for five minutes to remove the serum. The resultant serum was thoroughly subdivided into sterile microtubes and kept at -20 °C awaiting subsequent serological and immunological analysis.

TORCH Screening

TORCH infections- *Toxoplasma gondii*, Rubella virus, cytomegalovirus and herpes simplex virus- were tested in serum samples following the procedure as outlined by ⁸. The tests were conducted as per the instructions of the manufacturer and also in line with the normal laboratory practice. To conclude, ELISA detected IgG and IgM antibodies targeting each of the pathogens of the TORCH. Samples that were positive in *T. gondii* IgM were taken to mean that they were recently infected, whereas IgG positivity meant that they were previously infected or vaccinated. Each and every test was done in duplicate to ensure accuracy, and positive and negative controls were added to each experiment done.

Preparation of Histological sections: -

The placental tissue samples were processed into histological analysis using the procedure of ⁹ and amended where the need arises. In a nutshell, the fixation of tissue specimens in 10% neutral-buffered formalin was carried out in 24-48 hours to maintain cellular and tissue architecture. After that, the samples were thoroughly dried in a graded ethanol (70%, 80%, 90% and 100%) to remove all the water, cleared in xylene to remove all alcohol, and then embedded in paraffin wax to give the structure to sectioning.

The tissues embedded in paraffin were cut into sections of 5 μ m of thickness using a rotary microtome. Slices were next placed on clean glass slides. The xylene deparaffinization of the sections was followed by rehydration using the descending series of ethanol (100, 95, 70) to water. H&E-staining of sections was done to observe cellular and tissue structures. After being stained, the sections were then dehydrated, cleared with xylene, and then placed under permanent mounting medium with a coverslip.

Ready slides of each group were observed under the light microscope using 100x and 400x. The histopathological changes observed were tissue necrosis, degeneration, vacuolation, fibrosis, hemorrhage and integrity of villous and trophoblast. Comparison and contrasting of the infected and uninfected placental tissues were performed through representative picture taking.

Evaluation of Interferon-Gamma (IFN- γ) Levels by ELISA

The quantity of IFN- γ in serum samples was determined by the enzyme-linked immunosorbent assay

(ELISA) technique. In this work, a commercially available ELISA Kit of Human IFN- γ was created based on the recommendations of the manufacturer (Shanghai, China, 2022). In the experiment, 100 μ L of each serum sample was added to the microplate wells that had initially been coated with human IFN- γ -specific antibodies. Wells were afterwards incubated at 37 °C for 90 minutes and bound. The wells were washed three times with the help of the supplied wash buffer after incubation to eliminate unbound material. Biotinylated detection antibody (100 μ L) was added to each well, and the mixture was left to incubate at 37 °C for 60 minutes. It was then washed again, and streptavidin-horseradish peroxidase (HRP) conjugate was added, and the mixture was left to sit at room temperature (30 minutes).

The reaction of the color was then formed, with the addition of the substrate solution and running the reaction in the dark after fifteen minutes. A microplate reader was employed in the identification of the optical density at 450 nm, and the interruption of the reaction was performed by the stop solution. With the aid of the results of the absorbance, the concentrations of IFN- γ were determined by comparison with a standard curve drawn using known concentrations of recombinant human IFN- γ . The results of the triplicate measurements of the individual samples were expressed as picograms per milliliter (pg/mL).

Determination of Vitamin C Activity

The activity of vitamin C in the blood samples was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai, China, 2022) according to the instructions. In order to get rid of the confounding proteins in the experiment, 100 μ L of every serum sample was mixed with a protein-precipitating agent, typically metaphosphoric acid. The supernatant was then transferred to vitamin C detection-specific reagent-coated microplate wells. The plates were incubated at room temperature with the required length of time to allow the vitamin C in the samples to react with the detection reagents.

The conjugate of the enzyme was put in after the cleaning of the wells to remove residual content, and subsequently, another incubation period was used to facilitate the reaction between the enzyme and the substrate. A chromogenic substrate was then added to each well, which produced a color change based on the concentration of vitamin C in the sample. The stop solution of the kit was added to end the reaction, and the optical density at 520 nm was measured using a microplate reader. Through comparison of the sample absorbance values to a standard curve developed using known vitamin C concentrations contained in the kit, vitamin C concentrations were determined. The findings were expressed in milligrams per deciliter (mg/dl), and the samples were tested twice each.

Statistical Methods

The SPSS software (version 23) was used in the analysis of the data. The quantitative variables (vitamin C activity and interferon-gamma (IFN- γ)) were represented as mean and standard error. The ANOVA or one-way analysis of variance was used to evaluate the group differences. A post-hoc comparison of them was performed by the multiple range test of Duncan in case of any significant difference. We used the Chi-square (χ^2) test to analyze the categorical data (such as the distribution of *Toxoplasma gondii* infection among the groups). The statistically significant (P) value was taken as ≤ 0.05 ¹⁰.

RESULTS

The study was carried out from March 2023 to July 2023 at Kirkuk Teaching Hospital's Obstetrics and Gynecology Department. There were one hundred ladies in all, ages 20 to 40. The participants were divided into three groups: pregnant women without infection, women who had spontaneous births, and women who had abortions linked to *Toxoplasma gondii* infection.

Diagnosis by TORCH Test

The TORCH cassette test was used to determine the prevalence of *Toxoplasma gondii* infection. Women who lived in rural regions had greater infection rates than those who lived in urban areas, according to an analysis of the data based on the participants' place of residence. In particular, 22% of women in urban regions tested positive for *T. gondii*, compared to 28% of women in rural areas. 28% of participants in urban areas and 22% of individuals in rural areas had negative outcomes. An overall infection prevalence of 50% was obtained from the examination of 100 women, of whom 50 tested positive for *T. gondii* infection and 50 tested negative (Table 1).

These results point to a somewhat greater frequency of *T. gondii* infection in rural populations, which may be impacted by environmental exposure, hygienic habits, and lifestyle elements typical of rural living.

Table 1: Distribution of *Toxoplasma gondii* infection by place of residence

Countryside	22	22%	28	28%	50
City	28	28%	22	22%	50
Total	50	50%	50	50%	100

In relation to the total number of specimens (100), the percentages of positive and negative specimens were computed. The findings show that participants in rural and urban areas had similar infection distributions, with a slightly greater frequency in rural areas.

Histological Changes of the Placenta:

In comparison to uninfected placental tissues, histological analysis of placental tissues from women who had abortions linked to *Toxoplasma gondii*

infection showed significant pathological changes. Representative histological characteristics seen in the afflicted placentas are shown in Figure 1.

The major changes in the infected placental tissues were:

1. Severe chorionic villi necrosis.
2. The trophoblast and stromal cells exhibit vacuolation.
3. Degeneration of cells in villous structures.
4. Leukocyte infiltration which denotes an inflammatory reaction.

5. *T. gondii* bradyzoites are found within fibrous connective tissue.

6. The formation of fibrous tissue and extensive internal tissue bleeding.

These results imply that placental architecture is significantly disrupted in infected women with vascular damage, inflammatory infiltration, and loss of normal villous structure, all of which may be factors in unfavorable pregnancy outcomes. Conversely, uninfected women's placentas showed normal histological architecture devoid of necrosis, degeneration, vacuolation, and bleeding.

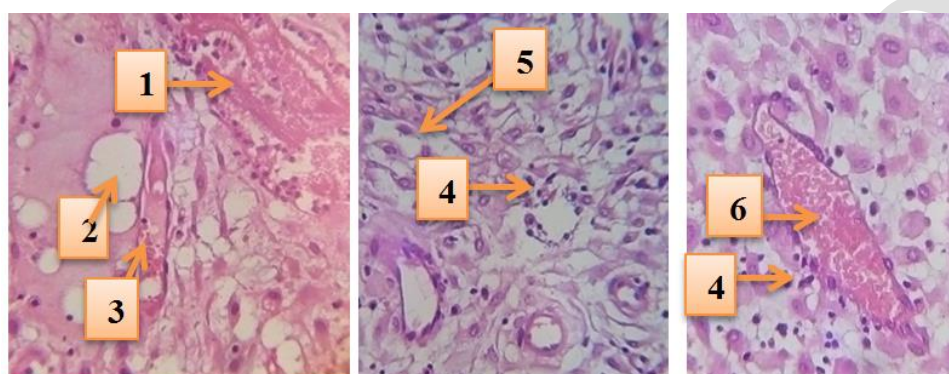


Fig. 1: Histopathological features of *T. gondii*-infected placental tissue (Hematoxylin and Eosin, 100×).

Interferon-Gamma (IFN-γ) Levels in Toxoplasma gondii-Infected Women

The serum IFN-γ concentration was significantly higher than that of uninfected women in those infected with *Toxoplasma gondii*. As indicated in Table 2, the mean value of IFN-γ for the aborted women infected by *T. gondii* was 359.44 ± 22.09 pg/mL, which was notably higher compared with the pregnant women devoid of the infection and women who delivered normally, 24.45 ± 1.13 pg/mL and 78.56 ± 4.57 pg/mL, respectively. These results show that IFN-γ is strongly induced in response to *T. gondii* infection, reflecting the activation of the Th1-mediated immune response. The difference between groups is statistically significant at $P \leq 0.05$.

Table 2: The mean of the cytokine IFN-γ concentration in both infected and uninfected women.

Mean (pg/ml) ±SD	N.	Groups
24.447 ± 1.126 A	27	pregnant women without infection
78.561 ± 4.567 A	23	women who had natural births
359.438 ± 22.089 B	50	abortions with <i>Toxoplasma gondii</i>

*The different letters indicate a significant difference between the means at $P \leq 0.05$.

Vitamin C activity:

Vitamin C activity in serum was determined in an attempt to determine its possible contribution to oxidative stress during *Toxoplasma gondii* infection. The findings did not statistically differ with regard to the three groups at $P \leq 0.05$. Pregnant women without infection had a slightly higher mean vitamin C level (1.501 ± 0.109 mg/dL) compared to women with natural deliveries (1.230 ± 0.079 mg/dL) and women who experienced abortions associated with *T. gondii* infection (1.311 ± 0.068 mg/dL). However, these differences were not statistically significant, indicating that vitamin C activity remained relatively similar across all study groups (Table 3).

Table 3: The mean of vitamin C activity in both infected and uninfected women.

Mean (mg/dl) ± SD	N.	Groups
1.501 ± 0.109	27	pregnant women without infection
1.230 ± 0.079 a	23	women who had natural births
1.311 ± 0.068 a	50	abortions with <i>Toxoplasma gondii</i>

*The similar letters indicate non-significant differences at $P \leq 0.05$

These findings suggest that while *T. gondii* infection may contribute to oxidative stress, the serum vitamin C levels were not significantly affected in this study population.

DISCUSSION

The results revealed that the prevalence of infection in the countryside was higher than in the city (place of residence) according to the TOURH Cassette test, where the percentage of parasite infection in rural areas was (28%), while it was (22%) in the city, estimated population prevalence rates vary greatly between different countries and between different geographical regions within a country ¹¹. The discrepancy is clear according to what was obtained through our current study about the place of residence between the countryside and the city, due to the association with a number of different factors, including cultural, health, livelihood, food habits, age, dealing with soil in the countryside, as well as the climatic conditions of temperature, high humidity, and raising domestic animals ¹². All of these factors increase the incidence of parasite infection in that area.

The results are consistent with the study by ¹³ conducted in Dyala city, and also with the study by ¹⁴ conducted in Tikrit city. The reason for the higher rates of infection in rural areas compared to urban areas is due to the higher chances of transmission in rural areas, which may be attributed to the irrigation of crops with contaminated water ¹⁵.

While, the results of the study are inconsistent with those of a study ¹⁶ about the prevalence of parasite infection, showing a higher percentage in the city than in the countryside, with a prevalence of 50.4% in Najaf. In contrast, cats in rural areas have larger areas to shed parasite egg sacs, resulting in a lower concentration of egg sacs and a decreased risk of infection among rural populations ¹⁷.

Histological study of a group of placenta specimens from aborted *T. gondii*-infected women illustrated the major pathological abnormalities, the results showed significant changes in the shape of the affected placenta tissue compared to the uninfected tissue, where there were: linked to the absence of tissue necrosis, degeneration or vacuolation, and fibrous tissue, serious internal tissue hemorrhage for histological changes in the placenta, the current study validates a prior study conducted by ¹⁸ that found bleeding, fibrinoids necrosis, and hydropic degeneration in trophoblast cells in the placenta histological section taken from women who had been aborted and infected with *T. gondii*. In a study of placenta tissue, positive immunoreactivity was also seen, indicating the possibility of parasite transmission from the placenta to the fetus and these abortion-causing histopathological alterations.

The association between parasitic infection and abortion is confirmed by the histology analysis that identified parasite stages in the placentas of *T. gondii*-infected females. It is not a secret that the placenta is a valuable route for various compounds that may penetrate the fetus and subsequently cause the

transmission of such diseases as *T. gondii* that may lead to abortion ¹⁹.

The most common infection mechanism in pregnant women can be placenta tachyzoite invasion, which consists of developing pathogens within placenta cells and subsequently into fetal tissue or fetus bloodstream ²⁰. Moreover, as this study revealed, inflammation and necrosis were present, all of which are generated either by infection, the immune system, or parasites ²¹. Moreover, it was found out that placental villitis was because by chronic inflammation, a non-specific immune reaction, and hence it is believed to be an immunological reaction, and the fetal-placental unit is made of different hostile species, one of which is *T. gondii* ²².

The results indicate that the IFN- γ in *T. gondii*-infected women was much higher compared to the natural birth uninfected group and the control group. Based on these new observations, defense against *T. Gondii* requires the involvement of interferon. This demonstrates the importance of interferon to the immune response of the body against the parasite. It uses a number of methods in fighting the parasite and preventing its proliferation, which eventually kills it or turns it into a tachyzoite into a bradyzoite by a number of effects ²³.

Macrophages produce tumor necrosis factor-alpha under the stimulation of IFN-gamma. The protein interacts with IFN- γ to enhance the production of nitric oxide and superoxide dismutase (SOD). Additionally, IFN- γ controls numerous other effector processes that fight *T. gondii*, such as p47 GTPase activation, iron deprivation, reactive oxygen intermediate (ROI) production, and tryptophan starvation ²⁴. The disruption caused by GTPase translocation to the PV permits the parasites to leave the protective vacuole ²⁵.

The development of acquired immunity against *T. gondii* is typified by the activation of CD8+ and CD4+T cells, and IFN- γ is important for resistance to the parasite in both the acute and chronic phases of infection ²⁶. These results come in acceptance with Bruna-Romero²⁷, who reported higher levels of IFN- γ concentration in women with abortions with *T. gondii* compared with uninfected groups.

According to the current investigation, there were non-significant differences in vitamin C activity between the groups at ($P \leq 0.05$), but there was no significant increase in the group of pregnant women without infection when compared with other study groups, Vitamin C has been shown to have a protective effect within the follicle; deficiencies in this vitamin have been linked to premature meiosis restarting, ovarian atrophy, and widespread follicular atresia²⁸.

The current results showed no significant increase in pregnant women without infection and women who had natural births, indicating that vitamin C activity rises physiologically during pregnancy and may lower the

risk of abortion because vitamin C likely assists in reducing the oxidative burst that occurs during the early stages of pregnancy²⁹. Additionally, the parasite's generation of oxidants like free radicals may be the cause of the decreased vitamin C levels in infected women; as an antioxidant, vitamin C protects the body from these radicals by oxidation itself^{30,31}.

A similar study by Al-Khshab³² showed a significant decrease in vitamin C. in abortions with *T. gondii* compared with pregnant women without infection and women who had natural births.

CONCLUSIONS

The percentage of infected women in the countryside was higher than in the city, and there were phenotypic abnormalities in the tissue of the placenta infected with *T. gondii* compared to uninfected women, and the concentration of IFN- γ in infected women was significantly higher compared to uninfected women (pregnant women and those with normal births), with a decrease in Not significant at $P \leq 0.05$ in vitamin C activity in infected women compared with uninfected women.

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Ethical Approval: Verbal consent was obtained from the patients' companions before collecting the samples. The research protocol was reviewed by the Research Ethics Committee of the College of Nursing, Kirkuk University, and approved under document number 1732 on 26-2-2023.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

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