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The Oxidative Stress and Platelet Activation in Patients Infected with *T. gondii*

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Abstract:
Background: *Toxoplasma gondii* is a highly frequent obligate intracellular protozoan parasite; it can cause serious problems to the public health especially pregnant females, however, the pathogenesis of this condition is not clear. **Objective:** was to evaluate the status and the inter-relationship of the oxidative stress and platelet activation in patients infected with *T. gondii*. **Methods:** Thirty patients infected with *T.gondii* (10 acute and 20 chronic cases) and 10 healthy subjects (control group) were included in this study. Serum levels of malondialdehyde (MDA), total glutathione (t GSH), reduced glutathione (GSH), oxidized glutathione (GSSG), redox potential (RP) and soluble P-selectin (sP-selectin) were measured. EDITA blood samples were used for complete blood picture with special emphasis on platelet count and mean platelet volume (MPV). **Results:** The mean platelet volume (MPV) in patients was significantly higher than the control group, however, platelet count showed no significant difference. The serum mean values of MDA, GSSG, RP and sP-selectin in patients were significantly higher than the control subjects. On the other hand, the levels of tGSH and GSHin patients were significantly lower than in controls. MPV and platelet count showed significant positive correlations with sP-selectin concentration. **Conclusion**: Although toxoplasmosis is mostly asymptomatic, the findings of the present study strongly indicate that the occurrence of oxidative stress could be a potential mechanism of subclinical inflammatory pathology and tissue damage in these cases.
Key Words: Toxoplasmosis, Oxidative stress, Glutathione, P-selectin, Platelet, and Malondialdehyde.

INTRODUCTION

Toxoplasma gondii is a highly frequent obligate intracellular protozoan parasite. It was reported that about one-third of the world population is infected with *T.gondii.* In most humans infected with *T. gondii* the disease is asymptomatic; however, *T.gondii* can cause serious problems to the public health especially pregnant females.(1)In Egypt, the prevalence of seropositive T*oxoplasma* cases amounts up to 67.4% among blood donors.(2)

The life cycle of *T. gondii* includes three different infectious stages: tachyzoites, which

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facilitate expansion during acute infection, bradyzoites, which maintain chronic infection and sporozoites, which are disseminated in the environment within the oocysts.[3]

Toxoplasmosis has been proved to be a source of large number of reactive oxygen species(ROS) and other biotoxic agents leading to increased oxidative stress.[2,4,5] Lipid peroxidation is a well-established mechanism of cellular injury in human, and used as an indicator of oxidative stress in cells and tissues.[6,7]Measurement of malondialdehyde (MDA) is widely used as an indicator of increased levels of lipid peroxidation due to oxidative stress which is associated with a variety of chronic diseases.[6,7]

The potentially harmful effects of ROS are controlled by the cellular antioxidant defense system including glutathione (GSH) and related enzymes.[3,7] Glutathione, an endogen-originated peptide which can be synthesized in the liver, is made up of glutamic acid, cysteine and glycine amino acids, and is an important antioxidant. It defends the cells against oxidative damage by undergoing reaction with free radicals and peroxides.[6,7]

During parasitic infection, including toxoplasmosis, platelet stimulation and activation occur as a result of direct contact with the parasite leading to the immediate expression of the adhesion molecule P-selectin (stocked in the platelet α-granules) on its surface.[8,9]

P-selectin (CD62) is a cell adhesion molecule (CAM) stored in the membranes of platelets' α-granules and in the Weibel-Palade bodies of endothelial cells. It is expressed on the cell surface upon granule exocytosis and plays an essential role in the initial recruitment of leukocytes to the sites of injury during inflammation.[10] In addition, inflammatory stimulation by ROS generation causes post-translational cell signaling up-regulation of P-selectin and this may represent an important physiological trigger of the micro-vascular

inflammatory response.[11]

Soluble P-selectin (sP-selectin) released from activated endothelial cells and platelets has been proposed as a useful biomarker in various pathologic states in which platelets and/or endothelial cells are activated.[11, 12]

Thus, the aim of the present study was to evaluate the status and the inter-relationship of the oxidative stress (levels of MDA and GSH) and platelet activation (levels of sP-selectin and mean platelet volume) in patients infected with *T. gondii*.

SUBJECTS& METHODS:

Study design: a case control study was conducted. Thirty patients infected with *T. gondii* were selected from patients attending the Parasitology Clinic at Medical Research Institute, Alexandria University. According to the type of anti-toxoplasma antibody detected by ELISA, patients were divided into acute (10 cases positive for IgM antibodies) and chronic (20 cases positive for IgG antibodies). Patients infected with other parasites were excluded from the study. Ten age and sex matched healthy individuals were taken as a control group; they were negative for IgG and /or IgM *T. gondii* antibodies. None of the studied subjects was on a special diet or taking any antioxidant (vitamin E, C, etc) or treated with antioxidant drugs. Patients and controls had no smoking or drinking habits.

Written Consents were taken from patients and control subjects to be enrolled in this study. The protocol of this work was approved by the Ethical Committee of the Medical Research Institute.

Venous Blood samples were taken from all subjects. EDITA blood samples were used for complete blood picture with special emphasis on platelet count and mean platelet volume (MPV).[13]Serum was prepared from blood after clotting at room temperature, aliquot and kept frozen at 70^{o} C according to the standard guidelines till used in the following tests:

1) Determination of total Glutathione (tGSH), Oxidized glutathione (GSSG)

and reduced glutathione (GSH):

The enzymatic method described by Griffith.[14] was used to measure the total glutathione (tGSH) and oxidized glutathione (GSSG) content.

a. **Assay for total glutathione (tGSH):**

This is a sensitive and specific enzymatic method which depends on the oxidation of GSH by 5.5-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2-nitrobenzoic acid (TNB). Oxidized GSSG is reduced enzymatically by the action of glutathione reductase and NADPH to regenerate GSH which reacts again. The rate of TNB formation is monitored at 412 nm and is proportional to the sum of GSH and GSSG present in the sample.

Procedure: Aliquots of 0.1 ml of 6mM DTNB, 0.7 ml of 0.3 mM NADPH, 0.18 ml distilled water and 10 µl of the test sample or standards were mixed and incubated for 15 minutes at 30^{o}c. The reaction was initiated by the addition of 10 µl of 50 µ/ml GSH reductase. The rate of formation of TNB was monitored by recording the change in absorbance at 412 nm per minute (ΔA/min).The total glutathione content in the serum samples was determined from a GSH standard curve, results were subsequently expressed as nmole/ml.

a. Assay for oxidized glutathione (GSSG):

The GSSG content is determined by the same assay as total glutathione, but here the reduced glutathione is bound by 2-vinylpyridine.

Procedure: 2µl of 2vinyl pyridine were added to 100µl serum sample. With mixing, 6µl of 50% (v/v) triethanolamine were added to the side of the test tube and the solution was vigorously mixed. The final pH should be 7 -7.5. The mixture was allowed to stay for one hour at room temperature. After incubation the mixture was assayed as in tGSH procedure. The GSSG content in the serum samples was determined from the standard curve; results were subsequently

expressed as nmole/ml.

c. Reduced glutathione (GSH):

It was estimated using the following equation:

GSH = tGSH –GSSG

d. Redox status as redox potential[15].

Using Nernst equation:

$$\Delta E = \Delta E^0 - (RT/nF) \ln Q$$

Where **Q** is the ratio $[GSHstc]^2$ * [GSSG]/[GSSGstc] * $[GSH]^2$, with [GSHstc] and [GSSGstc] being 1 M concentrations (stc= standard conditions), E^0 denotes the standard redox potential at pH 7.0 (240 mV for the GSH/GSSG couple), ***R***is the gas constant (8.31 J K^{-1} mol^{-1}),***T*** is temperature (303 K), ***F*** is the Faraday constant (9.65 × 10^4 C mol^{-1}), and ***n***is the number of electrons transferred (***n***=2 for GSH/GSSG).

1) Determination of MDA:

Malondialdehyde in serum was determined according to the method of Drapper and Hadley.[16]

Procedure: An aliquot of 0.1ml of the sample was pipetted into a tube containing an equal volume of sodium dodecylsulphate (SDS) solution (8.1% in distilled water). This was followed by the addition of 0.75ml of 20% acetic acid (PH was adjusted to 3.5 with 1 N NaOH), 0.75ml of TBA (0.8% in distilled water) and 0.3ml distilled water. The contents of the tubes were mixed with a vortex, incubated in a boiling water bath for 1 hour then cooled to room temperature. An aliquot of 0.5ml of distilled water was added to each test tube followed by the addition of 2.5ml n-butanol. The contents of the tubes were vigorously mixed with a vortex then rotated in a centrifuge at 2500xg for 10 minutes. A blank tube was prepared similarly but without serum. Absorbance was read at 532nm using a spectrophotometer (Spectronic-21) against blank. The concentrations (nmole /ml) of MDA in samples were obtained from a standard curve.

1) Determination of sP-selectin by ELISA.[17]

This assay employed the quantitative sandwich immunoassay technique. Standards

and samples were pipetted into the microplate wells (pre-coated with a monoclonal antibody specific for sP-selectin) together with a polyclonal antibody specific for sP-selectin which had been conjugated to horseradish peroxidase. After removal of unbound conjugated antibody, a substrate was added and color was developed which was proportional to the concentration of sP-selectin. A standard curve was constructed by plotting the mean absorbance for each standard against the corresponding concentration. The concentration of sP-Selectin (ng / ml) of each sample was calculated from the standard curve.

Statistical analysis

Statistical analysis was performed using the SPSS program version 12. Results were reported as a mean ± standard deviation (SD). Statistical analysis was performed by paired samples T-test. Pearson's correlation was carried out. Two-tailed p values ≤ 0.05 were considered significant.

RESULTS

I- Hematological Findings:

The mean values of different hematological parameters of *T. gondii* patients did not differ significantly from those of control subjects (Table 1). Although the platelet counts showed no significant difference, the mean platelet volume (MPV) in patients (acute & chronic) were significantly higher than in control group; there was no significant difference between acute and chronic patients (Table2).

Table (1): Hematological parameters in *T. gondii* patients and control subjects

Parameters		Control Group (n=10)	*All T. gondii* patients (n=30)
Hemoglobin (Hb) (g/dl)	Range	9.50 - 13.80	9.5 - 14.10
	Mean ± S.D	12.0 ± 1.1	11.8 ± 1.0
	P*		0.453
WBCs (X 10^3/L)	Range	4.4-10.9	4.9-9.4
	Mean ± S.D	7±1.7	6.9±1.6
	P		0.851
Red blood cells (RBCs) (×10^6/L)	Range	3.6 - 5.0	3.5 - 5.2
	Mean ± S.D	4.4 ± 0.5	4.4± 0.5
	P		0.672
Neutrophils (%)	Range	45.0 - 66.0	40.0 - 77.0
	Mean ± S.D	56.0 ± 6.4	60.3 ± 7.3
	P		0.069
Lymphocytes (%)	Range	26.0 - 47.0	15.0 - 54.0
	Mean ± S.D	35.7 ± 6.7	32.7± 7.2
	P		0.194
Monocytes(%)	Range	4.0 - 9.0	3.0 -10.0
	Mean ± S.D	6.3± 1.9	5.3± 1.8
	P		0.165
Eosinophils (%)	Range	1.0 - 3.0	0.0 -4.0
	Mean ± S.D	1.7 ± 0.7	1.8± 0.9
	P		0.920
Basophils (%)	Range	0.0 -1.0	0.0 -1.0
	Mean ± S.D	0.3 ± 0.5	0.1 ± 0.3
	P		0.236

*Paired samples T-test & significance was considered at level of P≤ 0.05.

Table (2): Mean values of platelet count and mean platelet volume (MPV) in *T. gondii* patients and control subjects

		Control gp (n=10)	*Toxoplasma gondii* patients		
			All Patients (n=30)	Chronic gp (n=20)	Acute gp (n=10)
Platelet count (×10^9/L)	Range	194-341	151-387	151-387	222-293
	Mean ± S.D	243.5±39.3	262.4±56.9	264.3±68. 4	258.7±25.6
	P1		**0.235***	**0.428**	**0.130**
	P2			**>0.05**	
MPV (fl)	Range	6.5-10.0	10.3-15.2	10.9-15.2	10.3-14.9
	Mean ± S.D	7.67±0.93	12.86 ± 1.51	13.1±1.5	12.2±1.4
	P1		**0.000**	**0.000**	**0.000**
	P2			**>0.05**	

P1: comparing the mean values of all patients to control group.
P1: comparing the mean values of chronic to acute patients.
*Statistical significance at P≤ 0.05.

II-Oxidative Stress parameters

1- Malondialdehyde (MDA)

The mean values of MDA in all, acute and chronic patients infected with *T. gondii* were significantly higher than that of normal control group. Also acute patients showed significantly higher mean MDA level than chronic patients (Table 3).

Table (3): Malondialdehyde concentration (nmole/ ml) in the sera of *T. gondii* patients and control subjects

	Control gp. (n=10)	*Toxoplasma gondii* patients		
		All patients (*n=30)*	Chronic gp. (n=20)	Acute gp. (n=10)
Range	0.21-0.57	0.68-12.5	0.83-9.7	0.68-12.5
Mean±S.D	0.377±0.12	5.460±3.75	4.6±3.03	7.2±4.5
P1		**0.000***	**0.000**	**0.000**
P2			**0.007**	

P1: comparing the mean values of all patients with control subjects
P2: comparing the mean values of chronic to acute patients.
*Statistical significance at P≤ 0.05.

2- Glutathione Parameters:

The mean values of total glutathione (tGSH) and reduced glutathione (GSH) in patients were significantly lower than the corresponding mean values in control subjects. Also the mean level of oxidized glutathione (GSSG) in patients was significantly higher than in control subjects. On the other hand the mean values of these parameters were comparable in acute and chronic patients (Table 4).

3- Redox potential

The mean values of the redox potential in total, chronic and acute patients infected with *T. gondii* were significantly higher than that of the control group. The mean value of this parameter was comparable in acute and chronic patients (Table 4).

Table (4): Redox potential level (mv) and concentration (nmole/ ml) of total glutathione (tGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) in *T. gondii* patients and control subjects

		Control group (n=10)	*Toxoplasma gondii* patients		
			All Patients (n=30)	Chronic group (n=20)	Acute group (n=10)
tGSH	**Range**	11.00-12.5	6.67-10.83	7.5-10.8	6.6-10.4
	Mean±S.D	11.42±0.414	9.17±1.00	9.3±0.85	8.9±1.27
	P1		**0.000**	**0.000**	**0.000**
	P2			**>0.05**	
GSH	**Range**	10.56-12.10	5.85-10.19	6.8-10.2	5.8-9.9
	Mean±S.D	11.01±0.43	8.5±1.06	8.6±0.89	8.4±1.37
	P1		**0.000***	**0.000**	**0.000**
	P2			**>0.05**	
GSSG	**Range**	0.26-0.44	0.48-0.96	0.48-0.96	0.48-0.88
	Mean±S.D	0.38 ± 0.05	0.64±0.15	0.65±0.15	0.59±0.14
	P1		**0.000**	**0.000**	**0.000**
	P2			**P< 0.038**	
redox potential	**Range**	(-52.8)-(-45.1)	(-42.4)-(-21.9)	(-41.2)-(-27.3)	(-42.4)-(-21.9)
	Mean±S.D	-48.14±2.28	-35.04 ±5.34	-34.8±4.62	-35.3±6.82
	P1		**0.000**	**0.000**	**0.000**
	P2			**>0.05**	

P1: comparing the mean values of total *T. gondii* patients to normal control group.
P2: comparing the mean values of chronic to acute patients.
*Statistical significance at P≤ 0.05.

III-Serum sP-selectin Concentration

The mean values of sP-selectin in all, acute and chronic patients infected with *T. gondii* were significantly higher than that of control group. On the other hand there was no significant difference between acute and chronic patients (Table 5).

Table (5): sP-selectin (ng/ ml) in sera of *T. gondii* patients and control subjects

	Control group (n=10)	*Toxoplasma gondii* patients		
		All Patients (n=30)	Chronic group (n=20)	Acute group(n=10)
Range	3.96-9.00	9.19-21.38	10.1-21.4	9.2-19.2
Mean ± S.D	7.28±1.85	14.72±3.85	15.5±3.96	13.2±3.24
P1		**0.000**	**0.000**	**0.000**
P2			**>0.05**	

P1: comparing the mean values of all patients to control group.
P2: comparing the mean values of chronic to acute patients.
*Statistical significance at P≤ 0.05.

IV. Correlation studies (Table 6)

Correlation studies between different parameters in all *T. gondii* patients (n=30) **s**howed positive correlation between sP-selectin concentration and MPV and platelet count (p=0.000, p=0.014 respectively)

On the other hand tGSH concentrations showed a negative correlation with redox potential values (p= 0.000) and a positive correlation with GSH (p=0.000), whereas GSSG showed positive correlation with redox potential (p=0.000) and negative correlation with GSH (p=0.013).

Table (6): Correlations between the studied parameters in all *T. gondii* infected patients (n=30)

	Platelet count	MPV	MDA	tGSH	GSSG	GSH	Redox potential
P-selectin	**r=0.445*** **p=0.014**	**r=0.991** **p=0.000**	r=-0.086 p=0.650	r=0.152 p=0.424	r=-0.079 p=0.678	r=0.154 p=0.415	r=-0.132 p=0.487
MPV	**r=0.411** **p=0.024**		r=-0.100 p=0.599	r=0.133 p=0.484	r=-0.041 p=0.828	r=0.131 p=0.489	r=-0.099 p=0.604
Platelet count			r=0.215 p=0.254	r=0.155 p=0.414	r=-0.210 p=0.266	r=0.176 p=0.352	r=-0.212 p=0.261
MDA				r=-0.294 p=0.115	r=-0.256 p=0.172	r=-0.24 p=0.199	r=0.028 p=0.884
tGSH					r=-0.326 p=0.079	**r=0.991** **p=0.000**	**r=-0.796** **p=0.000**
GSSG						**r=-0.45** **p=0.013**	**r=0.827** **p=0.000**
GSH							**r=-0.869** **p=0.000**

*Pearson's correlation and p values ≤ 0.05 was considered significant.

DISCUSSION

Toxoplasmosis has been proved to be a source of large number of free radicals and biotoxic agents leading to increased oxidative stress resulting in lipid peroxidation with consequent increased production of MDA.[18-20] The present finding of increased serum concentration of MDA in patients infected with *T. gondii* than in control subjects agreed with these studies.

The present finding of significantly

higher mean values of MDA in acute cases than that in chronic cases can be explained by the findings of Aziz et al.[20] They stated that production of the biotoxic agents was more in the acute than in chronic cases infected with *T. gondii*.

The potentially harmful effects of free radicals and ROS are controlled by the cellular antioxidant defense system. GSH is an important constituent of intracellular antioxidant protective mechanisms against a number of noxious stimuli including oxidative stress.[5] Therefore, the higher levels of redox potential and of oxidized glutathione (GSSG) in our *T. gondii* infected patients compared to the control group provide further evidence for the increased oxidative stress in toxoplasmosis.

On the other hand, the decrease in serum concentrations of both reduced glutathione (GSH) and total glutathione (tGSH) in *T. gondii* infected patients than in healthy controls agrees with a previous study[19] and this decrease in GSH and tGSH could be attributed to their depletion in response to increased oxidative stress and results in higher lipid per oxidation due to the lower antioxidant capacity of the cells.[21] In this concern, our results also agree with the study conducted by Azab et al.[2] that *T. gondii*-seropositive blood donors had significantly higher MDA level paralleled with significant decrease in the level of GSH-Px and tocopherol fractions compared with *T. gondii*-seronegative blood donors.

Regarding platelets, although their count in the present study in patients was comparable to that of the control group, the mean value of MPV was significantly higher in patients than in controls. It has been shown that MPV is the most accurate measure of the size and functional status of platelets, as larger platelets are more reactive, i.e. an increased MPV is an indicator of larger and more reactive platelets [22-24] and may represent a risk factor for overall vascular mortality, including myocardial infarction.[25]

The increased MPV (i.e. platelet activity) in our patients is likely to be due to their interaction with tachyzoites mediating acytotoxic effect against free tachyzoites in the presence [26] or absence [27] of IgE antibodies.

Changes that occur during platelet activation affect not only the membrane but also their internal structures, i.e. changes in their shape with formation of pseudopodia and degranulation and the release of P-selectin.[13] It was suggested that platelets are the major source of circulating sP-selectin in healthy individuals and endothelial cell activation can also be involved in the production of soluble P-selectin in plasma.[11,12]

P-selectin is important for initial recruitment, rolling and adhesion interactions between leukocytes and endothelial cells at the sites of inflammation or infection.[28] Also it has a role with its ligand (PSGL-1) in the generation of fibrin and thrombus formation with a state of hypercoagulability.[29,30] This may represent an important physiological trigger of the micro-vascular inflammatory response.[10]

In the present study, serum sP-selectin concentration in patients was significantly higher than in the control group. Our finding of a significant positive correlation between MPV and sP-selectin concentrations supports the previous hypothesis that increased production of sP-selectin is correlated with platelet activation.[31,32]

In addition, Taubert et al.[33] showed that, isolated tachyzoites of *T. gondii* invade bovine umbilical vein endothelial cells in vitro and activate these cells resulting in enhanced P-selectin and other adhesion molecules gene transcription. Thus, in vivo activation of endothelial cells by *T. gondii* could be an additional source for plasma P-selectin in our cases. On the other hand, endothelial cells express a variety of ROS-generating enzymes (ex. Xanthine oxidase), that enhance ROS release and this

can affect platelet activity leading to increased expression of P-selectin.[10]

From the findings of the present study we can conclude that, although toxoplasmosis is mostly asymptomatic, there is a continuous subclinical inflammatory pathology due to released free radicals and ROS that cause lipid peroxidation and cellular injury. In addition, they trigger platelets and endothelial cells resulting in secretion of various pro-inflammatory cytokines (including P-selectin). Accordingly, cases infected with *T. gondii* should be monitored and investigated for early detection of more serious complications, especially in immune-compromised cases. Also, treatment of these patients should not be neglected and simultaneous antioxidant and anti-parasite drugs should be prescribed for them.

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