



Immunological response and markers of cell damage in seropositive horses for *Toxoplasma gondii*



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ABSTRACT

Toxoplasmosis is an important parasitic disease affecting several species of mammals, but little is known about this disease in horses. This study aimed to investigate the levels of several immunological variables and markers of cell damage in the serum of seropositive horses for *Toxoplasma gondii*. Sera samples of adult horses from the Santa Catarina State, Brazil used on a previous study were divided into groups according to their antibody levels for *T. gondii* determined by immunofluorescence assay, i.e. 20 samples from seronegative horses (Group A – control), 20 samples from horses with titers of 1:64 (Group B), 20 samples of horses with titers of 1:256 (Group C), and five samples from horses with titers of 1:1024 (Group D). Positive animals (Groups B, C, and D) had higher levels of immunoglobulins (IgM and IgG), pro-inflammatory cytokines (TNF- α , IFN- γ , IL-1, IL-4, and IL-6) and protein C-reactive protein, as well as lower levels of IL-10 (anti-inflammatory cytokine) when compared to seronegative horses (Group A). The nitric oxide levels were also elevated in seropositive horses. Therefore, we have found humoral and cellular immune responses in seropositive horses, and a correlation between high antibody levels and inflammatory mediators. Markers of cell injury by lipid peroxidation (TBARS) and protein oxidation (AOPP) were elevated in animals seropositive for *T. gondii* when compared to seronegatives. Therefore, seropositive horses to *T. gondii* can keep active immune responses against the parasite. As a consequence with chronicity of disease, they show cellular lesions that may lead to tissue damage with the appearance of clinical disease.

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1. Introduction

Toxoplasmosis is a protozoal disease widely distributed in the American continent and affects many warm-blooded

vertebrates. This disease is caused by *Toxoplasma gondii*, an obligate intracellular parasite of the Sarcocystidae family [1,2]. Felidae are the definitive host and many vertebrate species can be intermediate host, both usually with asymptomatic disease [3]. When clinical signs occur, they may vary among the species involved resulting from a simple fever and mild lymph node enlargement to severe cases with nervous system involvement, blindness, and

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abortions [4–6]. *T. gondii* infection is characterized by a marked cellular immune response [7], which may induce fever [8]. Although clinical disease caused by the parasite is rare, toxoplasmosis is often associated with cases of immunosuppression by being an opportunistic parasite [9].

The immunity against *T. gondii* is complex and involves two types of response: humoral and cellular [10]. The humoral response is characterized by the production of specific immunoglobulins (Ig) by B lymphocytes, mainly IgG and IgM. On the other hand, cellular immune response is mediated by T lymphocytes, which secrete cytokines, well known inflammatory mediators capable of killing extracellular tachyzoites [11].

Among domestic animals, small ruminants are the main ones showing clinical signs of toxoplasmosis, mainly related to reproductive problems [6]. In Brazil, around 32% of horses are seropositive to *T. gondii* [12,13], but the seroprevalence may vary from 0% up to 90% in natural conditions [14]. However, most of them are chronically infected and asymptomatic. According to the literature, clinical signs in horses are different ranging from incoordination, abortions, excessive irritability [15], to hyperthermia, lack of appetite, sluggishness, diarrhea, ocular mucous secretion, and nasal discharge [16]. In view of this, our hypothesis is that equine immune responses against *T. gondii* are lasting, variable and a contributor factor for the disease pathogenesis and cellular lesion. In this context, the aim of this study was to investigate immunological variables and markers of cell damage in the serum of seropositive horses for *T. gondii*.

2. Materials and methods

2.1. Serum samples

Sera samples stored at -80°C of adult horses from the mountainous region (mountainous plateau) the Santa Catarina State, Brazil were used this study. The assessment of IgG anti-*Toxoplasma* was carried out by indirect immunofluorescence assay (IFA), according to the technique described by Camargo [17]. Briefly, the tachyzoites of *T. gondii* (RH strain) were used as antigens, obtained of peritoneal fluid containing parasites from experimentally infected mice. Anti-IgG antibodies of horse, conjugated with fluorescein were used as the secondary antibody in this reaction. A sample was considered as positive when fluorescence occurred on the entire tachyzoite surface and negative when fluorescence was absent, or only apical. Seropositive and negative samples were used as the controls. The sera were tested for antibodies at a dilution of 1:64, and positive samples were tested up to the maximum dilution, at which was possible to detect antibodies against the protozoa.

A total of 45 seropositive horses for *T. gondii* was used in this study, and negative samples for *Neospora* spp. and *Sarcocystis* spp. (analysis by serological tests). Seronegative samples for the three protozoa were used as negative control. Thus, the groups were formed as follows: 20 sera samples from not-infected horses (Group A – control), 20 of *T. gondii* infected horses (Group B – titers 1:64), 20 of

T. gondii infected horses (Group C – titers 1:256), and 5 of *T. gondii* infected horses (Group D – titers 1:1024) in order to evaluate immunoglobulins, cytokines, C-reactive protein levels, concentration of nitrite/nitrate (NO_x), thiobarbituric acid reactive substances (TBARS), and advanced oxidation protein products (AOPP). The samples were processed in duplicate for all those described below. The samples were stored for about six months, and thawed only on the day of immunological and biochemical processing. Group D comprised only five animals, because they are natural, and hard to be identified cases. The other groups were formed by 20 animals, in order to have a meaningful sampling for the variables studied for naturally infected animal, as this natural situation the variation could be great.

2.2. Immunoglobulin levels

Serum IgG and IgM levels were determined using immunonephelometry on the Behring Nephelometer BN II (Dade Behring – USA) with reagents from Dade Behring, and specific kits for IgG (Horse IgG(T) ELISA Quantitation Set[®], Bethyl Laboratories, USA), and IgM (Horse IgM ELISA[®], Kamiya Biomedical Company, Seattle). Briefly, all samples were diluted into specific diluents and measured after 10 min according to technical instructions in kit. Polystyrene particles were coated with a specific monoclonal antibody for each serum protein, forming an agglutinate that disperses the light irradiated in the presence of the protein. The intensity of scattered light depends on the amount of protein concentration in the sample, and the results are compared with known standard curves [18].

2.3. Cytokines levels

Cytokine quantification (TNF- α , INF- γ , IL-1, IL-4, IL-6, and IL-10) was assessed by ELISA assay using commercial Quantikine immunoassay kits (GSI Equine – Plasma/Serum DataSheet, Genorise Scientific, Inc., USA) according to the manufacturer's instructions. Briefly, 96-well microplates were sensitized with primary antibody at room temperature for 30 min; the sample was added and incubated for 30 min at 37°C . After washing, the secondary antibody conjugated with peroxidase was added to each well and incubated. The concentration of the cytokines was determined by the intensity of the color measured spectrophotometrically using a microplate reader.

2.4. C-reactive protein levels

The quantification of serum C-reactive protein (CRP) was performed using commercial kits of ultrasensitive CRP (BioTécnica, Minas Gerais, Brazil), following the manufacturer's protocol at semiautomatic analyzer Bio-2000. The test samples were treated with a specific antibody for horse in a suitable buffer. The turbidity induced by the formation of immune complexes was measured at 540 nm, and the values were then calculated automatically when compared to known standards. All the assay steps were performed automatically by spectrometry (BioTécnica, Minas Gerais, Brazil).

Table 1

Immunoglobulins (IgG and IgM), cytokines (TNF- α , IFN- γ , IL-1, IL-4, IL-6, and IL-10), and C-reactive protein (CRP) in sera samples of horses with different titers of antibodies for *Toxoplasma gondii*.

Variables	Group A (n=20)	Group B (n=20)	Group C (n=20)	Group D (n=5)
IgG (mg/dL)	153.0 \pm 11.0 ^d	181.1 \pm 9.2 ^c	202.6 \pm 8.5 ^b	828.8 \pm 28.3 ^a
IgM (mg/dL)	55.0 \pm 6.0 ^d	107.5 \pm 8.7 ^c	148.1 \pm 9.6 ^b	337.5 \pm 26.5 ^a
TNF- α (pg/mL)	124.0 \pm 7.0 ^d	159.6 \pm 10.3 ^c	191.1 \pm 9.2 ^b	261.5 \pm 15.5 ^a
IFN- γ (μ g/mL)	152.4 \pm 4.0 ^d	192.1 \pm 12.1 ^c	254.5 \pm 8.2 ^b	311.0 \pm 10.8 ^a
IL-1 (pg/mL)	62.0 \pm 5.0 ^d	72.5 \pm 6.3 ^c	118.2 \pm 9.5 ^b	146.5 \pm 15.2 ^a
IL-4 (pg/mL)	39.2 \pm 5.2 ^d	55.6 \pm 4.8 ^c	94.2 \pm 3.9 ^b	208.0 \pm 14.0 ^a
IL-6 (pg/mL)	77.0 \pm 10.0 ^d	103.6 \pm 9.4 ^c	149.6 \pm 4.8 ^b	197.5 \pm 19.2 ^a
IL-10 (pg/mL)	72.0 \pm 7.0 ^a	47.0 \pm 4.5 ^b	28.6 \pm 4.9 ^c	17.8 \pm 2.0 ^d
CRP (mg/dL)	0.45 \pm 0.2 ^d	2.0 \pm 0.6 ^c	16.2 \pm 5.2 ^b	38.3 \pm 13.2 ^a

Means and standard deviation followed by different letters in the same line differ statistically among themselves, the significance of 1% ($P < 0.01$). Group A: seronegative horses; Groups B, C and D seropositive horses with antibody titers of 1:64, 1:256, and 1:1024 for *T. gondii*, respectively.

2.5. Nitric oxide levels

Nitric oxide levels in serum of *T. gondii* seropositive horses were evaluated indirectly by NO_x quantification according to the technique described by Tatsch and cols [19], and its levels were expressed in micromoles per liter.

2.6. Lipid peroxidation and protein oxidation

Lipid peroxidation (determined by TBARS levels) was measured in sera samples as described by Jentzsch and cols [20]. Results were obtained by spectrophotometry at 535 nm and expressed as nanomoles of malondialdehyde per milliliter of serum. In addition, protein oxidation was assessed through measurement of AOPP concentrations by a method previously described by Hanasand and cols [21]. Results were expressed as micromoles per liter.

2.7. Statistical analysis

The data from these studies showed normal distribution checked with normality test. Immunoglobulins, cytokines, and C-reactive protein results were subjected to analysis of variance (ANOVA) and Tukey test. The relation of TNF level on the concentration of TBARS and AOPP in sera was evaluated by linear correlation. Values with probability (p) less than 1% were considered statistically different. Data were presented as mean values \pm standard deviation (SD).

3. Results

3.1. Immunological variables

The results of immunoglobulins, cytokines, and C-reactive protein in *T. gondii* seropositive horses were shown in Table 1. The levels of immunoglobulins (IgG and IgM),

cytokines (TNF- α , IFN- γ , IL-1, IL-4, and IL-6), and C-reactive protein were higher in positive horses compared to those negatives for the disease. It was also found differences in the levels of these immunoglobulins and inflammatory mediators among seropositive groups for *T. gondii* (Group B, C and D), i.e., samples from groups with the highest titer (Group D – 1:1024) also showed high level of pro-inflammatory cytokines and immunoglobulins compared to the others (Table 1). The levels of IL-10 in horses seropositives for toxoplasmosis was lower compared to seronegatives, and an inverse correlation between antibody concentration and levels of IL-10 was observed.

3.2. Markers of cellular injury

High levels of NO_x, TBARS and AOPP were observed in the serum of horses seropositives for *T. gondii* when compared to seronegatives (Table 2). Seropositive animals showed high levels of antibodies for *T. gondii* (Groups B, C and D), and high concentrations of markers of cell lesion (Table 2) and a positive correlation between them. As an example, a positive correlation between TNF levels and the concentration of TBARS ($r=0.94$) and the AOPP ($r=0.86$) was observed in this study.

4. Discussion

The present study determined significant differences in all variables analyzed on sera samples from horses *T. gondii* seropositives and seronegatives. The seropositive horses had no record of clinical disease; however showed different degrees of pro-inflammatory cytokines. Abortions are generally associated with inflammatory infiltrates in the placenta, but according to the literature horses were occasionally affected by abortion related to toxoplasmosis [14].

Table 2

Markers of cellular lesion in seropositive horses for *Toxoplasma gondii* compared to seronegatives: nitrite/nitrate (NO_x), lipid peroxidation (TBARS), and protein oxidation (AOPP).

Variables	Group A (n=20)	Group B (n=20)	Group C (n=20)	Group D (n=5)
NO _x (μ mol/L)	76.0 \pm 39.0 ^c	162.8 \pm 47.2 ^b	182.6 \pm 55.1 ^b	303.6 \pm 37.2 ^a
TBARS (μ mol MDA/L)	11.5 \pm 3.1 ^d	19.7 \pm 4.2 ^c	29.4 \pm 4.1 ^b	47.1 \pm 12.8 ^a
AOPP (μ mol/L)	9.1 \pm 3.8 ^c	13.7 \pm 3.9 ^{bc}	16.5 \pm 3.2 ^b	25.2 \pm 7.1 ^a

Means and standard deviation followed by different letters in the same line differ statistically among themselves, the significance of 1% ($P < 0.01$). Group A: seronegative horses; Groups B, C, and D seropositive horses with antibody titers of 1:64, 1:256, and 1:1024 for *T. gondii*, respectively.

As a result, we try to relate our findings with pathologic changes arising from parasitism.

Horses *T. gondii* seropositives showed high levels of immunoglobulins. Kishiyama [22] have reported the participation of immunoglobulins in the destruction and elimination of parasites, helping to reduce the spread of the etiological agent. The antibody levels were detected in indirect immunofluorescence is IgG and IgM, and both were increased, but probably other immunoglobulins were also elevated in these naturally infected animals.

IFN- γ is related to disease control, directly interfering with tachyzoite proliferation [23]. Titus and cols [24] stated that the first host response against infection is the secretion of cytokines such as TNF- α , IFN- γ , IL-1, IL-4, and IL-6, which in the current study were high in seropositive horses for *T. gondii* compared to seronegatives (Table 1). On the other hand, IL-10 concentration was low in positive animals, an interleukin with anti-inflammatory properties [25]. IL-10 is a pleiotropic cytokine, known by its inhibitory effect on the synthesis of pro-inflammatory cytokines like TNF- α [26,27] with positive and negative effects on the host. TNF- α can lead to tissue damage during *T. gondii* infection, and our study confirmed it by the positive correlation found between TBARS and AOPP. It is noteworthy that inflammation contributes to the phlogistic process causing exacerbation of the clinical signs and eventually host death [28,29].

In a study of patients with positive serology for toxoplasmosis, researchers found that high levels of C-reactive protein were associated with acute infection or recurrent [30]. In our study, this acute phase protein was higher (Table 1), as well as other immunological variables quantified in horses. In general, the increase of cytokines and C-reactive protein are related to a mechanism of host protection, in order to keep the infection under control. This hypothesis was confirmed when we compared concentrations of different immunological variables of horses with high titers for *T. gondii*, which might be a consequence of recent infection or reactivation of it.

Increased nitric oxide (NO) levels in serum from *T. gondii* infected mice have been described previously [31], as well as in other species, but there are no studies on seropositive horses for this protozoan. NO is an important molecule in different physiological activities, acting as an important inflammatory mediator. According to research, increased NO levels are directly related to the control of parasitemia inhibiting *T. gondii* replication [32]. However, the increase of NO can be toxic and can cause cellular damage in the brain, since the NO can generate peroxynitrite, a molecule with cytotoxic effect. In our study the NO_x levels were elevated, a possible consequence of the immune response against *T. gondii* infection, this concomitantly may have contributed to tissue damage.

Highly reactive ROS have been indicated in the pathogenesis of many protozoan infections including *T. gondii* [31]. In this study, seropositive horses showed an enhanced lipid peroxidation confirmed by increased TBARS levels that may be considered a sign of cellular lipid oxidation [33], since MDA is the main lipid peroxidation product often used as a marker for oxidative stress in several diseases [34] along with increased AOPP levels may

characterize protein oxidation [35,36]. So, despite the fact that seropositive horses are apparently asymptomatic, cell and tissue injuries are occurring, which may be a consequence of the constant inflammatory process. Important to point out: higher levels of antibodies and cytokines are correlated to higher levels of TBARS and AOPP.

Therefore, it is possible to conclude that seropositive horses for *T. gondii* maintain their immune responses, and as a consequence they show cellular lesions that may lead to tissue damage, clinical disease, and chronicity.

Commission of ethics and animal welfare

This research project was approved by the Ethics Committee on Animal Experiments of UDESC under protocol number 1.05.09.

Conflict of interest

The authors have declared no conflict of interest.

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