TLR2 AND TLR4 PARAMETERS LEVELS IN TOXOPLASMA-SEROPOSITIVE WOMEN WITH A HISTORY OF RECURRENT SPONTANEOUS ABORTION IN BABYLON CITY

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Abstract

Toxoplasma gondii (T. gondii) is an obligate intracellular parasite causing toxoplasmosis that is one of the most common infections of animals and human. This parasite has the ability to cross the placenta to the fetus during pregnancy causing congenital toxoplasmosis. Toll-like receptors (TLRs) recognize pathogen-derived molecules and influence immunity to control parasite infections. TLRs are important in mediating immune responses against various pathogens during pregnancy. However, uncontrolled TLR-triggered inflammation will endanger normal pregnancy, resulting in pregnancy loss. This study was aimed to investigate the alterations in the levels of TLR2 and TLR4 in the sera of the recurrent miscarriage women with toxoplasmosis and compare them with the results of age matched healthy women as control group. It also aimed to evaluate any correlations between TLR2 and TLR4 levels and miscarriage women age. Sixty patients with positive anti-T gondii (IgG) antibodies and 28 healthy individuals were included in this study. The level of TLR2 was measured using enzyme-linked immunoassay (ELISA) technique. The level of TLR2 was (56.55±7.395 ng/ml) while in control group was (57.985±6.18 ng/ml) and the level of TLR4 was slightly increase (6.322±4.84 ng/mL) compare with (6.045±5.40 ng/mL) of control group, but this increasing was non-significant. The level of serum TLR2 significantly decrease in women with age (34-43 years) it was (57.03±6.522 ng/ml) in compare with control group (66.07±3.177. In conclusion, the results indicated that the levels of TLR2 and TLR4 may have an important role to increase possibility of exposure to toxoplasmosis in women, because these receptors are believed to be important for immune responses against pathogens, therefore Women should be encouraged to perform tests for TLR2 and TLR4 parameter.

Key words: Toxoplasma gondii, miscarriage women, TLR2, TLR4.

Introduction

Toxoplasmosis is a parasitic disease that caused by Toxoplasma gondii is an obligate intracellular protozoan parasite that presents high rates of gestational and congenital infection worldwide being therefore considered a public health problem and a neglected disease (Saadatnia and Golkar, 2012). Toxoplasmosis is an important cause of miscarriage or adverse fetal effects such as neurological and ocular diseases and may also lead to late sequelae in the life of the infected newborn (Kravetz and Federman, 2005).

Most pregnant women (>90%) with acquired T. gondii infection do not experience obvious signs and symptoms and spontaneous recovery is the rule. Only a small proportion will develop clinical signs of the disease. The clinical presentation in pregnant women is not more severe than in non-pregnant women and most often occurs as an influenza-like illness (low-grade fever, malaise, lymphadenopathy), with an incubation period of 5 to 18 days following exposure. Pregnant women will rarely show visual changes due to toxoplasmic chorioretinitis. In immuno compromised pregnant women, T. gondii can cause severe encephalitis, myocarditis, pneumonitis, or hepatitis via acute infection or reactivation of a latent infection (Lappalainen and Hedman, 2004).

Transmission to the fetus occurs predominantly in
women who acquire their primary infection during pregnancy. Congenital transmission, in certain rare cases, has been detected in chronically infected pregnant women whose infection was reactivated because of their immunocompromised condition. Maternal-fetal transmission occurs between 1 and 4 months following placental colonization by tachyzoites. The placenta remains infected for the duration of the pregnancy and therefore may act as a reservoir supplying viable organisms to the fetus throughout pregnancy (Abolghasem et al., 2011).

Toll-like receptors (TLRs) may play important roles in the immune regulation of the female reproductive tract. TLRs, especially the surface ones, TLR2 and TLR4 have gained immense importance due to their extreme ability of identifying distinct molecular patterns from invading pathogens. These pattern recognition receptors (PRRs) not only act as innate sensor but also shape and bridge innate and adaptive immune responses. In addition, they also play a pivotal role in regulating the balance between Th1 and Th2 type of response essential for the survivability of the host. TLR2 and TLR4 have an important role in the immunity against T. gondii previous studies commonly showed the role of TLR2 and TLR4 in several pregnancy disorders, including premature rupture of membranes (PROM), bronchopulmonary dysplasia and preterm labor (Wujcicka et al., 2013; Rey et al., 2008).

Toll-like receptors 2 and Toll-like receptors 4 were identified as secondary factors that can also affect the risk of miscarriage, therefore this study aimed to determine the TLR2 and TLR2 immunological parameters in recurrent miscarriage women which infected with Toxoplasma gondii.

Materials and Methods

The current study was conducted in the Department of Microbiology and Biochemistry, College of Medicine, University of Babylon. The study period extended from April 2019 to end of February 2020.

Patients

One hundred and seventy five (175) aborted women were selected randomly to determine the role of T. gondii in their abortions. Patients were from Obstetrics and Gynecology Clinic, Emergency and consultant for the obstetrics and gynecology hospital and Al-Hilla surgical hospital in Babylon city, Iraq. The age of patients ranged between (14-43) years old. Blood samples was taken from patients and control, Control group, those included 28 apparently healthy women without past or present history of spontaneous abortion and not suffer any disease. A general information about each patient were recorded, included age, living area, main job, number of children, number of miscarriage, week of miscarriage and level of education.

Sample Collection

Five milliliter blood was collected from miscarriages women in the first and second trimester of pregnancy. Serum was separated immediately and divided into two parts. The first part was stored at – 20ºC until assayed for level of to determine some immunological parameters such as TLR2 and TLR4 the second part of serum was used immediately for detection of antibody.

Detection of Toxoplasma Antibody

The miscarriage women participating in this study were classified according to age into three groups:

- First Group: miscarriage women (n=20) aged 14-23, Control (n=16).
- Second Group: miscarriage women (n=27) aged 24-33, Control (n=9).
- Third Group: miscarriage women (n=13) aged 34-43, Control (n=3).

Toxoplasmosis usually is diagnosed on the basis of antibody detection. The test was done according to Stango et al., (1985). All sera samples were screened for T. gondii IgG and IgM antibodies by using rapid diagnostic immunochromatographic test (ToxIgG/IgM Rapid Test Cassette) according to the manufacturer’s instructions (Egyptian company for Biotechnology, Egypt).

Determination of TLR2 parameter

ELISA kit uses the Sandwich-ELISA principle. The micro ELISA Aplate provided in this kit has been pre-coated with an antibody specific to Human TLR-2. Samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human TLR-2 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TLR-2, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of (450) nm± (2) nm. The OD value is proportional to the concentration of Human TLR-2. The concentration of Human TLR-2 in the sample can calculate by comparing the OD of the samples to the standard curve.
Determination of TLR4 parameter

ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human TLR4. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human TLR-4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TLR-4, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of (450) nm+- (2) nm. The OD value is proportional to the concentration of Human TLR-4. The concentration of Human TLR-2 in the sample can Calculate by comparing the OD of the samples to the standard curve.

Statistical Analysis

Data were analyzed by using Statistical Analysis System (SAS)-2012 to study the effect of different factors in different parameters which were used in this study. Least significant difference test (LSD) (p value < 0.05) was used to compare between means of different groups in this study (SAS, 2012).

Results and Discussion

Distribution of T. gondii Infection in Recurrent Miscarriage Women

The current study included 175 miscarriage women who were enrolled and screened for the presence of anti-toxoplasma IgG and IgM antibodies, plus 28 natural healthy women (who did not suffer miscarriage) as control group. Both case group and control group are involved in the sample of the study. Toxoplasmosis infection appears to affect pregnancy in infected group compared with control group.

The seroprevalence of *Toxoplasma gondii* IgG and IgM antibodies result revealed that 60 (34.28%) of 175 miscarriage women were positive for anti-toxoplasma-antibody IgG and 115(65.71%) of 175 were negative for anti-toxoplasma-antibody-IgG, while revealed that there was no case of acute infection IgM antibody.

The results of this study confirm that the prevalence of toxoplasmosis was 34.28% when used LAT test; this result was in accordance with the results of Al-Masoudi (2015) who confirmed the infection rate was 49.7% when they used LAT method as a screening test for the detection of toxoplasmosis. LAT provides an excellent format for routine serological screening because of its high specificity, low cost and easy to use.

The result of this study was agree with other such as study in Kirkuk, Iraq, proportion of the examined patients’ sera showed evidence of infection (IgG), whilst no have evidence of current infection (IgM) (Kadir et al., 2013).

In present study the highest seroprevalence was in anti- *T. gondii* IgG, this is due to that in *T. gondii* infection the maturation of antibody (*T. gondii* IgG) in response to infection generally are slow (Al-masoudi, 2015).

However, seroprevalence of toxoplasmosis in the present study was similar to many studies around the world and some Arabic countries in women with Bad Obstetric History (BOH). Although, the result of present study is agreement with other studies in Iraq, that were 37.5% for IgG in Erbil (Anwar and Nuha, 2017). While, in Diyala province found high infection were 37 (46.26%) women out of eighty were infected with toxoplasmosis (Kadhim, 2014). A recent study in Iraq showed a different result than present study in which the ratio of infected female with toxoplasmosis was 330 (98.51%) (Saheb, 2018).

None of the women in our study were positive for Toxoplasma specific IgM antibodies, indicating that no one has acute infection, This is in accordance with the study done by (AL-Masoudi et al., 2018; Senthamarai et al., 2013; Ebad et al., 2011) who reported *Toxoplasma gondii* seroprevalence of 13.8%, 15.67% and 16.47% respectively. But this our result is far to those reported in study in Diyala, Iraq showed that 44(44%) were positive (IgG&IgM) which considered as a confirmed toxoplasmosis cases (Darweesh et al., 2018) and the study in Baghdad city shows that the median values of IgM –Toxoplasma antibodies titer was statistically significant. It is higher in the group of pregnant women with positive diagnosis (1.9±0.850), compared to those with negative diagnosis of toxoplasmosis (0.41±0.315) (Ali, 2016).

<table>
<thead>
<tr>
<th>Anti-Toxoplasma antibodies</th>
<th>Examine NO.</th>
<th>Positive NO.</th>
<th>Negative NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>175</td>
<td>60</td>
<td>115</td>
</tr>
<tr>
<td>IgM</td>
<td>175</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgM+IgG</td>
<td>175</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1: Distribution of anti-*T gondii* IgG and IgM antibodies for aborted woman using rapid immunochromatographic method.
This relatively high percent of toxoplasmosis, in current study may be due to many factors including the sample size which was only 175 This indicates that is considerable number of females in this society harboring the parasite, transmitter to other people and it is representing a real problem that should not be neglected and must receive attention from health authorities.

In acute infections of toxoplasmosis, IgG and IgM antibodies levels generally rise within one to two weeks of infection (Montoya and Remington, 2000). The presence of raised levels of T. gondii specific IgG antibodies specifies that infection has arose but does not discriminate between recent infection and infection learned 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 Auley, 1999).

The high prevalence of this disease in Iraq could be due to high number of risk factors and many sources of infection. The role of health education is an important factor in decrease the incidence of this infection.

The level of TLR2 and TLR4 was measured in miscarriage women with T. gondii positive sera and then compared with their levels healthy women sera to identify the effect of T. gondii infection on the serum TLR2 and TLR4 level.

Detection of TLR2 and TLR4 concentration levels among one hundred and seventy five miscarriages women were tested; sixty of them was with miscarriages due to toxoplasmosis and Twenty-eight apparently healthy women (with out of any miscarriages) were enrolled as a control group. In the table 2 showed that In miscarriage women infected with T. gondii the mean TLR2 level was (56.055 ± 7.395 pg/ml) in test group, the mean TLR2 value was (57.985 ± 6.18 pg/ml) in control group (healthy women). The difference between these values is non-statistically significant (Sig. Value = 0.234).

Values of TLR4 in the table 2 showed slightly increasing it was (6.322 ± 4.84) in (60) miscarriage women with T. gondii, in (28) healthy women mean value of TLR4 was (6.045 ± 5.408U/ml). The difference between these values is non-statistically significant (Sig. Value = 0.8102).

The observation of this study agree with Ihara et al. (2019) in Japan who showed that The interaction between T. gondii infection and TLR2 deficiency was not statistically significant, also study of Sereshki et al., (2019) in Isfahan, Iran showed that The flow cytometry results showed no significant differences in the percentage of TLR2 expression miscarriage women and control group.

Many a previous study about a role TLR2 in spontaneous abortion such as study of Razdaibiedina et al., (2018) in Ukraine who suggest that TLR2 SNPs are associated with imbalance in the system of innate immunity and, as a result, an increase in mother’s organism sensitivity to the infections and miscarriage risk, also study in Malaysia suggest that TLR2 plays a role innate immunity in bacterial and viral infection in the placenta, However, their role in protection against toxoplasma may be limited (Hayati et al., 2010), while Hartel et al., (2004) confirmed that TLR2 play important role in abortion in women.

Study of Parthiban and Mahendra, 2015 explained that TLR2 and the risk factor caused of spontaneous abortion, while Study of Razdaibiedina et al., (2018) in Ukraine confirm that the factors that can also be considered when predicting the risk of spontaneous abortion are TLR2 and TLR4.

There are many studies for TLR4 parameter as study of Kropf et al., (2004) demonstrate that TLR4 plays a role in the host defense against parasitic protozoan. he show that after infection with L. major, TLR4 contributes to both innate and adaptive immune responses. Iram et al., (2012). In his studies to understand the role of TLRs in gaining immune proficiency, he conducted a comprehensive study to evaluate the expression of TLR function in the development of human skin before and after birth and to compare it with adults. He was found that prenatal skin already expresses the same spectrum of TLRs as adult skin.

Toll-Like Receptore 4 activation may contribute to the risk of idiopathic miscarriage by disturbing the Th1/Th2 balance at the feto-maternal interface. First, functional TLR4 is expressed by the placenta (Holmlund et al., 2002; Abrahams et al., 2004 and Kumazaki et al., 2004). Second, TLR4 activation triggers the secretion of a range of Th1 cytokines experimentally implicated in

<table>
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<tr>
<th>Table 2: Distribution of TLR2 and TLR2 among Recurrent Spontaneous Aborted and control group.</th>
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</thead>
<tbody>
<tr>
<td><strong>No.</strong></td>
</tr>
<tr>
<td>Abortive women</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td></td>
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<tr>
<td>Sig. Value</td>
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</table>
TLR2 and TLR4 Parameters Levels in Toxoplasma-Seropositive Women with a History

infertility and pregnancy failure, including TNF-α, IL-1, IL-6 and IL-8 (Chaouat et al., 2004).

The expression of TLR4 may facilitate secretion of inflammatory cytokines such as TNF-α and INF-γ; elevated expression of these cytokines in the placenta plays a crucial role in inducing trophocyte apoptosis, therefore, TLR4 may play a role in RSA pathogenesis (Saini et al., 2011).

Toll-Like Receptor 4 serves as the first point of defense in the innate immune system. TLR4 is critical in the recognition of viruses, bacteria and parasites serving as a key immune system effector. Previous studies showed the role of TLR4 in several pregnancy disorders, including premature rupture of membranes (PROM), bronchopulmonary dysplasia and preterm (Rey et al., 2008) and Rey et al., 2008).

A combined involvement of TLRs was reported in the development of T. gondii infection in humans (Andrade et al., 2013). Some participation of both TLR2 and TLR4 in triggering an immune response after T. gondii infection was observed in human macrophages. Finally TLR2, TLR4, play roles in the recognition of T. gondii, course of infections with this parasite and also in pregnancy progression and disorders.

The study also covered study the relationship between TLR2 and TLR4 levels and miscarriage women age. In the table 3 showed that In Toxoplasmosis miscarriage women with aged (14-23 years) the mean TLR2 level was 55.03±7.792 ng/mL, while in control (healthy women) was 58.65±5.673 ng/mL. The difference between these values is non-statistically significant (Sig. Value = 0.129).

In Toxoplasmosis miscarriage women with aged (24-33 years) the mean TLR2 level was 55.94±7.262 ng/mL. As for miscarriage women with aged (34-43 years) the mean TLR2 level was 57.03±6.522 ng/mL, in control the mean TLR2 value was 66.07±3.177 ng/mL. Show in this age group there was decrease in the level of TLR2 among miscarriage women compared with healthy women (Sig. Value = 0.0397).

The results of ELISA TLR4 test in seropositive of miscarriage women in different women age: 14-23, 24-33 and 34-43 years old it was 5.507±4.457 ng/mL, 7.543±5.238 ng/mL and 6.568±5.605 ng/mL respectively. While in control group it was 6.747±6.39, 4.67±4.183 and 6.427±2.453 respectively. The statistical analysis of the positive results showed that there was no significant differences among different age groups as shown in table 4.

Study by Hirschfeld et al., (2007) show that the most common indication for miscarriage is advanced maternal age.

Results of study in Baghdad showed that Investigate the association between the AG genotype of TLR-4 rs4986790 and susceptibility for toxoplasmosis that may play a role in the induction of abortion (Turkey et al., 2019). Also Wujcicka et al., (2017) show the role of genotypes, haplotypes and multiple-SNP variants in the range of TLR4 single nucleotide polymorphisms (SNPs) in the development of Toxoplasma gondii infection among Polish pregnant women. Recruited 116 Polish pregnant women including 51 patients with toxoplasmosis and 65 age-matched controls, to investigate the association of 4 polymorphisms in TLR-4 with toxoplasmosis. Helmby and Grencis (2004). Provide conclusive evidence that TLR4 plays a key pathogenic role in chronic gastrointestinal nematode (Trichurismuris) infection. While Akbal et al., (2017) detected that serum TLR-2 and TLR-4 levels increased in HBV patients with aged

### Table 3: Means± SD of TLR2(ng/mL) in the serum of Toxoplasmosis abortive women according to age group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>1st Group 14-23 years</th>
<th></th>
<th>2nd Group 24-33 years</th>
<th></th>
<th>3rd Group 34-43 years</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Abortive</td>
<td>Control</td>
<td>Abortive</td>
<td>Control</td>
<td>Abortive</td>
<td>Control</td>
</tr>
<tr>
<td>TLR2</td>
<td>Mean</td>
<td>55.03</td>
<td>58.65</td>
<td>55.94</td>
<td>54.11</td>
<td>57.03↓</td>
<td>66.07↑</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>7.792</td>
<td>5.673</td>
<td>7.262</td>
<td>4.878</td>
<td>6.522</td>
<td>3.177</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>20</td>
<td>16</td>
<td>27</td>
<td>9</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Sig. Value</td>
<td>0.129</td>
<td>0.4877</td>
<td>0.0397*</td>
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</tbody>
</table>

### Table 4: Means± SD of TLR4 (nmol/ml) in the serum of Toxoplasmosis abortive women according to age group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>1st Group 14-23 years</th>
<th></th>
<th>2nd Group 24-33 years</th>
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<th>3rd Group 34-43 years</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Abortive</td>
<td>Control</td>
<td>Abortive</td>
<td>Control</td>
<td>Abortive</td>
<td>Control</td>
</tr>
<tr>
<td>TLR4</td>
<td>Mean</td>
<td>5.507</td>
<td>6.747</td>
<td>7.543</td>
<td>4.67</td>
<td>6.568</td>
<td>6.427</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>4.457</td>
<td>6.39</td>
<td>5.238</td>
<td>4.183</td>
<td>5.605</td>
<td>2.453</td>
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<td>16</td>
<td>27</td>
<td>9</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Sig. Value</td>
<td>0.4979</td>
<td>0.1455</td>
<td>0.9674</td>
<td></td>
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</tr>
</tbody>
</table>
between 18 and 70 years old. Liver injury in CHB may cause elevated TLR-2 and TLR-4 levels.

The researcher Murray et al. (2013) showed that TLR4 actively regulate host responses in L. donovani infection in the Liver and the researcher Won et al. (2016) showed that the role of TLR4 in apoptotic cell death in gynecological cancer cells; gynecological cancer is associated with infertility and spontaneous abortion.

**References**


