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RESEARCH ARTICLE

Iron Status, Malondialdehyde, and Nitric oxide Levels in Iraqi Infertile Women with IgG Seropositive Toxoplasma Gondii

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Abstract

The purpose of this study is to highlight the levels of IgG toxoplasma gondii *(T. gondii)* in infertile Iraqi women and its effect on iron status, malondialdehyde (MDA), and nitric oxide (NO). In order to do this, four groups were included in this study, infertile women with seropositive IgG *T. gondii* group (PP, n=25), infertile women with seronegative IgG *T. gondii* group (NP, n=25), healthy fertile women with seropositive IgG *T. gondii* as control group (PC, n=15) and healthy fertile women with seronegative IgG *T. gondii* as control group (NC, n= 15). All of the samples were tested as seronegative of IgM *T. gondii*. The results were as followes: Infertile women (PP) group have a significant levels of seropositive IgG *T. gondii* than fertile women (PC) group (P=0.008), while both (NC & NP) groups have seronegative IgG *T. gondii*. Serum iron and Hb concentration have been found to be with no significant differences ($p>0.05$), while significant increases were found in (TIBC, UIBC, transferrin and saturation of transferrin, $p<0.001$) in (NP vs. NC) group and (PP vs. PC) group, Also in (PP vs. NP) group and (PC vs.NC) group. The levels of malondialdehyde in serum of (NP & PP) were higher than (NC&PC) groups respectively. No remarkable changes were observed of (NC) group compared to (PC) group but a significant increase in MDA serum levels in PP group as compared to NP group, $p<0.05$. In addition to that levels of NO showed significant alterations in serum of infertile groups (NP & PP) as compared to that of their control groups (NC & PC) respectively ($p<0.05$), while no significant variation in sera of both (NP & NC) groups compared to (PP & PC) groups.

Keywords: *Toxoplasmosis, Infertility, Iron, Lipid peroxidation, Nitric oxide.*

Introduction

Toxoplasmosis is one of the common parasitic infections caused by coccidian protozoon, *Toxoplasma gondii* (*T. gondii*) [1, 2]. Some estimates suggest that about one-third of the world's population has been exposed to this parasite [3]. In Iraq, *T. gondii* prevalence was examined in different governments and it was found a different percentage of the seropositivity infection [4, 6]. Environmental pollution with the organisms due to situations of sanctions and series of wars that attacked the country as far as this disease was elevated after Iraq occupation.

Toxoplasma gondii elevated with a frequency of infection more than 40% compared to the 1980s in Iraq which was the frequency of the infected women with T. gondii did not exceed 2% of the women tested at that time by Iraqi laboratories [7].There is limited clinical evidence supporting the association between chronic *T. gondii* infection and the development of many disorders, including infertility, in both animals and humans. Toxoplaasma gondii can cause infertility in experimental animals, according to previous research [8].Zhou et al. found that T. gondii infection in infertile human couples was higher than that in fertile ones [9].Women with seropositive T. gondii are reported to take a longer time to conceive and to have more fertility problems than women with seronegative T. gondii.

Additionally, they become pregnant at an older age, more often needed in vitro fertilization [10].Previous researchers showed that iron may be contributed in ovulatory function and fertility [11,12]. Iron the most prevalent nutritional deficiency

worldwide, it's associated with the high-risk for women of reproductive age [11]. Researches proved the presence of iron-transporting proteins in ovarian cells [12, 13]. Moreover, to that both transferrin and its receptor are found in granulose cells and oocytes. These proteins have an important role in iron supplying for the ovum and follicle development [13].Ovulation, fertilization, embryo development, and implantation can be affected by oxidative stress (OS) [14,15].Thus, OS is considered a cause of female infertility. It is suggested that OS is caused by ROS overproduction rather than antioxidant depletion [16]. The impact of OS in decreasing the egg quality, fertilization and pregnancy rates in mouse and human has been previously reported. On the other hand, it is reported that antioxidant depletion leads to infertility and use of antioxidants could be helpful in the treatment of infertility [17]. There are several reports indicating that infection with various parasites is associated with a marked elevation in lipid peroxidation [18, 19].

Measurements of MDA, is widely used as indicator of lipid peroxidation, and increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both human and model system [20]. Fertilization may be defective by damaging the DNA of the oocytes and spermatozoa a result of increased levels of OS [21].Nitric oxide (NO) is one of the smallest known bioactive products of mammalian cells, can be produced by almost all cells [22]. Nitric oxide participation in signal transduction pathways might play a significant role in establishment and maintenance of pregnancy [23].

Generation of NO could impair endometrial function by either inducing cellular apoptosis [24], or through nitrosylation of key endometrial proteins impair their physiological function [25]. In our present research we detect the presence of specific antibodies IgG *T. gondii* in fertile and infertile women using ELISA techniques and study its effecting on iron status and oxidative stress.

Materials and Methods

Fifty unexplained infertile Iraqi women with age ranged from (20-41 years) were participate in this study. The study groups were divided as following: 25 infertile women with seropositive IgG *T. gondii* (PP) and 25 infertile women with seronegative *T. gondii* (NP). Thirty normally ovulating women having unless one baby matched for age served as control group. The control groups were also classified in subgroup: fifteen fertile women with seropositive *T. gondii* (PC) and fifteen fertile women with seronegative *T. gondii* (NC). All of the samples were found seronegative of IgM. All patients were attending to Kamal Al-Samurai hospital in Iraq during the period of November 2016 to Aprile 2017.All the participants were fasting for 12 hours before blood collection in the next morning. A required information was completed for each participant by asking them a special questionnaire. Infertile women with other causes of infertility (ovarian, tubal, galactorrhea, hormonal, infection, taking any hormonal medication, and abortion) were excluded from the study. In addition to that, females with autoantibodies, autoimmune diseases, and females who their couples are infertile were excluded from the study.

Detection of anti- *Toxoplasma gondii* antibody (IgG and IgM) by Enzyme linked Immunosorbent Assay (ELISA) technique: (Biotek ELISA) kit was used.

Blood Samples Collection

Eight milliliters of fasting whole blood was collected from each of infertile and fertile women, kept at room temperature for 1 hour in tubes without any anticoagulant. Ten minutes was used to centrifuged each tube at 2000×g, then clear serum was pipetted into clear dry test tube. All tubes were stored at (-20) ^{o}C for subsequent analysis.

Determination of Iron

Serum iron and total iron binding capacity (TIBC) were determined using a kit from HUMAN Company. Unsaturated iron binding capacity (UIBC) was calculated by subtracted the serum iron concentration from the TIBC. Transferrin and the percentage of saturation of transferrin with iron can be estimated indirectly from the TIBC value [26].

Determination of Malondialdehyde (MDA)

Malondialdehyde levels were determined using the method based on the reaction of MDA with thiobarbituric acid (TBA) at 95 C°.

TBA and MDA react to form a pink pigment with an absorption maximum at 532 nm [27].

Determination of Nitric oxide (NO) Concentration

Nitric oxide concentration was measured using Griess reaction which used sulfanilamide and naphthyl ethylene diamine dihydrochloride (NED) under acidic conditions. Sulfanilamide and NED compete for nitrite in the Griess reaction [28]. Standard NO curve was prepared using different concentrations (0, 5,10,20,30,40,60,70 and 100 µM) of stock sodium nitrite ($NaNO_2$), then used to obtain NO concentration in the serum of all the studied groups.

Results and Discussion

Table (1) revealed that infertile women (PP) group have high significant levels of seropositive IgG *T. gondii* (150.20±89.28 IU/mL) than fertile women (PC) group (82.71±30.01 IU/ml), *P*=0.008.

These results are in agree with the hypothesis that latent toxoplasmosis has some negative effects on the reproductive capacity of *T. gondii* infected women. Toxoplasma gondii-associated infertility mechanisms have a role in development of endometritis and fetal rejection due to local release of *T. gondii* from cysts located in the endometrial tissue during placenta formation. Impaired folliculogenesis in the ovaries, uterine atrophy and reproductive failure are occurred as a result of chronic toxoplasmosis [29]. Other results found an association between *T. gondii* and infertility. This finding encourages both prompting health education to prevent *T. gondii* infection in female population especially in childbearing age and further investigation to elucidate the causative relation between *T. gondii* infection and female infertility [30].

Table1: Levels of IgG *T. gondii* among all studied groups.

Groups	Toxoplasma gondii IgG (IU/mL)		*P*
	Range	(Mean ±SD)	
NC (n=15)	0.2-19.3	5.52±5.47	0.184
NP (n=25)	0.4-28.7	9.71±11.19	
PC (n=15)	41.0-109.2	82.71±30.01	0.008**
PP (n=25)	45.0-315.0	150.20±89.28	

NC: Negative*T. gondii* conrol, PC: Positive *T. gondii* control, NP: Negative *T. gondii* patients and PP: Positive *T. gondii* patients

Several studies found that iron have a role in ovulatory function and fertility [11]. To our knowledge there are few literatures dealing with iron in patients with Toxoplasma gondii [12, 32]. Therefore, to look for the impact of iron status in the present studied patients, serum iron status measurements were carried out here, which included iron concentration, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), transferrin concentration and transferrin saturation. The results present in Table (2) revealed that no significant differences (p> 0.05) in the concentration of serum iron (100.92±21.28 µg/dl vs. 95.70 ± 15.51 µg/dl) and Hb (12.51 ± 1.16 g/dl vs. 11.83 ± 1.25 g/dl), and significant differences (p < 0.05) in the serum of TIBC (371.49 ± 87.36 µg/dl vs. 268.58 ± 73.35 µg/dl), UIBC (270.57 ± 94.13 µg/dl vs. 172.87 ± 77.53 µg/dl), transferrin saturation(28.90 ± 9.86% vs. 39.64 ± 17.91%), and transferrin concentration (260.04 ± 61.15 mg/dl vs. 188.00 ± 51.35 mg/dl) between (NC vs. NP) .

Table 2: Mean values ± SD of iron status in the serum of all studied groups.

Parameters	NC n=15	NP n=25	*P*-value	PP n=25	PC n=15	*P*-value
Iron (µg/dl)	100.92±21.28	95.70±15.51	0.378	103.99±17.52	95.35±22.34	0.209
TIBC (µg/dl)	371.49±87.36	268.58±73.35	0.000**	276.09±46.45	509.79±156.13	0.000**
UIBC (µg/dl)	270.57±94.13	172.87±77.53	0.001**	172.10±44.23	414.39±159.97	0.000**
Transferrin(mg/dl)	260.04±61.15	188.00±51.35	0.000**	193.27±32.51	356.85±109.29	0.000**
Saturation of transferrin (%)	28.90±9.86	39.64±17.91	0.04*	38.45±8.44	20.68±8.78	0.000**
Hb (g/dl)	12.51±1.16	11.83±1.25	0.100	12.88±0.68	12.30±1.05	0.065

NC: Negative*T.gondii* conrol, PC: Positive *T. gondii* control, NP: Negative *T. gondii* patients and PP: Positive *T. gondii* patients. (**The difference is significant at the 0.01 level)

The same results were found between (PC vs. PP) groups: serum iron (103.99 ± 17.52 µg/dl vs95.35 ± 22.34 µg/dl) *p*> 0.05, Hb (12.88 ± 0.68 g/dl vs. 12.30±1.05 g/dl) *p* > 0.05, TIBC (276.09±46.45µg/dl vs. 509.79 ±156.13 µg/dl) *p*>0.001,UIBC (270.57±94.13µg/dl vs.172.87±77.53 µg/dl) *p*>0.001, transferring saturation (38.45±8.44% vs. 20.68±8.78%),

p>0.001 and transferrin concentration (193.27±32.51mg/dl vs. 356.85±109.29mg/dl) *p*>0.001. The results in Table (3) show that there is no significant variation in Fe and Hb levels of PP vs. NP group and PC vs.NC groups (p>0.05), but a highly significant increase in TIBC, UIBC, transferrin and saturation of transferrin serum levels in PP vs. NP group and PC vs.NC group (p<0.001).

Table 3: Mean values ± SD of iron status in the serum of positive IgG *T. gondii* groups among negative IgG *T. gondii* groups

Parameters	PC n=15	NC n=15	*P*-value	PP n=25	NP n=25	*P*-value
Iron (µg/dl)	103.99±17.52	100.92±21.28	0.669	95.35±22.34	95.70±15.51	0.949
TIBC (µg/dl)	276.09±46.45	371.49±87.36	0.001**	509.79±156.13	268.58±73.35	0.000**
UIBC (µg/dl)	172.10±44.23	270.57±94.13	0.001**	414.39±159.97	172.87±77.53	0.000**
Transferrin(mg/dl)	193.27±32.51	260.04±61.15	0.001**	356.85±109.29	188.00±51.35	0.000**
Saturation of transferrin (%)	38.45±8.44	28.90±9.86	0.008**	20.68±8.78	39.64±17.91	0.000**
Hb (g/dl)	12.88±0.68	12.51±1.16	0.292	12.30±1.04	11.84±1.25	0.157

NC: Negative*T.gondii* conrol, PC: Positive *T. gondii* control, NP: Negative *T. gondii* patients and PP: Positive *T. gondii* patients. (**The difference is significant at the 0.01 level)

Different concentrations of TIBC were associated with Iron-metabolism disorder. In malignancies, hemochromatosis, and chronic inflammatory TIBC is often decreased while it increases in iron deficiency. Transferrin saturation values in excess of 60 percent may be indicative of hemochromatosis or iron overload and 16 percent generally indicate iron-deficiency anemia [33]. The presence of iron-transporting proteins in ovarian cells was proved by the evidence of several researches [12, 34].

Data demonstrated that granulose cells are capable to synthesize transferrin, which could be translocating to the oocyte. It has been suggested that ovarian transferrin and transferrin receptors might not participate in local iron metabolism [12]. Measurements of MDA, is widely used as indicator of lipid peroxidation, and increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both human and model system [20].

The results indicate that NP &PP groups have significant increase in serum level of MDA (9.016±4.44& 14.57±12.09 nmol/L respectively) as compared to NC&PC groups (6.13±3.03&7.95±2.00 nmol/L respectively, *p* <0.05), (Table 4). Macrophages, neutrophils and granulose cells in the graphian follicle are the source of reactive oxygen species, during follicular maturation oocytes are well protected against toxic injury due to oxidative stress by important antioxidants.

The results of our study are in agreement with previous report which concluded significantly higher concentration of MDA in serum of infertile women than in fertile women [35]. Increase in MDA in infertile women and decrease in total antioxidant capacity (TAC) in a recent study indicates an increase in oxidative stress. Veenas, B. S.et al. have found significantly higher concentration of MDA in serum of infertile women than in fertile women [16].

Table 4: The characteristics of MDA and nitric oxide in all studied groups

Parameters	NC n=15	NP n=25	*P*-value	PC n=15	PP n=25	*P*-value
MDA nmol/L	6.13±3.03	9.016±4.44	0.033*	7.95±2.00	14.57±12.09	0.043*
NO µmol/L	6.81±3.04	10.62±5.41	0.034*	7.31±2.37	9.96±5.00	0.031*

NC: Negative*T.gondii* conrol, PC: Positive *T. gondii* control, NP: Negative *T. gondii* patients and PP: Positive *T. gondii* patients. (*The difference is significant at the 0.05 level)

Also, it was found a significant increase in MDA serum levels in PP group (14.57±12.09 nmol/L) as compared to NP group (9.016±4.44 nmol/L, *p*<0.05), while there is no significant variation in MDA levels of (NC) group compared to (PC) group (*p*>0.05), Table (5).

Table 5: Mean values ± SD of MDA and nitric oxide in the serum of positive IgG *T. gondii* groups among negative IgG *T. gondii* groups

Parameters	NC n=15	PC n=15	*P*-value	NP n=25	PP n=25	*P*-value
MDA nmol/L	6.13±3.03	7.95±2.00	0.063	9.016±4.44	14.57±12.09	0.036*
NO µmol/L	6.81±3.04	7.31±2.37	0.619	10.62±5.41	9.96±5.00	0.658

NC: Negative*T.gondii* conrol, PC: Positive *T. gondii* control, NP: Negative *T. gondii* patients and PP: Positive *T. gondii* patients. (*The difference is significant at the 0.05 level)

Toxoplasma tissue cysts could increase the generation of free radicals which may exceeds the cellular defenses resulting in oxidative stress [36]. The increase of MDA level in Toxoplasma gondii patients demonstrates the increase of lipid peroxidation. In a previously study it was suggested that decreased activity of the defense system protecting the tissues from free radical damage is one of the main reasons for high MDA levels in the patients infected with toxoplasmosis. The potentially harmful effects of reactive oxygen species are controlled by the cellular antioxidant defense system [37]. Results obtained by Al-Azzauy [38] showed an agreement with our study when he found an increase in MDA level in the erythrocyte of Toxoplasma gondii patients as compared with normal healthy control.

These findings are also in agreement with Karaman, U. et al. [39] and Yazar et al. [40]. Antioxidants consumption may increase as a result of elevation of lipid peroxidation [21]. Defense role of antioxidants which directly damaged by OS effects on graffian follicles and disrupting the ova and it may be damage the ovum DNA, leads to disorder and defect in fertilization and congenital malformations in the embryo [41].

Pathophysiology of unexplained infertility, endometriosis, polycystic ovarian syndrome (PCOS), tubal and peritoneal factor infertility can be associated with oxidative stress [42]. Nitric oxide production in our studied groups was determined and the results indicate that NP &PP groups have significant increase in serum level of NO (10.62±5.41&9.96±5.00 µmole/L respectively) as compared to NC&PC groups (6.81±3.0 &7.31±2.37 µmole/L respectively, $p < 0.05$), (Table 4). The level of NO in serum of our studied groups showed that there are no significant differences in sera of NO levels in both NP&PP (10.62±5.41 & 9.96±5.00 µmole/L respectively) groups and NC&PC (6.81±3.04 &7.31±2.37 µmole/L respectively) groups, Table (5). These results mean that IgG antibody of toxoplasma gondii had no effect on NO production. Al-Azzauy [38] showed increased level of serum nitric oxide in the Toxoplasma gondii patients. Arginine metabolism produce NO and one of the most effective O^{-2} free toxins. There are also previous studies reporting an increase in the NO level in parasitic diseases [43, 44].It can be stated that the NO level increase as a defensive mechanism to protect the patient against the harmful effects of the parasite. The incompatible of our results with the above finding may be due to the production of NO occurred during acute infection.

Nitric oxide (NO) is a molecule that incorporates in many physiological processes of female reproductive system. The role of endothelial isoform of nitric oxide synthase (eNOS) enzyme in female infertility was suggested by Najafi et.al. Who demonstrated the localization of (eNOS) enzyme in glandular and luminal epithelium, vascular endothelium and stromal in both fertile women and women with unexplained infertility [45, 46].

The association of OS with various gynecologic and obstetric conditions related to infertility suggests a potential role for oral antioxidant supplementation [42]. The levels of vitamin D were determined by our team in both fertile and infertile women with and without T. gondii on the same groups of the present study [47]. We find that the levels of MDA and IgG obtained from this study were significantly negative correlated with vitamin D in PP group ($r = -0.415$, $P = 0.039$ and $r = -0.397$, $P = 0.05$) respectively, while no significant correlation found in NC, PC and NP groups (Table 6).

Table 6: Correlation parameters of Vitamin D with MDA and IgG in PP group

Parameters	Vitamin D	
	r	P
MDA (nmol/L)	-0.415*	0.039
IgG *T. gondii* (IU/mL)	-0.397*	0.05

* Correlation is significant at the 0.05 level

Conclusion

Lipid peroxidation associates with IgG and vitamin D deficiency in infertile women with seropositive IgG *T. gondii*. A recent research studied oxidative stress markers and demonstrated their optimum levels in the female reproductive system and improves their role in success rate of assisted reproductive techniques and infertility management [48]. Therefore, we recommend that female must measurement IgG and IgM as important step in treatment of infertility.

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