POSSIBLE CELLULAR EXPRESSION OF IFN-γ IN WOMEN WITH ABORTION INFECTED WITH *TOXOPLASMA GONDII*

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SUMMARY : This study included one hundred and twenty six women with spontaneous abortion (6 of them with induced abortion). Venous blood was collected from these women, and serum was obtained for the performance of the ELISA test for the detection of anti-Toxoplasma IgM to indicate the acute T. gondii infection. Paraffin embedded blocks of the trophoblastic tissue obtained from each patient were prepared. The trophoblastic tissue was investigated for the presence of T. gondii antigen by using immunohistochemical analysis, using specific monoclonal antibodies for T. gondii. According to the immunohistochemical analysis, the patients were divided into three groups, (group 1) 26 positive for Toxoplasma, (group 2) 26 negative for Toxoplasma, and (group 3) 6 negative for Toxoplasma (induced abortion group).

The results indicated a high frequency of the T. gondii infection among women with abortion, 23 of 120 women (19.17%) have IgM Abs against T. gondii by ELISA test. The use of ELISA test for the detection of anti-Toxoplasma IgM was highly specific (100%) but not highly sensitive (88.46%). This could explain the use of more sensitive techniques for the detection of T. gondii infection like immunohistochemical analysis. The results of the IHC revealed that 26 of 120 women (21.66%) had Toxoplasma antigen within the trophoblastic tissue. The sensitivity and specificity of IHC were 100%, 96.91%, respectively.

The current study showed that there was no significant difference between the mean age of positive and negative groups, while there was a highly significant difference between the mean age of positive and induced abortion groups. Similarly, the mean age between negative and induced abortion groups showed a highly significant difference.

In this protocol, the majority of patients within the positive and negative groups were found to have no previous abortions, while patients with previous abortions constituted a less percent. Among the induced abortion group, it has been found that all the six cases had no previous abortions.

The mean gestational age among the three groups was compared in this study, where it revealed a highly significant difference between the positive and induced abortion groups. The same result was found between the mean gestational age of negative and induced abortion groups. In addition, it revealed the lack of significant difference between the mean gestational age of positive and negative groups. The majority of abortions within the positive group for T. gondii fall in the period of 12 weeks (41%) followed by 8 (15%) and 10 (12%) weeks of gestational age.

Levels of IFN-γ were investigated by using immunohistochemical analysis. The results revealed a highly significant difference regarding the mean percent of IFN-γ when compared between the positive and negative groups. In this work, t-test revealed a highly significant difference regarding the mean percent of IFN-γ between positive and induced abortion groups. Similarly, a significant difference was found when the mean percent of IFN-γ was compared between the negative and induced abortion groups. The results of immunohistochemical analysis of IFN-γ within positive group showed that there were negative and highly significant correlations with gestational age but not with number of previous abortions.

In conclusion, the data of this study strengthen the possibility that IFN-γ may explain the role of type 1 cytokines in the pathogenicity of abortion in the positive group for T.gondii.

Key words: Interferon-γ, Toxoplasma gondii, Abortion

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INTRODUCTION

Toxoplasmosis is caused by the intracellular parasite *Toxoplasma gondii* that infects up to a third of the world's population. Infection is mainly acquired by ingestion of food or water that is contaminated with oocysts shed by cats or by consuming contaminated meat containing tissue cysts. In addition, infection may be acquired by contact with cat feces containing oocysts (1, 2). Toxoplasmosis can be transmitted to the fetus *in utero* through transplacental transmission (3).

Primary infection is usually sub-clinical, but in some patient cervical lymphadenopathy or ocular disease can be present. Infection acquired during pregnancy may cause severe damage to the fetus. In immunocompromised patients, reactivation of latent disease can cause life-threatening encephalitis (4). Congenital infection occurs only when mothers first encounter *T. gondii* during pregnancy (5, 6).

High seroprevalence of *T. gondii* has been found in many countries such as UAE (7) and Egypt (8). In Iraq, many studies were accomplished concerning the seroprevalence of toxoplasmosis by using different techniques including ELISA, IFAT and IHAT (9, 10). Moreover, *T. gondii* is often difficult to find in tissue sections, but is more likely to be present in sections of brain and placenta. Identity can be confirmed by immunohistochemistry, while the polymerase chain reaction may be used to identify parasite DNA in tissues (11).

Toxoplasma infection stimulates humoral immune response as antibody production, which includes IgM and IgG, in addition to cell mediated immunity. Cell mediated immune responses are essential for the host control of intracellular infections, so the protection against toxoplasmosis is mediated by cellular defense (12). Resistance to *T. gondii* is mainly mediated by type 1 cytokines, such as IFN-γ which is central in resistance to *T. gondii* infection, whereas type 2 cytokines, such as IL-4 and IL-10, are associated with increased susceptibility to infection (13, 14). Susceptibility of the pregnant host to toxoplasmosis may be due to a type 2 cytokine bias that is maintained during gestation (15). *T. gondii* is a strong stimulus of type 1 cytokines, perhaps reflecting an advantage in keeping the host alive during infection. On the other hand, despite the protective role of type 1 cytokines during *T. gondii* infection, there is likelihood that strong type 1 response induced early during *T. gondii* infection will induce abortion early in pregnancy (16, 17).

A type 2 cytokine bias has been identified in normal murine placenta and is associated with successful implantation maintenance of early pregnancy, and suppression of local inflammatory responses (18). On the other hand, type 1 cytokines cause inflammatory immune reactions and graft rejection mechanisms which lead to the abortion of the conceptus (19).

MATERIALS AND METHODS

Subject selection

One hundred and twenty pregnant female patients attending the Obstetrics and Gynecology department of Al-Kadhimyia Teaching Hospital in Baghdad/ Iraq, between December 2004 and August 2005 were the subjects of this study. They were all admitted to the hospital for spontaneous abortion for evacuation. Six other females were also included with elective termination of pregnancy, due to maternal cardiac diseases, induced abortion, as a control group. Consent of two senior gynecologists and physician was given regarding this issue. The abortion for the six women was the first.

A questionnaire sheet (Figure 1) was filled out for each patient studied. The gestational age was calculated for each patient from data of the last menstrual period.

According to the results of the immunohistochemical analysis for the detection of *Toxoplasma* antigen, the patients were divided into three groups:-

Group 1: - 26 positive for *Toxoplasma*.

Group 2: -26 negative for *Toxoplasma* (chosen from the 120 females and were matched according to their age and gestational age with those that were positive).

Group 3: - 6 negative for *Toxoplasma* (induced abortion).

Note: all the 120 patients and the 6 with induced abortions were subjected to all the tests mentioned in the next sections.

Figure 1: Questionnaire Sheet

Case number:	Date:	
Patient Name:	Age:	
Address:	Urban	Rural
Date of LMP:	Gestational age	
Number of previous pregnancies		
Number of previous abortions		
Parity		
Summary of obstetrical history (Abortions in details)		
G1	G2	G3
Repeated abortion consecutive	or not	
Previously diagnosed for toxoplasmosis		
Previous treated for toxoplasmosis		
Are there congenital abnormalities in fetus?		
Contact with animals		
Educational level		
Preexisting medical diseases		
Uterine abnormalities		
Family history of genetic diseases		

Sample collection

From each female patient included in this study, the following samples were collected:

Blood

Five ml of venous blood were collected by disinfecting the anticubital fossa with 70% ethanol (Riedel de Haen) and using a disposable syringe with a 23- gauge needle, after applying a tourniquet. The blood was placed in a plain tube and left to stand for one hour at room temperature for clot formation. The tube was centrifuged for 10 minutes at 4°C at 450X g for serum collection. The serum was then aspirated by using a Pasteur pipette and dispensed into sterile glass tubes (1 ml in each) and stored at -20°C until used.

Trophoblastic tissue

Trophoblastic tissue was collected from curettage and placed in 10% formaldehyde. Two to three paraffin embedded blocks were prepared for each patient. Staining with haematoxylin and eosin was carried out to decide which block can be used in the study (only sections that contained trophoblastic tissue were included in the study).

Enzyme Linked Immunosorbent Assay for the detection of IgM antibodies for *Toxoplasma* gondii in serum

Reagents

Materials provided with the kit: (BioCheck, Inc. Foster City, CA)

* Microtiter Wells: purified *Toxoplasma* antigen coated wells (12 X 8 wells).
* Enzyme Conjugate Reagent
* Sample Diluent
* Negative Control
* Cut-off Calibrator
* Positive Control
* Wash Buffer Concentrate (20X)
* TMB Reagent
* Stop Solution: IN HCl

Reagent preparation

* All reagents should be allowed to reach room temperature (18-25°C) before use.
* One volume of Wash Buffer (20X) was diluted with 19 volumes of distilled water

Assay procedure

* The desired numbers of coated wells were placed into the holder.
* 1:40 dilution of test samples, Negative Control, Positive Control, and Calibrator were prepared by adding 5 µl of the sample to 200 µl of Sample Diluent. They were mixed well.
* 100 µl of diluted sera, Calibrator, and Controls were dispensed into the appropriate wells. For the reagent blank, 100 µl Sample Diluent were dispensed in 1A well position. The holder was tapped to remove air bubbles from the liquid and mixed well.
* The wells were incubated at 37°C for 30 minutes.
* At the end of incubation period, liquid from all wells was removed. The microtiter wells were rinsed and flicked 4 times with diluted Wash Buffer (1X) and then one time with distilled water.
* 100 µl of Enzyme Conjugate were dispensed into each well. They were mixed gently for 10 seconds.
* The wells were incubated at 37°C for 30 minutes.
* Enzyme Conjugate was removed from all wells. The microtiter wells were rinsed and flicked 4 times with diluted Wash Buffer (1X) and then one time with distilled water.
* 100 µl of TMB Reagent were dispensed into each well, mixed gently for 10 seconds.
* The wells were incubated at 37°C for 15 minutes.
* 100 µl of Stop Solution (1N HCl) were added to stop the reaction.
* *Mixing gently was performed for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely, and before reading, it should make sure that there are no air bubbles in each well.*
* The Optical Density (O.D.) was read at 450 nm within 15 minutes with a microwell ELISA reader (Tietertek multiskan, Finland).

Calculation of results

a. The mean of duplicate cut-off calibrator values X_C was calculated.

b. The mean of duplicate positive control (X_P), negative control (X_n) and patient samples (X_S) were calculated.

c. The *Toxoplasma* IgM Index of each sample was calculated by dividing the mean values of each sample (X) by calibrator mean value, X_C.

Interpretation of results

Negative: Toxo M Index less then 0.90 is negative for IgM antibody to *T. gondii*.

Equivocal: Toxo M Index between 0.91-0.99 is equivocal. Sample should be retested.

Positive: Toxo M Index of 1.00 or greater is positive for IgM antibody to *T. gondii* and indicates the probability of current or recent toxoplasmosis.

Calculation of the sensitivity and specificity of the ELISA test

The calculation of sensitivity and specificity of ELISA test was done according to the following equations compared to the IHC:

$$\text{Sensitivity} = \frac{a}{a + c}$$

$$\text{Specificity} = \frac{d}{b + d}$$

Where a : True positive patient
b : False positive patients
c : False negative patients
d : True negative patients

Immunohistochemical analysis for the detection of *Toxoplasma gondii* antigen and IFN-γ protein in paraffin embedded sections

Materials

1. Immunophosphatase secondary detection kit (Chemicon International, USA).

The following Reagents are included in the kit:

a. Blocking Reagent: normal goat serum in phosphate buffered saline (PBS) containing carrier protein.

b. Secondary Antibodies: biotinylated goat anti-mouse IgG and goat anti-rabbit IgG in PBS, containing carrier protein.

c. Streptavidin AP: Streptavidin Immunophosphatase diluted in TBS.

d. Fast Red Chromogen A: Fast Red Violet diluted in TBS.

e. Fast Red Chromogen B: Naphthol AS phosphate.

f. Haematoxylin: Mayer's haematoxylin counter stain.

g. Rinse Buffer (20X): TBS

h. Phosphate Buffered Saline tablets: 1 tablet dissolved in 100 ml deionized distilled water to make (1X) PBS.

Alternative Staining materials using:

a. Secondary anti-mouse antibody conjugated with peroxidase enzyme (Sigma).

b. DAB chromogen: 3, 3-diaminobenzidine in a chromogen solution.

c. Tris buffer pH=7.6 diluted for DAB.

d. H_2O_2 substrate (3% concentration).

e. Haematoxylin as a counter stain.

2. Monoclonal Antibodies:

a. Mouse anti-human *T.gondii* antigen: IgG1-K to *T.gondii* RH strain, applied in 1:100 dilution (Chemicon, USA).

b. Rat anti-human IFN-γ: RMMG-1 (clone), IgG1 to human IFN- , applied in 1:50 dilution (Chemicon, USA).

3. Absolute ethanol (Merck, Germany).

4. DPX mounting medium, is a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (BDH, England).

Preparation of reagents

1. Rinsing buffer was diluted 20X in distilled water.

2. Absolute ethanol was diluted in distilled water to prepare 95% and 70% concentrations of ethanol.

3. Primary antibody was diluted by using common antibody diluted in concentrations according to each monoclonal antibody.

4. Peroxidase conjugated secondary antibodies were diluted 1:100 in common antibody diluent.

5. DAB solution: Dissolving DAB at a concentration of 0.6 mg/ml Tris buffer immediately before use.

6. H_2O_2 should be added to DAB diluted chromogen solution to give a final concentration of 0.01%.

7. Chromogen Reagent was prepared by adding an equal volume of Fast Red Chromogen A and Fast Red Chromogen B in a mixture bottle. The volume of Chromogen reagent required for total number of slides was determined as being approximately 200 µl per slide.

Preparation of tissue sections

a. Paraffin embedded sections were cut into 5 µm thickness, placed on positively charged slides and left overnight to dry at room temperature.

b. To determine the signal specificity, negative control slides were included. In the first run, the negative control slides included sequential omission of reactive components in the test; the primary (monoclonal antibody), the secondary antibody (the biotinylated link), the conjugate and the substrate. Then, in each immunohistochemistry run, the negative control slides were obtained by omitting the primary antibody and applying antibody diluent alone (20). This was under identical test conditions (i.e. on the same slide).

c. Positive control slides were also included for each immunohistochemical run.

Immunohistochemistry procedure

1. Slide baking: the slides were placed in a vertical position in a drying oven (hot air oven) at 65°C over night.

2. Deparaffinizing the tissue sections: the slides were immersed sequentially in the following solutions at room temperature for the indicated times:

-Xylene for 5 minutes.

-Absolute ethanol for 5 minutes.

-95% ethanol for 5 minutes.

-70% ethanol for 5 minutes.

-Distilled water for 5 minutes.

3. After draining and carefully blotting around the specimen to remove any remaining liquid, the slides were placed in the humid chamber then 100 µl of a protein-blocking reagent were applied onto the tissue to cover the whole specimen then incubated at room temperature for 15 minutes. Then the slides were rinsed gently with distilled water then drained and blotted as before.

*(in the alternative staining method, (peroxidase secondary detection system) 100 µl of H_2O_2 at a concentration of 3% were added plus the protein-blocking reagent in the same step).

4. 100 µl of the diluted primary antibody were applied onto the tissue after the slides were placed in the humid chamber then incubated at 37°C for 1 hour. After that, the slides were rinsed with (1X) rinse buffer for a minimum of 15 seconds then the slides were drained and blotted as before.

5. 100 µl of alkaline phosphatase secondary antibody were applied onto the tissue after the slides were placed in the humid chamber then incubated at 37°C for 10 minutes. After that, the slides were rinsed with (1X) rinse Buffer for a minimum of 15 seconds then the slides were drained and blotted as before.

*(In the alternative staining method, diluted peroxidase secondary antibodies were used instead of the alkaline phosphatase secondary antibody).

6. 100 µl of streptavidin-alkaline phosphatase conjugate were applied onto the tissue after the slides were placed in the humid chamber then incubated at 37°C for 10 minutes. After that, the slides were rinsed with (1X) rinse Buffer for a minimum of 15 seconds then the slides were drained and blotted as before (This step is not done in DAB staining).

7. 100 µl of chromogen reagent were applied to the tissue in a dark place for 10 minutes at room temperature. The slides were washed in distilled water for 5 minutes and then drained and blotted gently.

*(In the DAB staining method, 100 µl of DAB solution were applied to the tissue in a dark place for 10 minutes at room temperature).

8. The tissue was stained by 100 µl of counter (Haematoxylin) stain which was placed onto the tissue and incubated for 30 seconds at room temperature. Slides were drained gently.

9. Slides were washed in distilled water then drained and cleaned gently.

10. A drop of mounting medium (DPX) was placed onto the tissue section and then quickly covered with a cover slip and left to dry.

11. Slides were examined by light microscope at X400 magnification. Immunostaining was scored according to cut-off value.

Interpretation of result

* In the immunophosphatase secondary system :

The positive and negative tissue controls should be examined first to ascertain that all reagents are functioning properly. The presence of a red reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell nuclei. If the positive or negative tissue controls fail to demonstrate appropriate staining, any results with the test specimens should be considered invalid.

* In the peroxidase secondary detection system (the alternative staining method) the presence of a brown reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell nuclei.

Scoring

The expression of both IFN-γ was measured by counting the number of positive trophoblastic cells which gave red cytoplasmic

staining in the immunophosphatase secondary detection system or brown cytoplasmic staining system under light microscope.

The extent of the IHC signal in the villi was determined in 10 fields (X400 magnification). In each field, the total number of villi were counted and the extent of cytoplasmic staining of the trophoblast cells in a given villous was determined as a percent.

The total staining score was divided by the number of whole villi per field in 10 fields (21) so the percentage of positively stained villi in the 10 fields was calculated for each case by taking the mean of the percentage of the positively stained villi in the 10 fields.

C*alculation of sensitivity and specificity of the IHC test*

Calculation of sensitivity and specificity of IHC test was done according to the following equations in comparison to the ELISA test:

$$\text{Sensitivity} = \frac{a}{a + c}$$

$$\text{Specificity} = \frac{d}{b + d}$$

Where a : True positive patients
b : False positive Patients
c : False negative Patients
d : True negative Patients

Statistical Analysis

The data were statistically analyzed depending on the nature of the character, according to Snedecor and Cochran (22).

* The *t*-test of significance was used for the quantitative data, the lowest level of significance chosen to be when the probability (p) was less than or equal to 0.05 ($p \leq 0.05$).

* The relationship between the indicators was measured quantitatively by using the correlation coefficient which runs between complete positive and negative correlations, respectively.

* Means with their standard errors (SE) were calculated to reflect the size and precision of the estimated values.

RESULTS

This study involved 26 positive women for *T.gondii* (group 1), their mean age was 24.62±1.31, 26 negative women for *T.gondii* (group 2), their mean age was 24.38±1.3, and 6 induced abortions (group 3), their mean age was 32.0±1.21. According to the current results, t-test showed that there was no significant difference ($p>0.05$) between the mean age of positive and negative groups (because they were age matched) (Table 1A), while there was a highly significant difference ($p<0.01$) between the mean age of positive and induced abortion groups (Table 1B). Furthermore, there was a highly significant difference ($p<0.001$) between the mean age of the negative and induced abortion groups (Table 1C).

Frequency of number of abortions

The number of previous abortions in positive group ranged between (0-4) abortions, in which 15/26 cases (57.6%) had no previous abortion, 7/26 cases (26.9%) had

Table 1: Comparison of age (years) between positive (A), negative (B) (for *T.gondii*) and induced abortion (C) groups among women with abortion.

A

Group (Age)	No.	Mean ± SE	Median	Range	Significance level
Positive group	26	24.62 ± 1.31	25.5	16-42	p>0.05
Negative group	26	24.38 ± 1.3	22	16-42	

B

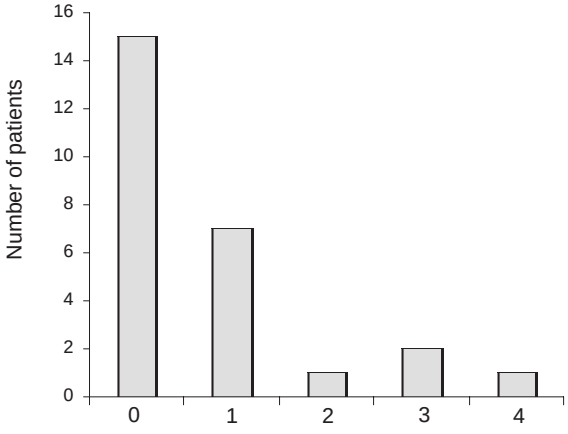
Positive group	26	24.62 ± 1.31	22.5	16-42	p<0.01**
Induced group	6	32.0 ± 1.21	31.5	29-36	

C

Negative group	26	24.38 ± 1.3	22	16-42	p<0.001**
Induced group	6	32.0 ± 1.21	31.5	29-36	

**High significant difference, SE: Standard Error

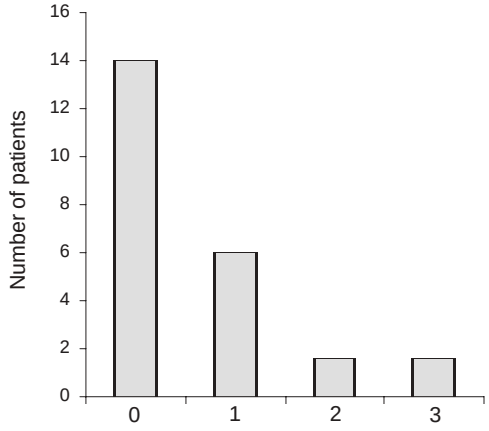
Figure 2: Frequency of previous number of miscarriages within positive group for *T. gondii.*



one previous abortion, 1/26 case (3.8%) had two previous abortions, 2/26 cases (7.6%) had three previous abortions, 1/26 case (3.8%) had four previous abortions (Figure 2). In the negative group, the number of previous abortions ranged between (0-3), in which 15/26 cases (57.6%) had no previous abortion, 7/26 cases (26.9%) had one previous abortion, 2/26 cases (7.6%) had two previous abortions, 2/26 cases (7.6%) had three previous abortions (Figure 3). Accordingly, the majority of the patients within the positive and negative groups were found to have no previous abortion, while patients with previous abortions constituted a lesser percent.

Among induced abortion group, it has been found that all the six cases have no previous abortions.

Figure 3: Frequency of previous number of abortions within negative group for *T. gondii.*



Gestational age (weeks)

The mean gestational ages (GA) for the positive, negative, induced abortion groups were 12.58±0.72, 12.77±0.78, and 10.33±0.61 respectively, were compared. It showed that there was no significant difference ($p>0.05$) between the mean gestational ages of the positive and negative groups (Table 2A), while there was a highly significant difference ($p<0.01$) between the mean gestational age of the positive and induced abortion groups (Table 2B) and between the mean gestational age of the negative and induced groups (Table 2C).

Distribution of abortion among gestational weeks

According to Figure 4, the majority of abortion cases fall in the period of 12 weeks (41%) followed by 8 (15%), 10 (12%) weeks of gestational age within the positive group for *T. gondii*. The frequency of the gestational period between 10 and 12 weeks was found within the induced abortion group.

The sensitivity and specificity of ELISA and immunohistochemistry (IHC) for the detection of *T. gondii* infection among women with abortion

The results showed that 23 of 120 women (19.17%, Figure 5) have IgM antibodies against *T. gondii* by ELISA method, while 26 of 120 women (21.66%, Figure 6) have *Toxoplasma* antigen within the trophoblastic tissue by IHC method.

The sensitivity and specificity of ELISA and IHC kits had been calculated and presented as 100%, 88.46% sensitivity of IHC and ELISA methods, respectively, and 96.91%, 100% specificity of IHC and ELISA, respectively (Table 3).

IFN-γ% among the three groups

In Table 4A, t-test showed that there was a highly significant difference ($p<0.001$) in the mean percent of IFN-γ between positive and negative groups. The t-test revealed a highly significant difference ($p<0.001$) in the mean percent of IFN-γ between positive and induced abortion groups (Table 4B). In Table 4C, t-test showed that there was a highly significant difference ($p<0.001$) in the mean percent of IFN-γ between negative and induced abortion groups.

A point worth mentioning, the three cases which were negative by ELISA and showed to be positive by immunohistochemistry, were statistically ($p<0.05$) higher in the IFN-γ percent than those who were true negative by ELISA. It is also worth while to note that there were two

cases negative by ELISA and immunohistochemistry, but at the same time their level of IFN-γ % was higher than the other negative cases.

Correlations within positive group

The current results revealed a highly significant negative correlation between IFN-γ and the gestational age (r = -0.919, p<0.001) (Table 5).

DISCUSSION

To the best of our knowledge, the present study is the first locally conducted immunohistochemical study to determine *Toxoplasma gondii* antigen in trophoblastic tissue and to determine the cellular expression of IFN-γ identified as being an important parameter that may reflect some of the immunological changes that occur in trophoblastic tissue in women with abortion.

Frequency of Toxoplasmosis

In the current study, the frequency of toxoplasmosis among women with single or repeated abortions was found to be 19.17% (23 positive out of a total of 120 cases) by using ELISA technique for the detection of specific IgM in serum. However, the frequency was found to be 21.66% (26 positive out of a total of 120 cases) by using immunohistochemical analysis for the detection of *Toxoplasma* antigen in trophoblastic tissue.

In Iraq, a similar result was obtained by Al-Sorchee (23) who demonstrated that 30.7% of women with abortion have positive IgM by using ELISA technique. Abbas (24) showed that 21.5% of women with first abortion have positive IgM by ELISA test. However, Al-Khafajy (10) demonstrated that 43.7% of women with abortion have positive IgM by ELISA test. This relatively high percent in the last mentioned study may be due to many factors including the sample size which was only 60 and the patients were selected from the Central Health Laboratory in Baghdad/ Iraq who had abortion and with suspension of toxoplasmosis during pregnancy (by history and physical examination). Therefore, this type of sample selection might reflect this high percent. In the present study, the relatively high frequency of toxoplasmosis in women with abortion could be due to the sample selection. The samples were collected from Al-Kadhimyia Teaching Hospital which is a reference hospital for the surrounding rural areas where they have habits in favor of acquiring toxoplasmosis by eating unwashed raw

Table 2: Comparison of gestational age (weeks) between positive (A), negative (B) (for *Toxoplasma gondii*) and induced abortion (C) groups among women with abortion.

A

Groups (GA)	No.	Mean ± SE	Median	Range	Significance level
Positive group	26	12.58 ± 0.72	12	8-24	p>0.05
Negative group	26	12.77 ± 0.78	12	8-24	

B

Positive group	26	12.58 ± 0.72	12	8-24	p<0.01**
Induced group	6	10.33 ± 0.61	10	8-12	

C

Positive group	26	12.77 ± 0.78	12	8-24	p<0.01**
Induced group	6	10.33 ± 0.61	10	8-12	

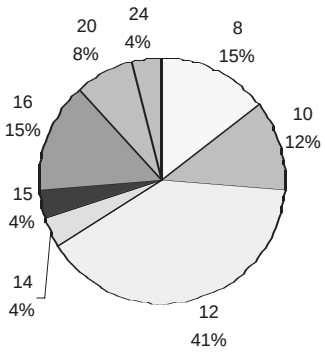
** Highly significant difference, SE: Standard Error, GA: Gestational Age

Table 3: Sensitivity and specificity of the IHC (for the detection of *T. gondii* antigen) and ELISA (for the detection of anti-*Toxoplasma* IgM) tests used for women with abortion.

	IHC	ELISA
Sensitivity	100 %	88.46 %
Specificity	96.91 %	100 %

IHC: Immunohistochemistry
ELISA: Enzyme Linked Immunosorbent Assay

Figure 4: Frequency (number within the circle) of gestational weeks (numbers outside the circle) in the positive group for *T. gondii* (26 patients) among women with abortion.



vegetables or unpadded fruits. In addition, in the rural cities there is close contact with cats and consequent exposure to sporulated oocysts by ingestion of these oocysts that contaminate soil during farming, or eating undercooked meat contaminated with cysts. Moreover, the low level of education in the women about the risk factors for toxoplasmosis may play an important role in the high rate of infection (25).

Other studies had variable results but most were concerned with the prevalence in the total population, where in USA it was found to be 60.4% (26) and in Iraq was found to be 22.06% of the total population (27).

ELISA versus immunohistochemistry

ELISA method for detecting IgM anti-*Toxoplasma* antibody in the serum and immunohistochemistry method for the detection of *Toxoplasma* antigen within the trophoblastic tissue were used in this study depending on the fact that the diagnosis of toxoplasmosis in humans is made indirectly by serological methods or directly by PCR assay, isolation of the organism, histology, or by some combination of the above (28). In addition, diagnosis of *T. gondii* infection which is based on the clinical appearance and serology is not always easy (29).

Different techniques have been developed for the diagnosis of toxoplasmosis, one of which is serology (ELISA) (30). Many other serological techniques have been applied in the diagnosis of toxoplasmosis including IFAT (31) and

Table 4: IFN-γ % in positive, negative (for *T. gondii*) and induced abortion groups among women with abortion.

A

	Positive			Negative			Significance level
	Mean ± SE	Median	Range	Mean ± SE	Median	Range	
IFN- γ %	74.50 ± 3.0	76.5	37-95	34.46 ± 2.61	40	7-50	p<0.001**

B

	Positive			Induced			Significance level
	Mean ± SE	Median	Range	Mean ± SE	Median	Range	
IFN- γ %	74.50 ± 3.0	76.5	37-95	7.33 ± 1.12	7	4-11	p<0.001**

C

	Negative			Induced			Significance level
	Mean ± SE	Median	Range	Mean ± SE	Median	Range	
IFN- γ %	34.46 ± 2.61	40	7-50	7.33 ± 1.12	7	4-11	p<0.001**

** Highly significant difference, SE: Standard Error

Figure 5: Percent of positive and negative cases for IgM against *T. gondii* by ELISA test in 120 cases of abortion.

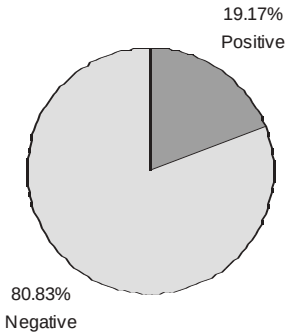
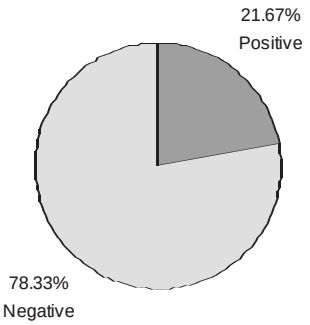


Figure 6: Percent of positive and negative cases of *T. gondii* antigen by immunohistochemcal analysis in 120 cases of abortion.



IHAT (32). The sensitivity and specificity of each differs according to the antigen used and antibody titer (33). ELISA was found to be more sensitive and specific than IHAT (8). The current study showed that the sensitivity and specificity of ELISA test were 88.46% and 100%, respectively.

T.gondii infection is seen in the villous trophoblast from placenta associated with congenitally infected infants (34). Congenital toxoplasmosis may result in abortion, still birth, and mental retardation (35). This issue was demonstrated in this study by detection of *Toxoplasma* antigen within the trophoblastic cells from patient who had abortion by the use of IHC. This method is both convenient and sensitive and is used with fixed tissue that may also exhibit degree of decomposition (36). Since immunohistochemistry involves specific Ag-Ab reaction, it has apparent advantages over traditionally used special and enzyme staining techniques that identify only a limited number of proteins, enzymes, and tissue structures. Therefore, immunohistochemistry has become a crucial technique and widely used by many medical research laboratories as well as clinical diagnosis (37). In the present study, the immunohistochemistry method showed a sensitivity of 100% and specificity of 96.91% for the detection of *T. gondii* antigen in the trophoblastic tissue of women with abortion.

Table 5: Correlations between different parameters within positive group for *T. gondii* among women with abortion.

		No. of abortions	Gestational age (weeks)	Age (years)	IFN - γ (%)
No. of previous abortions	r	1.000	-0.0657	-0.045	0.1306
	p	0.000	0.75	0.827	0.525
Gastational age (weeks)	r		1.000	-0.015	-0.919
	p		0.000	0.942	0.000**
Age (years)	r			1.000	-0.052
	p			0.000	0.800
IFN-γ (%)	r				1.000
	p				0.000

*Significant difference, **Highly significant difference,
r : correlation coefficient (r value), p : probability (p value).

A point to be noted in this study, is the presence of three cases which were negative by ELISA test but were positive by IHC which could be explained depending on the fact, that in acute *Toxoplasma* infection, IgM antibody levels generally rise within one to two weeks of infection (38, 39) but IgM level might be below the detection level of the kit used (the detection was very early in the disease) (40-42) or the lack of serum IgM in the three cases may suggest that their infection occurred because of reactivation of chronic latent infection (43). Furthermore, false negative cases may explain the positivity of the previous three cases when IHC was used because of the accuracy of ELISA kit used which was 97.4% (44). So the detection of the antigen in the tissue by using IHC was considered the confirmatory method. Regardless of the cost of this test, it was found to be 100% sensitive when compared to ELISA test.

Association of age and gestational age with Toxoplasmosis

The prevalence of antibodies specific to *T. gondii* is directly proportional to the age of the population, indicating that infection is acquired throughout life. On the other

hand, there was a highly significant difference between the mean age of both positive and negative groups and the mean age of the induced abortion group. One important limitation to our study was that, unfortunately, we had only six cases in the induced abortion group for obvious reasons, and this may explain the previous highly significant difference.

Frequency of abortions and Toxoplasmosis

It has been proposed that during pregnancy, systemic maternal immune response is biased in favor of a T_{H2} cytokines (45) and also in women with first abortion (46). Moreover, T_{H2} cytokines pattern of pregnancy induces the susceptibility to toxoplasmosis infection, together with the risk of placental infection and congenital transmission (15). Consequently, the result of the current study confirms previously mentioned data in which the majority (57.6%) of the positive group was found to have no previous abortions, while patients with previous abortions constituted a smaller percent.

Frequency of gestational weeks and Toxoplasmosis

When a mother acquires the infection with *T. gondii* during gestation, the organism may be disseminated hematogenously to the placenta. When this occurs, infection may be transmitted to the fetus transplacentally. If the mother acquires the infection in the first trimester and the infection is not treated, approximately 17% of fetuses are infected and disease in the infant is usually severe or spontaneous abortion may occur (47) because if infection occurs in the first trimester, hormone levels are low and there is little T_{H2} bias, the chance of transmission to the fetus is low, although the chance of abortion is high (17).

The risk of fetal infection with *T. gondii* is 2% if the infections occurred between the 3rd and 10th week's gestation, and 7% between 11 and 14 weeks (48). This observation could support the result in this work which showed that 15% of the positive patients had *T. gondii* infection in the 8th gestational week, 12% in the 10th gestational week, and 41% in the 12th gestational week. This indicates that the majority of the positive group was in the late first trimester. These different rates of transmission are most likely related to placental blood flow, the virulence and the amount of *T. gondii* acquired and the immunological ability of the mother to restrict parasitemia (43, 47).

Possible role of IFN-γ in spontaneous abortion caused by *T. gondii*

Evidence from murine and human pregnancy showed that since T_{H1} type cytokines mediated pregnancy loss, a shift towards T_{H1}-type immunity during *T. gondii* infection may help to explain pregnancy failure (17, 49). Thus, a considerable amount of evidence suggests that T_{H1} cytokines might well be implicated in adversely affecting pregnancy, directly by interfering with trophoblast survival and function, and indirectly by activating cell-mediated immune effecters (50).

The pro-inflammatory cytokine, IFN-γ was targeted as a reflection for type 1 immune response in this study, because of its T_{H1} polarizing effect due to its potential role in generating T_{H1} cells, mediating their effectors functions and regulation of T_{H1}/T_{H2} balance (51). Evidences had shed the light on the possible role of IFN-γ in pregnant women during *T. gondii* infection and showed that there was a concurrent increase in concentration of IFN-γ in placenta when there was a strong T_{H1} dominant response against *T. gondii* which resulted in abortion (52). This could be a potential explanation that evolved to enlighten the highly significant difference in the mean percent of IFN-γ between positive and negative groups. Evidence supporting this result is that, the administration of one of the T_{H1} cytokines like IFN-γ, TNF-α or IL-2 to normal pregnant mice causes abortion (53). IFN- γ and TNF-α inhibit the proliferation of human trophoblast cells *in vitro* (54) and are toxic to human trophoblast cells (55). Furthermore, IFN- γ and TNF-α induce apoptosis in trophoblast cells by the increase of Fas expression and enhance trophoblast sensitivity to Fas-mediated apoptosis (56, 57). Apoptosis is thus initiated when Fas is expressed on the maternal immune lymphocytes surface and contacts Fas L on placental cells (58). Once Fas-FasL interaction occurs, a series of caspases are activated that cleave specific substrates and, via the action of nucleases, degrade cellular DNA resulting in cell "suicide" (59) and this could reveal an explanation for the mechanism of abortion (60) In addition, IFN-γ increases production of NO by trophoblast cells (61, 62). NO has also been implicated as an apoptotic trigger during *T. gondii* infection. The mechanism by which NO induces apoptosis is not clear, but it may involve the effects of peroxynitrite formation from NO and super oxide in mitochondria (63). This may cause damage to placental trophoblast cells or other fetoplacental targets resulting in

embryo death and resorption (64). The previously mentioned mechanisms highlighted the potential role of IFN-γ in abortion caused by *T. gondii* infection and may be the cause of abortion in the cases enrolled in the current study.

In this work, the statistical analysis revealed the presence of a highly significant difference in the mean percent of IFN-γ between negative and induced abortion groups (the mean percent of IFN-γ in negative group was about 25% higher than in induced abortion group). This result could be explained based on the fact that the presence of other infections cannot be ruled out in the negative group which can cause abortion by the presence of high levels of IFN-γ (65). Accordingly, a highly significant difference was found in the mean percent between positive and induced abortion group. Since the induced abortion was done deliberately to terminate the pregnancy unlike the spontaneous abortion which has occurred in the positive group for *T. gondii* therefore one would expect no shift from T_{H2} towards T_{H1} immunity.

Acute toxoplasmosis causes placental inflammation and an inflammatory infiltrate consisting of lymphocytes, macrophages, and neutrophils (66, 67). This could support the results of high levels of IFN-γ in the positive group when compared to the negative and induced abortion groups, since the signal of IFN-γ, reflects the presence of IFN-γ in the inflammatory cells in addition to the signals which came from the trophoblastic cells. Unfortunately, the mechanisms responsible for triggering the local host response to toxoplasmosis are not fully understood. Moreover, such a high level of IFN-γ could be anticipated on the following bases: that *Toxoplasma* strain characteristics exert a profound effect on the host immune response, like hyper-induction of IFN-γ was found during RH strain infection, in contrast to ME49 strains infection (68). In fact, in the current study IgG_1k isotype of RH strain was detected. Furthermore, presence of other infectious agents cause a shift of immune response during pregnancy from T_{H2} to T_{H1} which can be observed as an abortion process (69). Conversely, regarding the level of IFN-γ in the negative group which was lower than the positive group; simultaneously, it was high when compared to the induced abortion group. This could base on the observation previously mentioned about the presence of other infectious agents which can cause abortion by the presence of high level of IFN-γ.

IFN-γ acts as a phagocytic stimulator at the maternofetal interface. This pathway includes the expression of IFN-γ R1 on the trophoblast cells (62). The expression of IFN-γ R1 is a fundamental characteristic of trophoblast cells throughout pregnancy (70). IFN-γR1 is immunolocalized mainly to spread trophoblast cells which suggests that trophoblast phagocytic activity in the presence of IFN-γ is a receptor mediated response (62). It is possible that signaling through this receptor enhances the uptake or delivery of *T. gondii* by trophoblast cells by the stimulating phagocytic activity (71). The molecular mechanism involved in IFN-γ stimulated phagocytosis includes that IFN-γ may trigger an autocrine cascade in trophoblast cells. IFN regulatory factor-1 (IRF-1) is highly expressed in the mouse trophoblast (72). This IFN-γ induced transcription factor activates the promoters of many interferon regulated genes (73). This may be a key to understanding the responsiveness of the trophoblast to IFN-γ. This activity declines during normal gestation (74).

How did IFN-γ correlate with the positive group for *T. gondii*?

There was no correlation identified between any of the parameters and the number of previous abortions. While, Al-Obaidi (75) showed that IFN-γ was expressed in lower levels in women with first abortion when compared to those of recurrent abortion. Such a difference could be anticipated on the difference in the number of the investigated cases. IFN-γ was found in the first trimester of pregnancy, but was barely detectable in the second, and undetectable in the third trimester (18). This may explain the presence of a highly significant negative correlation between the mean percent of IFN-γ and gestational age of the positive group for *T. gondii*.

An interesting finding in this study was that two cases who showed negative results by ELISA and IHC, simultaneously, expressed high levels of IFN-γ when compared to other negative cases. The explanation for this result may be depending on the fact that we cannot rule out the presence of other infectious agents of STORCH (Syphilis, Toxoplasmosis; Others which are Bacterial vaginosis, *Trichomonas vaginalis*, Group B streptococci, *Escherichia coli*, *Ureaplasma urealyticum*, *Haemophilus influenzae*, *Varicella*, *Listeria monocytogenes*, Rubella, Cytomegalovirus, H^5 Herpes, HIV, Hepatitis B, Human papilloma virus, Human parvovirus) since all these infectious agents include a shift of immune response during pregnancy from T_{H2} to T_{H1} which could be observed clinically as an abor-

tion process (69). The fact that in the current study, the detection for antigen by IHC is for the RH strain, could explain these two cases as being of ME49 strain that stimulated the elevation of IFN-γ but was undetectable by the current method, in which ME49 strain is type II low virulence parasite strain (76). In contrast, infection with type I high virulence RH strain led to widespread parasite dissemination and rapid death in mice (68).

IFN-γ, a friend or a Foe

Data from studies performed *in vitro* and *in vivo* suggest that IFN-γ is essential for resistance to acute *T. gondii* infection. IFN-γ can activate macrophages to inhibit or kill *T. gondii* without collaboration of any other lymphokine. This cytokine increases the expression of MHC-I products on cells (77) and by doing so may enhance the recognition of antigen on infected cells by immunized animals. This mechanism would be mediated by induction of CD^{+8} cytotoxic T-lymphocyte (CTL). IFN-γ acts in synergy with the TNF-α to induce the expression of iNOS, which results in the production of nitric oxide, which has been shown to kill intracellular *T. gondii* (78).

Despite the numerous studies that show the presence of IFN-γ producing cells in the pregnant uterus and, that IFN-γ can be beneficial to pregnancy if secreted at appropriate times, concentration, and location (79). In mice, high, *in vivo* dose of IFN-γ ($3x10^5$ IU/ml) are deleterious to early embryo while *in vitro* treatment reduces trophoblast out growth, limiting invasive potential (80). In this study, this cytokine was considered an abortion inducing factor. IFN-γ balance with other pro-and anti inflammatory cytokines, and the stage of gestation at which it is produced are fundamental in defining whether IFN-γ plays a physiological or pathological role (62). Hence, IFN-γ is important for reducing maternal infection with *T. gondii* by inhibition of tachyzoite replication. However, it seems that IFN-γ enhances, directly or indirectly, the transplacental transmission of *T. gondii* (71); directly includes the stimulation of phagocytic activity through IFN-γ receptor in murine trophoblast or indirectly by the enhancement of adhesion receptor expression by IFN-γ on the trophoblast cell surface, which might enhance transplacental passage of *Toxoplasma*. Since IFN-γ as a pro-inflammatory cytokine can cause up-regulation the adhesion molecules such as ICAM-1 and VCAM-1 (81), there was a correlation between the expression of IFN-γ and adhesion molecules in women with abortions (75).

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