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Assessing the Potentiality of Using CXCL9 as A Predictive Biomarker for Acute and Chronic Toxoplasmosis, and Study the Correlation Between CXCL9, Toxoplasmosis and Thyroid Disorder in These Cases

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Abstract

Background: Chemokine (C-X-C motif) ligand (CXCL9) has an important role recruiting the T-lymphocytes and immune response after infection by inducing T-cells accumulation around the areas associated with infections. However, this role is poorly known in relation with *Toxoplasma gondii* infection and also in association with thyroid hormones, which the present study is focused on.

Methods: Eighty-seven women were included in this study for the period between September 2021 and February 2022. Blood samples of uninfected healthy pregnant, in addition to aborted and pregnant women infected with toxoplasmosis, were collected. Sera were then obtained and stored at -10°C. Toxo-latex agglutination test was done, followed by detection of IgM and IgG antibodies, which were identified in sera of cases and controls using a commercially available enzyme-linked immunoassay. Finally, an ELISA test for CXCL9 and thyroid hormones tests were performed as well.

Results: CXCL9 levels showed a non-significant increase in comparison with the control group. This increase was observed during the first 5 months of abortion and pregnancy. Thyroid hormones T3 and TSH were significantly higher in the aborted and pregnant women than the control group. Whereas T4 results revealed lower levels in the same group in comparison to the control group, specifically during the first 5 months of abortion and pregnancy for most of the groups.

Conclusion: Determining CXCL9 chemokine levels in female patients infected with *Toxoplasma gondii* might play an important role in early diagnosis of Toxoplasmosis, especially during the first 5 months of abortion or pregnancy as well as thyroid hormones levels can be influenced or affected by the parasite infection, which can provide an indicator of thyroid dysfunction in general.

Keyword: CXCL9 Chemokine, *Toxoplasma gondii*, Thyroid hormone.

تقييم إمكانية استخدام CXCL9 كمؤشر بيولوجي تنبؤي لداء المقوسات الحاد والمزمن ودراسة العلاقة ما بين ال CXCL9 وداء المقوسات واعتلالات الغدة الدرقية في الحالات المصابة

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الخلاصة

ليجيند الكيموكاين Chemokine ligand (CXCL9) (C–X–C motif) يلعب دوراً مهماً في إجتذاب

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الخلايا اللمفاوية T–Lymphocytes والاستجابة المناعية بعد العدوى عن طريق إحداث تراكم للخلايا اللمفاويه حول المناطق المصاحبة للعدوى ؛ هذا الدور غير معروف بشكل جيد خاصة فيما يتعلق بعدوى المقوسات *Toxoplasma gondii* وعلاقة ذلك بهرمونات الغدة الدرقية والذي ركزت عليه الدراسة الحالية.

طريقة العمل: تضمنت الدراسة الحالية 87 امرأة للفترة ما بين أيلول 2021 وشباط 2022. تم جمع عينات الدم من النساء المجهضات والحوامل ثم تم الحصول على الأمصال وتخزينها في −10 درجة مئوية. بعد ذلك، تم تحديد الأجسام المضادة IgM و IgG في الأمصال المأخوذة من عينات النساء الحوامل والمجهضات المصابات بداء المقوسات Toxoplasmosis ومجموعة السيطره باستخدام مقايسة مناعية مرتبطة بالإنزيم والمتوفره تجاريًا. أخيرًا ، تم إجراء اختبار ELISA لـ CXCL9 واختبارات هرمونات الغدة الدرقية أيضًا.

النتائج: أظهرت نتائج الـ CXCL9 زيادة غير معنوية مقارنة بمجموعة السيطرة ولوحظت هذه الزيادة خلال الأشهر الخمسة الأولى من الإجهاض والحمل. كانت هرمونات الغدة الدرقية T3 و TSH أعلى بكثير من مجموعة السيطرة ، بينما أظهرت نتائج T4 مستويات أقل مقارنة بمجموعة السيطرة على وجه التحديد خلال الأشهر الخمسة الأولى من الإجهاض والحمل لمعظم المجموعات.

الاستنتاج : قد يلعب تحديد مستويات CXCL9 الكيميائي في المرضى الإناث المصابات بالتوكسوبلازما دورًا مهمًا في التشخيص المبكر لداء المقوسات وخاصة خلال الأشهر الخمسة الأولى من الإجهاض أو الحمل، كذلك مستويات هرمونات الغدة الدرقية يمكن أن تتغير أو تتأثر بعدوى الطفيلي وهذا يمكن أن يعد مؤشرا على وجود خلل في الغدة الدرقية بشكل عام.

Introduction:

Toxoplasma gondii is a protozoan parasite having a worldwide distribution that can widely infect a range of warm-blooded mammals. *T. gondii* infects almost a third of the world's population and endemic areas have extraordinarily high *T. gondii* seroprevalence [1, 2, 3]. The infection is normally asymptomatic in people, although it can occasionally cause complications that affect the brain, eyes or fetuses of pregnant women [4] and in immunocompromised patients. It can, however, also produce a number of life-threatening clinical problems [5]. People are infected mostly by eating raw or inadequately cooked meat (especially lamb and pig) with infectious tissue cysts or by consuming sporulated oocysts from feline feces in contaminated vegetables, fruits or water [6]. *T. gondii* develops through three main particular forms: oocyst (contains sporozoites), tachyzoite and tissue cyst (contain bradyzoites). The sexual reproduction generates oocysts that are developed specifically in the intestine of infected felines [7]. As the infection is acute, *T. gondii* spreads throughout the host body as a tachyzoite (the fast-replicating form of the parasite) that is targeted by the immune response of the host. As infection progresses, *T. gondii* converts into the chronic stage of infection via transition to the slow replicating bradyzoite [8 , 9]. Toxoplasmosis is another infection that can be passed through placenta during pregnancy [10]. Although, in the majority of women toxoplasmosis is usually asymptomatic, infection during pregnancy can lead to disease transfer through the placenta resulting in serious complications such as stillbirth, abortion, various levels of mental or physical disorders, blindness and hydrocephalus [11, 12]. In pregnant women, the prevalence of *T. gondii* infection is examined in various parts of the world and it is estimated to be 14-77% [13]. The highest rate of congenital toxoplasmosis is found to occur in the third trimester of pregnancy while the most severe infection is seen in the first and second trimesters which can result in stillbirth and abortion [13, 14].

As mentioned above neurological and ophthalmological dysfunctions are considered the severe disorders caused by the infection with *T. gondii,* and thus early diagnosis is critical to provide rapid therapeutic mediation and clinical management. Serological-based detection of *T. gondii* specific IgM and IgG antibodies are the most common methods used for toxoplasmosis diagnosis in women. Toxoplasmosis diagnosis is usually done by detecting

specific IgM antibodies, a rise in the titer of *T. gondii*-specific IgG antibodies or seroconversion. Although these methods may give differential diagnosis of acute, chronic or reactivated acquired toxoplasmosis, they have little relevance for early diagnosis of toxoplasmosis because seroconversion and a rise in IgG titers rarely appear in that phase. Therefore, the most regularly used serological marker in detecting acute infection has been *T. gondii*-specific IgM antibodies [15]. From this perspective, it is applicable to suggest complimentary and early diagnostic methods to obtain an accurate clinical management and practical initiation of therapeutic intervention, such as the detection of the immunological biomarker chemokine CXCL9 (CXC Motif Chemokine ligand 9).

Chemokines play a fundamental role in inflammatory responses production due to the induction of chemotaxis of leukocytes. Chemokines are powerful mediators of embryogenesis and neoangiogenesis during pregnancy and are crucial for enrolling NK, macrophages, T cells and dendritic to maternal decidua [16, 17]. Increasing evidence has reported a relationship between maternal inflammatory status and disease pathogenesis in pregnancy [18, 9]. Chemokines play an important role as pro-migratory factors for immune cells and employ these cells in the inflamed and damaged areas to reduce infectious elements and reconstruct the infected tissues. Chemokines are mainly divided into four subfamilies: CC, CXC, CX3C and C [20].

CXCL9 is one of the pro-inflammatory and inducible chemokines [21, 22] and is also comparably described by a broad range of cell types in response to some members of cytokine family, including, IFN-α/β and IFN-γ. It also recruits T-cells and NK cells into the inflamed areas, in addition to inflammation and injury related pathologies [23].

It has been reported that due to the direct exposure of thyroid gland by *Toxoplasma gondii*, a correlation exists between thyroid gland dysfunction and *Toxoplasma gondii* infection, and that the propagation and multiplication of the parasite in thyroid tissue have been found alter thyroid hormones levels. Furthermore, reactivation of latent toxoplasmosis might result in changing levels of thyroid hormones, particularly those which occur after or within 6 months of recovery from the beginning of toxoplasmosis [24]. It has also been mentioned that a mild increase of thyroid hormone production in pregnancy is associated with the latent toxoplasmosis [12]. Any disorder in the thyroid gland may cause several effects on different metabolism processes in the body. Hypothyroidism is the most common disorder of thyroid gland dysfunction which is characterized by low concentration of T4 and T3 hormones, accompanied by a high rate of TSH. This disease is common in adults, especially the women. Immune destruction of thyroid gland tissue is the cause of most cases in adults for instance, Hashimoto's disease and other cases caused by congenital children. It happens due to either lack of iodine in the food or after gland surgeries or because of radioactive iodine gland treatment [25].

The current study aimed to evaluate the performance of the immunological biomarker CXCL9 during early diagnosis of toxoplasmosis and to investigate the relationship between the subjected chemokine and thyroid hormones in female patients with *T. gondii* infection.

Materials and Methods:

Samples Collection: Eighty-seven women were included in this study. Blood samples were collected at Al-Yarmook teaching hospital and then separated and stored at -10°C until they had been analyzed.

The study included the following groups: control group which took in only healthy pregnancies (n=20 including five healthy pregnant women in their 1-5 months of pregnancy, and 15 healthy pregnant women in their 6-9 months of pregnancy), 1-5 months aborted patients infected with toxoplasmosis, and non-aborted infected pregnant women (n=49) which in turn included (10 patients with acute toxoplasmosis, 35 patients with chronic toxoplasmosis and 4 non-aborted pregnant women infected with chronic toxoplasmosis), 6-9 months aborted patients infected with toxoplasmosis, and non-aborted infected pregnant women (n=18) which included (3 patients with acute toxoplasmosis, 4 patients with chronic toxoplasmosis, and 11 non-aborted infected pregnant women) (Table 1).

Table 1: The number of samples in each group that were included in this study

Groups	N	Aborted Women Acute Toxoplasmosis (IgM)	Aborted Women Chronic Toxoplasmosis (IgG)	Pregnant Women Chronic Toxoplasmosis (IgG
1-5 Months	49	10	35	4
Healthy 1-5 Months	5	-	-	-
6-9 Months	18	3	4	11
Healthy 6-9 Months	15	-	-	-

Samples Testing: Following the samples collection, *Toxoplasma* agglutination test was first done according to the manufacturer's instructions. *Toxoplasma gondii*- latex agglutination test (from Spin react company-Spain) was based on antigen – antibody reaction where the agglutination occurs when soluble *T. gondii* antigen coating with latex particles are mixed with samples that contain antibodies anti *Toxoplasma* [26]. Then, all agglutination test positive sera were screened for the presence of IgM and IgG antibodies against *T. gondii* by using mini VIDAS kits (Vitek Immuno Diagnostic Assay System from Biomerieux Company). The VIDAS TOXO IgM (TXM) assay was used to detect IgM which is an enzyme-linked fluorescent immunoassay (ELFA) that is done in an automated instrument. While VIDAS TOXO IgG (TXG) assay was used to detect IgG. The principle of the assay is having two steps, enzyme immunoassay sandwich method with a final fluorescence detection (ELFA). The instrument controlled all assay steps and assay temperature. After completion of the assay, the instrument automatically analyzed and generated the test results and printed a report for each sample.
Moreover, human CXCL9 (C-X-C motif chemokine 9) ELISA kit was used to detect CXCL9.

The principal work of the ELISA kit is a sandwich enzyme-linked immune-sorbent assay procedure. Anti- CXCL9 antibody was pre-coated onto the well plates. In addition, the biotin conjugated anti- CXCL9 antibody was used to detect antibodies. O.D. absorbance was read at 450nm using a micro-plate reader, and then the CXCL9 concentrations were calculated.
Finally, thyroid hormones T3, T4 and TSH quantitative determinations in human serum were done using AccuBind ELISA microwells Kit from Monobind Inc, USA. This assay is a microplate enzyme immunoassay method where serum is first added to a micro plate well. Then, the enzyme either T3, T4 or TSH conjugate is added, and later the reactants are mixed. A dose response curve is used to ascertain the concentrations of T3, T4 or TSH in unknown specimens.

Statistical Analysis:

Statistical Analysis System- SAS (2012) program was implemented to identify the effects of different factors on the study parameters [27]. T-test was used for a significant comparison between means. Furthermore, Chi-square test was used to significantly compare between the percentages (0.05 and 0.01) probabilities. In addition, correlation coefficient was estimated among variables in this study.

Results:

The present study showed no significant increase in CXCL9 concentrations as shown below in Table 1, particularly in the first five months of pregnancy. The results agree with a previous study that showed elevation in CXCL9 serum levels in patients with toxoplasmosis. This elevation indicated that CXCL9 is critical for the recruitment of T-cells into the brain and prompting T-cells accumulation into the areas where tachyzoites grew to prevent reactivation of chronic *T. gondii* infection [28]. Furthermore, the current finding was in constant with a study that was also conducted in Iraq on pregnant women infected with toxoplasmosis. The study revealed that there were high serum chemokine levels in the blood of those women in comparison to the controls [29]. Therefore, CXCL9 might play a critical role in controlling *T. gondii* proliferation by inducing the immune T-cells into different organs.

Table 2: Shows the concentrations of human CXCL9 (pg/ml) for the aborted and pregnant women infected with toxoplasmosis in comparison to healthy pregnancies.

Groups	Mean ± SE of CXCL9 (pg/ml)		
	Aborted Women Acute Toxoplasmosis (IgM)	**Aborted Women Chronic Toxoplasmosis (IgG)**	**Pregnant Women Chronic Toxoplasmosis (IgG)**
1-5 Months abortion	271.93 ±62.58	257.75 ±52.50	233.08 ±27.43
Healthy 1-5 Months	166.33 ±32 .83	166.33 ±32 .85	166.33 ±32.83
T-test(P-value)	106.74 NS (0.399)	157.80 NS (0.508)	104.87 NS (0.176)
6-9 Months abortion	182.67 ±36.22	254.33 ±57.03	222.21 ±63.04
Healthy 6-9 Months	272.77 ±67.68	261.93 ±82.73	272.77 ±87.68
T-test(P-value)	106.91 NS (0.660)	115.82 NS (0.969)	139.54 NS (0.667)
NS: Non-Significant.			

While another study which had been done on patients suffering from toxoplasmic retinochoroiditis, showed no significant differences between CXCL9 concentrations in the patients serum and the control group [30]. This data suggested that the *T. gondii*-induced pathogenesis was stimulated by a pro-inflammatory response which was probably induced by macrophages/monocytes and T-cells [15].

The current results also included detecting the thyroid hormones levels in blood serum related to toxoplasmosis as shown in Tables 2, 3 and 4. It can be seen in the given results that T3 and TSH levels significantly increased in most of the weeks during pregnancy or abortion for the patients infected with toxoplasmosis. Whereas T4 levels showed a significant decrease in the first 5 months in the aborted women infected with toxoplasmosis in comparison to the other women groups. The recent results agreed with [31] study conducted in 2014, which established a strong link between damage, caused to thyroid gland by the *Toxoplasma* parasite [32]. Furthermore, these results agreed with [33, 34] which reported a significant increase in T3 and TSH, and significant decrease in T4.

Table 3: Shows the concentrations of human T3 in aborted and pregnant women infected with toxoplasmosis, in comparison to healthy pregnancies.

Group	Mean ± SE of T3 (ng/ml)		
	Acute Toxoplasmosis (IgM)	Chronic Toxoplasmosis (IgG)	Pregnant - Chronic Toxoplasmosis (IgG)
1-5 Months Abortion	2.42 ±0.20	3.04 ±0.12	2.34 ±0.25
Healthy 1-5 Months	1.16 ±0.33	1.16 ±0.33	1.16 ±0.33
T-test(P-value)	0.808 ** (0.0049)	0.698 ** (0.0001)	1.028 * (0.029)
6-9 Months abortion	1.80 ±0.37	2.73 ±0.22	2.23 ±0.21
Healthy 6-9 Months	2.27 ±0.26	2.36 ±0.27	2.27 ±0.27
T-test(P-value)	1.408 NS (0.491)	1.430 NS (0.595)	0.780 NS (0.909)
* (P≤0.05), ** (P≤0.01), NS: Non-Significant.			

Table 4: Shows the concentrations of human T4 in aborted and pregnant women infected with toxoplasmosis in comparison to healthy pregnancies.

Groups	Mean ± SE of T4 (µg/dl)		
	Aborted Women Acute Toxoplasmosis (IgM)	Aborted Women Chronic Toxoplasmosis (IgG)	Pregnant Women Chronic Toxoplasmosis (IgG)
1-5 Months Abortion	15.08 ±0.62	18.16 ±0.38	18.65 ±0.56
Healthy 1-5 Months	19.05 ±1.02	19.05 ±1.02	19.05 ±1.02
T-test(P-value)	2.449 ** (0.0039)	2.180 NS (0.409)	2.06 NS (0.757)
6-9 Months Abortion	19.78 ±0.65	20.10 ±0.83	17.54 ±2.09
Healthy 6-9 Months	18.22 ±1.17	18.33 ±1.12	18.22 ±1.17
T-test(P-value)	5.889 NS (0.583)	3.715 NS (0.523)	4.60 NS (0.766)
** (P≤0.01), NS: Non-Significant.			

Table 5: represents the concentrations of human TSH in aborted and pregnant women infected with toxoplasmosis, in comparison to healthy pregnancies.

Group	Mean ± SE of TSH (µIU/ml)		
	Acute Toxoplasmosis (IgM)	Chronic Toxoplasmosis (IgG)	Pregnant - Chronic Toxoplasmosis (IgG)
1-5 Months Abortion	1.722 ±0.47	1.033 ±0.15	1.012 ±0.36
Healthy 1-5 Months	0.640 ±0.16	0.640 ±0.16	0.640 ±016
T-test(P-value)	0.793 * (0.041)	0.624 NS (0.338)	0.872 NS (0.365)
6-9 Months Abortion	1.43 ±1.02	2.46 ±0.85	1.50 ±0.35
Healthy 6-9 Months	1.38 ±0.61	1.32 ±0.58	1.37 ±0.61
T-test(P-value)	1.071 NS (0.970)	1.401 NS (0.440)	1.056 NS (0.879)
* (P≤0.05), NS: Non-Significant.			

Discussion:

Toxoplasmosis, caused by *T. gondii*, is usually asymptomatic. Infection during pregnancy can lead to transplacental transmission of this parasite to the fetus [35] resulting in serious complications such as stillbirth, abortion, various levels of mental or physical disorders [10]. *T. gondii*-specific IgM and IgA detection that do not cross the placenta is regarded as one of the good markers of congenital infection. However, these biomarkers cannot detect more than

75% of the infected babies and show low sensitivity. Furthermore, it is proposed that *T. gondii*-maternally specific IgG crosses the placenta and the clearance of this maternally transferred IgG takes place between 6-12 months, hence rendering that it is not a useful laboratorial biomarker for congenital toxoplasmosis [35]. Therefore, this study demonstrated to use one of the chemokine, which is CXCL9, as one of the predictive biomarker for toxoplasmosis infection, token together, the effect of the immune response against this disease, on thyroid hormone dysfunction.

As the infection is acute, *T. gondii* spreads throughout the host body as a tachyzoite which is targeted by the immune response of the host. As infection progresses, *T. gondii* converts into the chronic stage of infection via transition to the slow replicating bradyzoite [7, 8]. Throughout the chronic infection, the immune response to *T. gondii* is sustained which is evident by the elevated *T. gondii*–specific IgG [37] and some cytokines such as IFN- γ and TNF-α in the sera in order to control the replication of tachyzoite in the acute and chronic infection cases [38].

Cytokines are key factors of the innate and adaptive immune system and are considered one of the soluble mediators of immunity. They interact with various types of immune cells to manipulate host body's immune cell physiology for a counter-attack on the foreign body, particularly toxoplasmosis. One of these cytokines is CXCL9 [39] which considered one of the chemokines that play an important role in recruiting T- and NK cells into the inflamed and insulted areas [22]. High expression level of CXCL9 was also found in retina of one the patients who was infected with ocular toxoplasmosis [27].

Another study revealed that CXCL9 displays high accuracy to distinguish the healthy infants from *T. gondii* infected infants. Furthermore, the same study proved the accuracy of CXCL9 in addition to CXCL10 to diagnose toxoplasmosis [40]. [39] found out high CXCL9 expression in retina of an patient infected with ocular toxoplasmosis that reached 8000 fold when they used real time PCR compared with uninfected patients. All of the above-mentioned results agree with the results of the current study.

Furthermore, the current results showed a significant increase in thyroid hormones levels with an increase in CXCL9 levels in toxoplasmosis women compared with the control. Many studies agreed with these results. One of them stated that hypothyroidism increased with susceptibility increase to infectious diseases because of interfering the immune system components with the central regulation of thyroid hormone levels. In addition, the same study mentioned that thyroid gland dysfunction leads to autoimmunity and chronic cellular immune processes lead to thyroid cells destruction, and that cause functional thyroid tissue reduction. Also [41] study mentioned that the circulating CXCL9 and CXCL11 increased in patients with thyroiditis, and the expression of these chemokine was induced by the Th1 cytokine, IFN-γ and TNF-α in cells, tissues and primary thyroid cells.

On the other hand, the results of this study confirmed the correlation between the thyroid hormone dysfunction and toxoplasmosis, particularly the significant increase in thyroid stimulating hormone (TSH) and triiodothyronine (T3) in all study groups. It showed that the infection with *T. gondii* has a direct effect on the thyroid gland mechanism according to its ability to activate and divide within thyroid tissue, which drives an imbalance in the gland work and increases the hormones levels that are produced from the thyroid gland [34].

In conclusion, the results of this study show an interesting relationship between CXCL9 (which is produced as a response of immune system against foreign bodies including *T. gondii*)

and thyroid gland dysfunction. This study concluded the hypothesis that during early pregnancy, and as part of the mechanism of maternal immune tolerance of the fetus as a semi-allograft, there is an initial shift from Th1 to Th2. This shift helps *T. gondii* to escape the control of the immune system [12]. After that, *T. gondii* acquire the ability to invade thyroid gland and then their multiplication and propagation in thyroid tissue changes leading to thyroid hormones alteration that ends in TSH increase. Abnormal production of T3 and T4 occurred in the group of infected women patients with toxoplasmosis in this study, particularly when *T. gondii* reached hypothalamus [33].

Finally, the non-significant increase and decrease in CXCL9 may be due to the limitation of the sample size which requires another study to confirm these results by using a large sample size.

Conclusion

The current study aligns with other research that was done on many different types of *Toxoplasma gondii* suggesting that CXCL9 is a crucial chemokine in detecting and testing the immunity and responses of human body against *T. gondii*. Together, these findings advocate the importance of recruiting the agents of the cell-mediated immune response as biomarkers with a potential to confirm early diagnosis of toxoplasmosis and contribute to an accurate clinical management and effective therapeutic mediation. In addition, the results revealed that *T. gondii* could lead to change or damage the function of thyroid gland in human body. The increase in T3 and TSH thyroid hormones production in toxoplasmosis provides new clue to the complex pathogenesis of *Toxoplasma*-associated thyroid diseases. However, further studies are required to understand the relationship between *T. gondii* and thyroid dysfunction.

Ethics: The local ethical committee of Department of Biology (Ref. CSEC/0921/0057), College of Science, University of Baghdad approved this work.

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