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Full Length Research paper

Correlation between apoptosis and *Toxoplasma* in abortion induction: Relevance of caspase 8

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A total of 120 women with spontaneous abortion and 6 women with induced abortion from medical causes were enrolled in this study. Enzyme-linked immunosorbent assay (ELISA) test, for the detection of specific immunoglobulin M (IgM) and immunohistochemistry method for the detection of antigen in tissue were used as diagnostic methods for *Toxoplasma gondii*. Twenty-six (26) women were found to have *Toxoplasma* infection by immunohistochemistry (IHC) method, while 23 women were found to have *Toxoplasma* infection by ELISA method (the same samples that had *T. gondii* infection by IHC). From the remaining negative cases by IHC, of the 120 cases, 26 women were selected as patient control group. Those patient control cases were matched with those 26 positive by the age and gestational age. Venous blood samples were collected from all patients for the detection of specific anti-*Toxoplasma* IgM in the serum using the ELISA test. At the same time, trophoblastic tissue during curettage was collected for immunohistochemical analysis for the detection of *T.gondii* antigen and evaluation of caspase 8. The results obtained indicate a high frequency 19.17% of the infection by ELISA method and 21.67% by immunohistochemistry among women with abortion. This may be due to multiple pathways of parasite exposure and transmission. The sensitivity and specificity of ELISA was 88.46 and 100%, respectively, while the sensitivity and specificity of IHC was 100 and 96.91%, respectively. The frequency of the number of miscarriages was studied. It was observed that within the positive group, the highest percentage of patients was among those who had no previous miscarriages and to a lesser percentage that had a previous single miscarriage. The same result was found within the patient control group, while the entire six control groups had no previous miscarriages. The frequency of gestational weeks during which abortion had occurred was studied. The highest frequency within the positive group was 12 gestational weeks, then, 8 gestational weeks. While the frequency of gestational weeks was between 10-12, within the control group. The highest percentage of caspase 8 was within the positive group for *T. gondii* then lower than this was within the patient control for *T. gondii* and the lowest percentage was within the control group. This may indicate that *T. gondii* infected cells undergo apoptosis more than non-infected cells, while the cells of the control group was with the lowest level of apoptosis because of the absence of apoptotic trigger (for example, *T. gondii*). The results obtained show that there was a significant difference in the percent of caspase 8 between the positive and control groups. A significant difference in the percent of caspase 8 was also found between the patient control group and the control group, while there was no significant difference in the percent of caspase 8 between the positive and patient control group.

Key words: *Toxoplasma gondii*, caspase 8, apoptosis, miscarriage.

INTRODUCTION

Toxoplasmosis is caused by the intracellular parasite *Toxoplasma gondii* and may be contracted by consuming contaminated meat or by coming in contact with cat feces containing oocysts. *T.gondii* infects a large proportion of the world's population. Individuals at risk include fetuses, newborns, and immunologically impaired individuals (Hökelek, 2005). If a pregnant woman contracts toxoplasmosis, it may be passed through the placenta to the fetus, resulting in congenital toxoplasmosis, which is a cause of mortality and malformation (Lambert et al., 2005).

High *T.gondii* seroprevalence has been found in

many countries including Iraq (www.WrongDiagnosis.com™, 2005). A study was made by Jasim (1979) and this showed that 27.64% had positive reaction by indirect haemagglutination test (IHAT); and 23.14% by indirect fluorescent antibody test (IFAT), more in females than males and more with older age group (Abbas, 2002). Seroprevalence has been reported to be 78% among pregnant women in Ibadan, Nigeria (Onadeko et al., 1992), 44% among persons living in the drier regions of Somalia and 83% in the population of the South Delta in Nigeria (Dubey and Beattie, 1988). *T. gondii* infection stimulates both the humoral immune response as antibody production, which includes IgM, and IgG in addition to cell mediated immunity (CMI). CMI response is essential for host control of intracellular infections like *T. gondii* (Suzuki et al., 1988).

Different methods can be used for the diagnosis of *T. gondii* including isolation of the parasite and several serological tests that are available for the detection of *T. gondii* antibodies. In one type, the observer judges the given color of tachyzoites under a microscope, such as with the dye test (DT) and IFA test. Another depends on the principle of agglutination of *Toxoplasma* tachyzoites, red blood cells or latex particles, such as with the direct agglutination test (DAT) and indirect haemagglutination test (IHA) and latex agglutination (LA) test, respectively. With the enzyme-linked immunosorbent assay (ELISA), the degree of color change defines the quantity of specific antibody in a given solution. Also, nucleic acid recognition methods such as several polymerase chain reaction (PCR)-based assays have been developed for detection of DNA from *T. gondii*. Immunohistochemistry can also be used for the diagnosis of toxoplasmosis (http//www.OIE.int., 2004). During infection of cells, *T.gondii* either inhibits the apoptosis of the cells infected by this parasite or on the other hand it induces apoptosis (Nishikawa et al., 2002).

For normal pregnancy to be established, a Th2 type immune response must be induced by the maternal immune system at the maternal-fetal interface (Parslow et al., 2001). Women who undergo spontaneous abortion may have a stronger Th1-response (Makhseed et al., 2001). So, infection of a pregnant mother by *T. gondii* may induce a Th1 CMI and hence lead to apoptosis, then abortion.

MATERIALS AND METHODS

Subject selection

One hundred and twenty (120) 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 miscarriages evacuation. 6 other females were also included with elective termination of pregnancy due to maternal cardiac disease (induced abortion). They were considered as a control group. Consent of two senior gynecologists and a physician was given regarding this issue. The abortion was the first for the six women.

The gestational age was calculated from the data of the last menstrual period. A questionnaire sheet (Figure 1) was filled out for each patient studied. 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 patient controls (negative for *T. gondii*) had been chosen from the remaining 120 females and were matched according to their age and gestational age with those who were positive and Group 3, 6 negative for *Toxoplasma* (induced abortion), were considered as a control group. All samples were subjected to enzyme linked immunosorbent assay (ELISA), immunohistochemistry for the detection of *T. gondii* antigen within the trophoblastic tissue and only those three groups mentioned above were subjected to the remaining investigation (evaluation of caspase 8).

Sample collection

Blood and trophoblastic tissue samples were collected from each female patient included in this study.

Blood

Five (5) ml of venous blood were collected by disinfecting the anticubital fossa 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 1 h at room temperature for clot formation. For serum collection, the tube was centrifuged for 10 min at 4°C at 450 × g. The serum was then aspirated 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 the evacuation of retained pieces during the procedure of 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 was included in this 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, USA; 2004) include microtiter wells purified *Toxoplasma* antigen coated wells (12x8 wells), enzyme conjugate reagent, sample diluents, negative control, cut-off calibrator, positive control, wash buffer concentrate (20X), TMB reagent and stop solution.

Reagent preparation

All reagents were allowed to reach room temperature (18-25°C)

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Abbreviations: ELISA, Enzyme-linked immunosorbent assay; **IgM,** immunoglobulin M; **IHC,** immunohistochemistry.

Case number : ……. Date: ……

Patient name : …….. Age: ……..

Address : ……. Urban: …… Rural: ……..

Gravidity : ……..

Parity : ……..

Date of last menstrual period : ……

Gestational age at time of abortion : ……

Parity : ……

Summary of obstetrical history (abortion in details) : ……..

Repeated abortion consecutive or not : ……..

Previously diagnosed for toxoplasmosis : ……

Previous treatment for toxoplasmosis : ……..

Are there congenital abnormalities in fetus? ……..

Contact with animals : ……

Educational level : ……..

Family history of genetic diseases : ……

Pre- existing medical diseases : ……

Figure 1. Questionnaire sheet for each patient included in a study concerning toxoplasmosis in women with miscarriage.

before use. One volume of wash buffer (20X) was diluted with 19 volumes of distilled water.

Assay procedure

The desired number of coated wells was placed into the holder. 1:40 dilutions 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 into 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 min. 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 once with distilled water.100 µl of enzyme conjugate were dispensed in each well. They were mixed gently for 10 s. The wells were incubated at 37°C for 30 min. Enzyme conjugate was removed from all wells. The microtiter wells were rinsed and flicked 4 times with diluted wash buffer (1X) and then once 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 min. 100 µl of stop solution (1N HCl) were added to stop reaction. Mixing gently was performed for 30 s. We ensured that all the blue color changed to yellow color completely before reading the result, we also ensured that there were no air bubbles in each well. The optical density (O.D.) was read at 450 nm within 15 min with a microwell ELISA reader (Titertek Multiskan, Finland).

Calculations of results

The mean of duplicate cut-off calibrator values Xc was

calculated. The mean of duplicate positive control (Xp), negative control (X n) and patient samples (Xs) were also calculated. The *Toxoplasma* IgM index of each sample was calculated by dividing the mean values of each sample (X) by calibrator mean value, Xc.

Interpretation of results

Toxo M index less than 0.90 is negative for IgM antibody to *T.gondii* while Toxo M index between 0.91-0.99 is equivocal. Sample were retested**,**Toxo M index of 1.00 or greater is positive for IgM antibody to *T. gondii* and indicates the probability of current or recent *Toxoplasma* infection. Calculation of sensitivity and specificity of the ELISA test is as follows:

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

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

Where, a, true positive; b, false positive; c, false negative; d, true negative.

Immunohistochemical analysis for the detection of *Toxoplasma gondii* antigen and Caspase-8 (FLICE) proteins in paraffin embedded sections

Materials

i. 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 antibody: biotinylated goat anti-rabbit and anti-mouse IgG in PBS, containing carrier protein, (c) Streptavidin alkaline phosphatase: Streptavidin, immunophosphatase diluted in PBS, (d) Fast red chromogen A: Fast red violet diluted in PBS, (e) Fast red chromogen B: Naphthol AS phosphate, (f) Haematoxylin: Mayer's haematoxylin counter stain, (g) Rinse buffer: TBS, (h) Phosphate buffered saline tablets, 1 tablet dissolved in 100 ml deionized distilled water to make 1X phosphate buffered saline (PBS). Or alternative staining materials: (a) Secondary anti-mouse antibody conjugated with peroxidase enzyme (Sigma), (b) The DAB solution (3, 3 Diamino-benzidine tetrahydrochloride) (Sigma), (c) Tris buffer pH 7.6 diluent for DAB, (d) H_2O_2 substrate (3% concentration), and (e) Haematoxylin as counter stain.
ii. Monoclonal antibodies: (a) Mouse anti-human *Toxoplasma gondii* antigen (Chemichon, USA): Isotype: IgG_{1-k} to *Toxoplasma gondii* RH strain, (b) Mouse anti-human Caspase-8 protein [FLICE] (Chemicon, USA): Isotype: IgG_{2b} to human Caspase 8.
iii. Deionized distilled water.
iv. Xylene (AnalaR, England).
v. Absolute ethanol (Merck, Germany).
vi. DPX mounting medium, which is a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (BDH, England).
vii. Positively charged microscope slides, Fisherbrand Superfrost/ Plus (Fisher Scientific, USA).

Preparation of reagents

Rinsing buffer was diluted 20×in deionized distilled water. Absolute ethanol was diluted in distilled water to prepare 95 and 70% concentrations of ethanol. Primary antibody was diluted by using the common antibody diluent in concentrations as either 1:100 for anti-*Toxoplasma gondii*, Monoclonal Abs. or 1:50 for anti-caspase-8, Monoclonal Ab. Peroxidase conjugated secondary Ab was diluted 1:100 in common antibody diluent. Dissolving DAB solution, at a concentration of 0.6 mg ml^{-1} Tris buffer. Immediately before use, hydrogen peroxide (H_2O_2) was added to DAB diluted chromogen solution to give a final concentration of 0.01%. 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

Paraffin embedded sections were cut into 5 µm thickness, placed on Fisherbrand positively charged slides and left overnight at room temperature to dry. It is recommended that one positive tissue control is to be included for each set of test conditions in each staining run. Negative tissue control should be used which has been fixed and processed identical to that of the patient sample(s) with each staining run. Each lot of primary antibody should be tested to verify antibody's specificity by testing on a series of known positive and negative tissues with known immunohistochemical characteristics (Elias, 1989; Herman and Elfont, 1991).

Immunohistochemistry procedure

Dewaxing

Paraffin embedded sections were placed inside a hot air oven at 65°C overnight, then dipped in xylene and ethanol containing jars in the order of Xylene for 5 min, Fresh xylene for 5 min, absolute ethanol for 5 min, fresh absolute ethanol for 5 min, 95% ethanol for 5 min, and 70% ethanol for 5 min. Slides were washed in running water for 5 min, then drained and blotted gently. 100 µl of a protein-blocking reagent were placed onto the section and incubated for 10 min in a humid chamber at room temperature. Then slides were drained and blotted gently. In the alternative staining method, 100 µl of H_2O_2 of 3% concentration were added and protein blocking reagent at the same step. 100 µl of diluted primary antibody were placed onto the section and incubated for 1 h at 37°C in a humid chamber. After incubation, the slides were drained and blotted gently. Slides were rinsed with 1× rinse buffer for a minimum of 15 s, then drained and blotted gently. 100 µl of alkaline phosphatase or diluted peroxidase secondary antibody were placed onto the section and incubated for 10 min at 37°C in a humid chamber. Slides were drained and blotted gently. In the alternative method, diluted peroxidase was used instead of alkaline phosphatase. Slides were rinsed with 1×rinse buffer for a minimum of 15 s, then drained and blotted gently. 100 µl of streptavidin-alkaline phosphatase conjugate (this step is not done in DAB staining method) were placed onto the section and incubated for 10 min at 37°C in a humid chamber. Slides were drained and blotted gently. Slides were rinsed with 1× rinse buffer for a minimum of 15 s then drained and blotted gently. 200 µl of chromogen reagent were applied to the spot in a dark place for 10 min at room temperature (or fresh DAB solution). Slides were washed in running water for 5 min and then drained and blotted gently. The spot was stained by 100 µl of counter (haematoxylin) stain which was placed onto the spot and incubated for 30 s at room temperature, after which slides were drained gently. Slides were washed in distilled water then drained and cleaned gently. A drop of mounting medium (DPX) was placed onto the wet spot and the spot quickly covered with a cover slip and thereafter, slides were let to dry. Slides were examined by light microscope at ×400 magnification. Immunostaining was scored

according to the cut-off value. This cut-off for positivity was that either any trophoblast villous containing *Toxoplasma gondii* is considered to be positive or for caspase 8, the extent of immunostaining in the villi was determined in 10 high power fields (x 200 magnification). The total number of villi was counted and the extent of circumferential cytoplasmic staining and syncytio-trophoblast in a given villus was determined as a percent. The total staining score was divided by the number of whole villi per high power field in 10 fields (Black et al., 2004).

Interpretation of the results

Immunophosphatase secondary detection system

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

Peroxidase secondary detection system

The presence of a brown reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain was pale to dark blue coloration of the cell nuclei. Non-specific staining, where present, was of a diffuse appearance. Sporadic light staining of connective tissue was also observed in sections from excessively formalin fixed tissues. Intact cells were used for interpretation of staining results. Necrotic or degenerated cells often stain non-specifically. Patient or test specimens stained with the primary antibody was examined last. Positive staining intensity was assessed within the context of any non-specific background staining of the negative reagent control. The absence of a specific positive staining reaction was interpreted as no antigen detected.

Calculation of sensitivity and specificity of immunohistochemistry

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

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

Where, a, true positive; b, false positive; c, false negative; d, true negative.

RESULTS

Frequency of number of miscarriages

Within the patient control group, it was observed that the highest percentage (57.69%) of patients was among those who had no previous miscarriages and to lesser percentage (26.92%) those that had a previous single miscarriage (Figure 2). Within the positive group, it was found that the highest percentage (57.69%) was of those patients who had no previous miscarriages and to a lesser percentage (26.92%) those that had a previous single miscarriage (Figure 3). Among the control group (induced abortion group), all the six cases had no previous abortions.

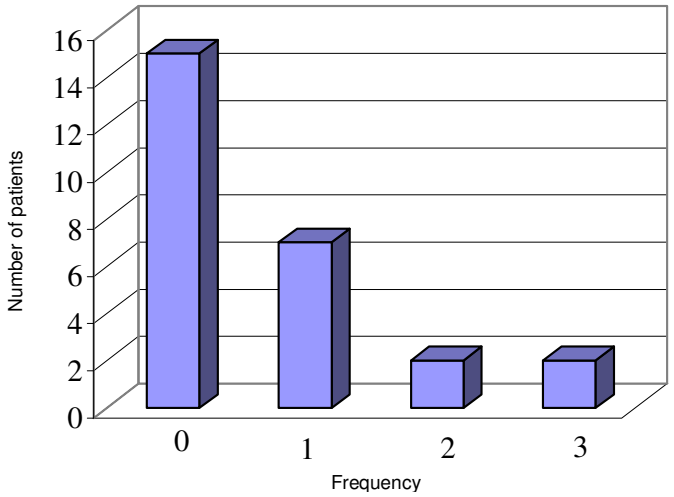

Figure 2. Frequency of previous number of miscarriages within the patient control group.

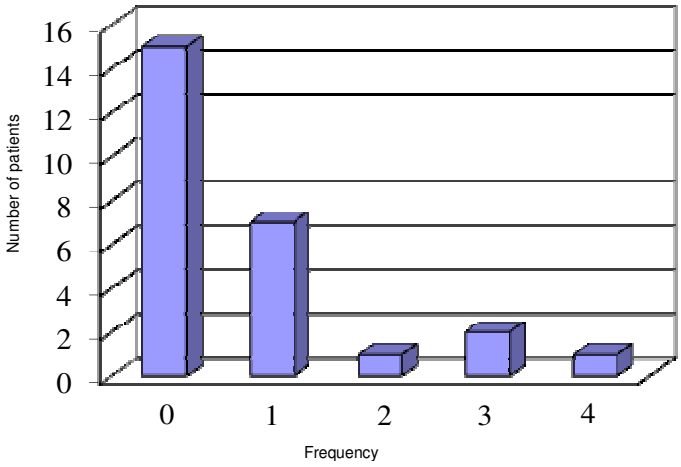

Figure 3. Frequency of previous number of miscarriages within positive group for *T. gondii*

Frequency of gestational weeks

The highest frequency within the positive group for *T.gondii* was 12 gestational weeks (41%), 8 and 16 gestational weeks (15%), 10 gestational weeks (12%), 20 gestational weeks (8%), 14 and 15 and 24 gestational weeks (4%) (Figure 4). The frequency of gestational weeks was between 10-12 weeks within the control group.

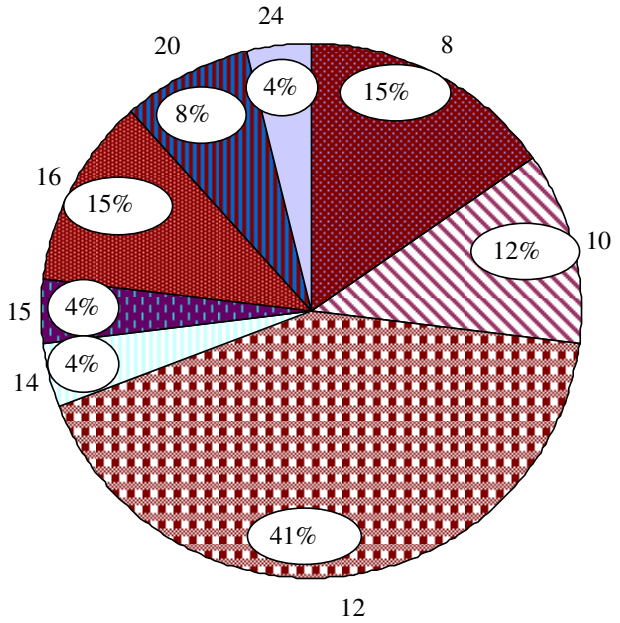

Figure 4. Frequency (numbers within the circle) of gestational weeks (numbers outside the circle) in the positive group for *T. gondii* (26 patients) among women with miscarriages.

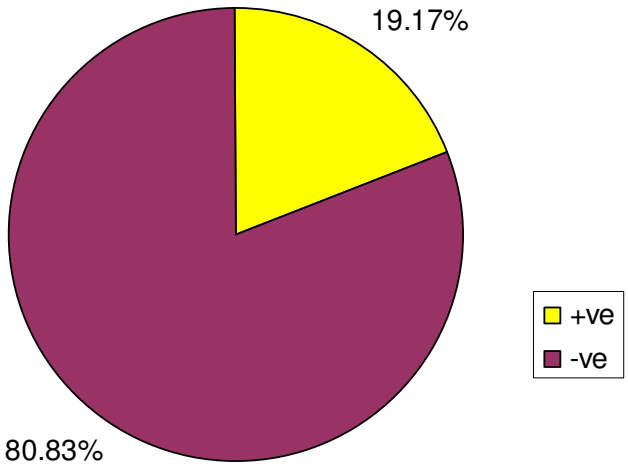

Figure 5. Percent of positive and negative cases for IgM against *T. gondii* by ELISA test in 120 cases of miscarriages.

The sensitivity and specificity of ELISA and immunohistochemistry (IHC) for *T.gondii* among women with miscarriage

The results show that 23 of 120 women (19.17%) (Figure 5), have IgM antibodies against *T. gondii* by ELISA method, while 26 of 120 women (21.67%) (Figure 5) have *Toxoplasma* antigen within the trophoblastic tissue by IHC method. The sensitivity and specificity of ELISA kit and IHC kit had been calculated and presented as 100 and 88.46% sensitivity of IHC and ELISA methods, respectively and 96.91and 100% specificity of IHC and ELISA, respectively (Table 1).

Table 1. 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 miscarriages.

Test	IHC (%)	ELISA (%)
Sensitivity	100	88.46
Specificity	96.91	100

IHC, Immunohistochemistry; ELISA, enzyme linked immunosorbent assay.

Percentage of caspase 8 among the three groups

A highly significant difference ($p<0.01$) was found between the percentage of caspase in those who were positive for *T. gondii* and the control group (Table 2). A highly significant difference ($p<0.01$) was found between the percentage of caspase in the patient control group and the control group (Table 3). No significant difference ($p>0.05$) was found between the percentage of caspase in those who were positive for *T. gondii* and the patient control group (Table 4). The highest percent of caspase 8 was within the positive group then lower than this within the patient control group and the lowest percentage was within the control group. It should be noted that the mean percentage of caspase 8 in those three cases that were negative by ELISA but positive by IHC were statistically higher than those who were true negative by both methods. A point worth mentioning was that there were two cases negative by ELISA and immunohistochemistry, the results of the percentage of caspase 8 of which were similar to the true positive.

Correlations within the positive group

Regarding the parameters (number of abortions, gestational age, age of patient, caspase 8) within the positive group, no statistical significant correlation was found between them (Table 5).

DISCUSSION

To the best of our knowledge, the present study is the first locally conducted immunohistochemical study to determine *Toxoplasma* antigen in trophoblastic tissue, and the use of the caspase 8 as a parameter that reflects the apoptotic process. *T. gondii* infects a large proportion of the world's population but uncommonly causes clinically significant disease. However, certain individuals

Table 2. Percentage of caspase 8 in patients positive for *T.gondii* and the control group.

Enzyme	Positive			Control group			Significance level
	Mean±SE	Median	Range	Mean±SE	Median	Range	
Percentage of caspase 8	30.29±3.06	32	5-60	16.17±3.4	17.5	5-25	$p< 0.0078$ *

*,significant difference; SE, standard error.

Table 3. Caspase 8% in patient control group and control group.

Enzyme	Patient control			Control group			Significance level
	Mean±SE	Median	Range	Mean±SE	Median	Range	
Percentage of caspase 8	33.73±3.08	31.5	10-66	16.17±3.4	17.5	5-25	$p < 0.0017$*

*Significant difference; SE, standard error.

Table 4. Percentage of caspase 8 in patients positive for *T.gondii* and patient control group.

Enzyme	Positive			Patient control			Significance level
	Mean±SE	Median	Range	Mean±SE	Median	Range	
Percentage of caspase 8	30.29±3.06	32	5-60	33.73±3.08	31.5	10-66	$p>0.05$

SE, standard error.

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

Parameter		Number of abortions	Gestational age (weeks)	Age (years)	Percentage of caspase 8
Number of abortions	r	1.0000	-0.0892	-0.1151	-0.1305
	p	0.0000	0.665	0.576	0.525
Gestational age (weeks)	r		1.0000	-0.0746	0.1404
	p		0.0000	0.7170	0.4940
Age (years)	r			1.0000	0.1252
	p			0.0000	0.5420
Percentage of caspase 8	r				1.0000
	p				0.0000

are at high risk for severe or life-threatening disease due to this parasite. Individuals at risk include fetuses, newborns, and immunologically impaired individuals (Hökelek, 2005).

The frequency of toxoplasmosis

The frequency of toxoplasmosis among women with abortion in the current study was found to be 21.67% (26 positive out of a total of 120), using the immunohisto-chemical analysis for the detection of antigen. However, using the ELISA technique for the detection of specific IgM, the frequency was found to be 19.17% (23 positive out of a total of 120). Abbas (2002) showed that 21.5% of women with first abortion had positive IgM by ELISA, while for women with no abortion, the positivity rate were 7.5%, which was comparable to the current results. Another study in Iraq was done by Al-Khafajy (2004), which demonstrated that 43.7% of women with abortion have positive IgM by ELISA. This relatively high percent when compared to the current 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 who had abortion and with suspicion of toxoplasmosis during pregnancy (by history and physical examination). Therefore, this type of selection might reflect this high percentage.

In Iraq, 22.06% of the total population is infected with *T. gondii* (www. WrongDiagnosis.com™, 2005). If this figure is compared to the current study which is (21.67%), then the results of the current study could be considered to be high because 22.06% represents toxoplasmosis among the total population with its different age groups and distribution (rural and urban areas) and for both sexes (males and females), while in the current study only a single sex, adult age, and small sample of the community had been studied (women with miscarriage). The relatively high frequency of this disease for patients with miscarriage in the current study 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. The rural citizens have habits in favor of acquiring toxoplasmosis by eating unwashed raw vegetables or unpadded fruits. In addition, the close contact with cats and the consequent exposure to sporulated oocysts by ingestion of these oocysts from cat feces that contaminates soil (during farming), or eating under cooked meat contaminated with cysts (Hökelek, 2005; Holland, 2003). It is worthwhile mentioning that in countries like France, where raw meat is popular, the prevalence may be higher. In Paris, for example, it may be as high as 50% (Roberts and Janovy, 1996). Prevalence rate of toxoplasmosis is approximately 1 in 4 or 22.06% or 60 million people in USA (www. WrongDiagnosis.com™, 2005). Because *T. gondii* can be transmitted from a recently infected mother to her fetus, a rapid and accurate diagnosis of the infection is critical for establishing proper clinical care (Li et al., 2000). When a pregnant woman is found to be infected with *T. gondii*, the next step is to determine whether the fetus is infected. Physicians most often use polymerase chain reaction (PCR) testing of amniotic fluid to diagnose congenital toxoplasmosis (Hohlfeld et al., 1994; Foulon et al., 1999).

ELISA versus IHC

ELISA method for detecting IgM anti-*Toxoplasma* antibody in the serum and the immunohistochemistry method for 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 a polymerase chain reaction (PCR), isolation of the organism, histology, or by some combination of the above (Remington, 2001). In addition, diagnosis based on clinical appearance and serology is not always easy. However, molecular methods do not depend on an immune response, and allow direct detection of the parasite in biological samples. Thus, they can be used to establish a diagnosis when serological tests are not definitive (Switaj et al., 2005). *T.gondii* infection is seen in the villous trophoblast from placentas associated with congenitally infected infants (Fox, 1993). Congenital toxoplasmosis may result in abortion, stillbirth, mental retardation, and retinal damage (American Medical Association, 2004). This issue was demonstrated in this current study by detecting *Toxoplasma* antigens within the trophoblastic cells from patients who had abortion, by using immunohistochemistry method. It is note worthy to mention that the sensitivity of many diagnostic tests is improved if immunohistochemical studies are employed (Holliman, 1996). In fact in the current study, two diagnostic methods were used, the ELISA for the detection of specific IgM and the immunohistochemical analysis for the detection of antigen. The sensitivity of the ELISA test was found to be 88.46% which was improved to 100% by the immunohistochemical analysis.

The combination of these two tests may put the physician on solid ground concerning the status of the patient. Immunohistochemistry is more reliable as a diagnostic test because of the detection of the antigen regardless of its virulence or its ability to stimulate antibody production and because of the fact that the presence of different strains, investigating the strain differences in Iraq is worth considering reflecting the reality of the current results. For further studies, comparison of the result of the current study with PCR testing of amniotic fluid which was found to be safer and more sensitive than fetal blood sampling (Foulon et al., 1999), and allows earlier confirmation of fetal infection (Hohlfeld et al., 1994; Li et al., 2000) is worth considering. However, false-positive and false-negative tests may occur with PCR tests (Remington, 2001).

Immunoglobulin M (IgM), IgG, or both

The ELISA method for the detection of IgM anti-*Toxoplasma* antibodies was chosen in this study and not IgG because acute toxoplasmosis usually is diagnosed on the basis of IgM antibody detection. In acute infections, IgG and IgM antibodies levels generally rise within one to two weeks of infection (Montoya and Remington, 2000). The presence of elevated levels of *T. gondii* specific IgG antibodies indicates that infection has occurred but does not distinguish between recent infection and infection acquired in the distant past. Detection of *T. gondii* specific IgM has been used as an aid in determining the time of infection. A negative IgM test result with a positive IgG result usually indicates infection at least six months previously. However, the interpretation of *T. gondii* specific IgM-positive result is complicated by the persistence of IgM antibodies up to 18 months after infection (Wilson and McAuley, 1999), and by false-positive reactions in commercial tests. IgM-

positive test results should be confirmed by a *Toxoplasma* reference laboratory (Wilson et al., 1997). The laboratory may also be able to narrow the time of infection through the use of specific tests, for example, IgG avidity test (Jenum, 1972) or a serologic profile for example, Sabin-Feldman dye test, IgA ELISA, IgE ELISA, or differential agglutination (Liesenfeld et al., 2001). Nevertheless, the detection of the antigen in the tissue was considered the confirmatory method in this study. Regardless of the cost of this test, it was found to be 100% sensitive when compared to the ELISA. The reason for misdiagnosing the three cases by ELISA in this study could be due to the fact that IgM might be below the detection level of the kit used (very early in the disease or a period of switching from IgM to IgG) (Wilson and McAuley, 1999).

Toxoplasmosis during pregnancy

For the entire cases positive for *T. gondii* in the current study, their pregnancy ended with abortion. This issue was investigated where levels of many sex hormones, most notably estrogens and progesterone, are vastly increased during pregnancy, and consequently their effects on the immune system can be profound. The normal physiological role of these changes would appear to be to protect the developing fetus from the mother's immune response. Although this hormonal manipulation of the immune system serves to prevent the fetus from being rejected, it also has consequences for parasitic infection. The ability of pregnancy to affect the immune system and indeed of the immune system to affect pregnancy has two important consequences for parasitic infection. First, pregnancy will favor the survival of many parasites that require a type 1 response to control them. Second, parasitic infections that induce a strong type 1 response will adversely affect pregnancy. Both of these scenarios have been demonstrated with the protozoan parasites *T. gondii* and *Leishmania major* (Roberts et al., 2001).

Is there any relation between the gestational age and the infection by *T. gondii* ?

In the current study, the highest percent of abortion was found to be in the first trimester (12 gestational weeks and below) for those who were positive using monoclonal antibodies against *T. gondii* antigen in tissue. This coincides with the fact that the rate of clinical pregnancy loss is known to decrease with gestational age from 25% at 5-6 weeks to 2% after 14 weeks (Campbell and Monga, 2000). Moreover, this result coincides with the issue that 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 or during vaginal delivery. 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. If the mother acquires infection in the third trimester and the infection is not treated, approximately 65% of fetuses are infected, and involvement is mild or unapparent at birth. These different rates of transmission are most likely related to placental blood flow, the virulence of the infecting strain, amount of *T. gondii* acquired, and the immunologic ability of the mother to restrict parasitemia (Hökelek, 2005). Moreover, if infection occurs in the first trimester, when hormone levels are low and there is little Th2 bias, the chance of transmission to the fetus is low, although the chance of abortion is high. Conversely, infection during the third trimester, when there is a strong Th2 bias, is unlikely to induce abortion but more frequently results in congenital transmission. There is every likelihood that the Th1 response induced early during *T. gondii* infection will induce abortion early in pregnancy. In contrast, during the late stages of pregnancy, the strong Th2 bias and the diminished NK cell, macrophage, and $CD8^+$ T-cell function may facilitate parasite survival and increase the likelihood of congenital transmission (Roberts et al., 2001). However, studies indicate that induction of a strong type 1 cytokine response at the fetal-maternal interface may also result in rejection of the fetus. Thus, such a response could contribute to spontaneous abortion during acute toxoplasmosis in a pregnant female (Denkers and Gazzinelli, 1998).

Is caspase 8 important to the trophoblastic tissue?

In the current study, the caspase 8% was measured and it was estimated in all the three groups with different rates of expression according to status of the case (whether infected or not) that is, in all conditions, caspase 8 is expressed in the trophoblastic tissue. This finding goes with the results of Black et al. (2004) in that in the villous trophoblast, the events may act as prerequisites for fusion, with the need for other cell type-specific molecules to be directly involved in the fusion process of trophoblast. The role of caspase 8 in cytotrophoblast fusion in cultured placental tissue was studied. Antisense oligonucleotides against caspase 8 and peptide inhibitors against the active caspase 8 protein were utilized to test the hypothesis that expression and activity of caspase 8 are required for villous cytotrophoblast fusion. The data demonstrate that the down regulation of caspase 8 protein expression as well as the inhibition of caspase 8 protein activity reduce the level of syncytial fusion, and result in an accumulation of multilayered mononucleated cytotrophoblast rather than a functional syncytial layer (Black et al., 2004). However, in the current study, the caspase 8% is highly expressed in *T.gondii* infected trophoblastic cells. This may suggest that caspase 8 in

those cells was increased as a consequence of the apoptotic process undergone by those cells. As a fact, caspases influence differentiation of a variety of cells (Ishizaki et al., 1998; De Maria et al., 1999; Pandy et al., 2000). An important point should be mentioned regarding caspases, proteases of the caspase family constitute the central executioners of apoptosis. Several recent observations suggest that caspases and apoptosis-regulatory molecules exert important functions beyond that of cell death, including the control of T-cell proliferation and cell-cycle progression (Los et al., 2001). This function is not well understood but mentioning such a point obliges the researcher to think in different directions and not become restricted to what is proved by ancestors only.

In the current study, does *T. gondii* overcome the host?

The highest percentage of caspase 8 was detected within the *T. gondii* infected trophoblastic cells in comparison to *T. gondii* uninfected trophoblastic cells (*T. gondii* induces apoptosis within the infected cells). However, caspase 8 positive cells cannot be considered as only the trophoblastic cells can be related to the inflammatory cells invading the area (Gavrilescu and Denkers, 2003). At the same time, the other remaining infectious agents of STORCH that were not investigated (S, *Syphilis*; T, *Toxoplasmosis*; O, Others which are Bacterial *vaginosis*, *Trichomonas vaginalis*, Group B Streptococci, *Escherichia coli*, *Ureaplasma urealyticum*, *Haemophilus influenzae*, Varicella, *Listeria monocytogenes,* R; *Rubella*, C; *Cytomegalovirus*, H[5]*; Herpes*, HIV, Hepatitis B, Human papillomavirus, Human parvovirus) cannot be ruled out, since all these infectious agents induce a shift of the immune response during pregnancy from Th2 to Th1 and apoptosis which can be observed clinically as an abortion process (Campbell and Lees, 2000). The three cases which were negative by ELISA and positive by IHC had their figures of caspase 8 comparable with the true positive results by both methods. This raises the suspicion that those cases might be false negative by ELISA method. The percentage of caspase 8 were estimated within the induced abortion group and found to be the least among the three groups studied currently. This may be because in the induced abortion group, their trophoblastic tissue is normal (the immune response is similar to the immune response of the normal pregnancy). In case of induced abortion, there is absence of inflammatory stimuli which lead to triggering of apoptosis, so the microenvironment is not suitable for induction of apoptosis (which can be detected by the percentage of caspase 8) (Parslow et al., 2001).

***T. gondii* and apoptosis**

Several contrasting views regarding the modulation of apoptosis following *T. gondii* infection exist that is, *T. gondii* either inhibit or trigger the apoptosis (Nishikawa et al., 2002). In the current study, the level of caspase 8 was higher in *T. gondii* infected cells more than the uninfected cells. This finding goes with the fact that high levels of host cell apoptosis is associated with several protozoan infections including *T. gondii*, particularly among immune cells (Gavrilescu and Denkers, 2003). At the same time, this may give an impression that one might be dealing with a virulent strain of *T.gondii* despite of not knowing which strain one is dealing with (Gavrilescu and Denkers, 2001; Mordue et al., 2001). Apoptosis, resulting from inflammatory cytokine overproduction, is associated with high-virulence *Toxoplasma* strain but not with low-virulence *Toxoplasma* strain. *T. gondii* strains are remarkably clonal, and virulence in the mouse model is strongly influenced by the genotype of the parasite. Studies demonstrate that the ability of type I strains to uniformly cause lethal infections in mice was not due to direct destruction by the parasite. Instead, infections with the type I RH strain were characterized by their rapid ability to reach tissue burdens associated with lethality ($\sim 10^6$ parasites/g tissue). In contrast, low dose infections with the type II PTG strain were delayed in reaching this threshold, and lethality was only observed with a higher initial inoculum. Unexpectedly, lethal infections were associated with over induction of inflammatory cytokines rather than an insufficient immune response. Lethality was associated with excessive levels of Th1 cytokines, particularly IL-18 and IFN- γ, in the serum. Findings indicate that acute virulence in *T. gondii* is associated with over stimulation of Th1 cytokines, which paradoxically are also required for protection. Following primary infection, type I and type II strains of *T. gondii* did not differ substantially in their ability to disseminate *in vivo* or to reach similar tissues. However, a key property of the RH strain of *T. gondii* is the dramatic increase in serum cytokine levels after low dose challenge, whereas the PTG strain required a much higher challenge dose (10^5 parasites). This difference may be due to the ability of a low dose of RH strain parasites to rapidly reach tissue levels of $\sim 10^6$ parasites/g tissue (within 4 days), whereas PTG strain parasites only reached this level at 6–8 days post infection. This difference was overcome by administering a high initial dose of PTG strain parasite, resulting in a faster increase in tissue burdens and a lethal outcome. Collectively, these results suggest that the early interaction of the parasite with the innate immune response is critical to triggering the lethal cascade of cytokines. The basis for the dramatic difference in virulence between type I (RH) and II (PTG) strains of *T. gondii* is presently unknown, but is probably due to the 1–2% underlying genetic differences between these strain types (Howe and Sibley, 1995). It should be noted that in this current study the detection by IHC was for RH strain. Another study showed that virulence in *T. gondii* is strongly influenced by the genotype of the parasite. Type I strains uniformly cause rapid

death in mice regardless of the host genotype or the challenge dose. In contrast, the outcome of infections with type II strains is highly dependent on the challenge dose and the genotype of the host. The findings establish that over stimulation of immune responses that are normally necessary for protection is an important feature of acute toxoplasmosis (Mordue et al., 2001). Some studies reveal that the neighboring non-infected cells by *T. gondii* in the same tissue undergo apoptosis. Lethal toxoplasmosis also induced extensive necrosis/apoptosis of non-infected cells within lymphoid tissues. Apoptosis was selective for α βT cells and NK cells, and was predominantly mediated through TNF-α, as shown by the significant reduction in apoptotic cells in TNFR KO mice challenged with RH strain of *T. gondii*. It has previously been reported that direct infection by *T. gondii* prevents activation of apoptosis within the parasitized host cell (Nash et al., 1998). Nonetheless, splenic $CD4^+$ T cells isolated from mice challenged with high doses of ME49 (PTG) strain parasites undergo apoptosis *in vitro*, resulting in diminished immune responsiveness (Khan et al., 1996). Collectively, these studies indicate that infection by *T. gondii* protects the resident cell from apoptosis, but cell death is efficiently activated in non-infected cells. In contrast, during acute toxoplasmosis, apoptosis occurred only late in infection. Combined with the similar outcome of infection in TNFR KO mice, which succumb at the same rate despite exhibiting less apoptosis, these findings indicate that apoptosis is probably a secondary consequence of pathogenesis during toxoplasmosis rather than causal (Hotchkiss et al., 1999).

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