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Serum levels of nitric oxide and protein oxidation in goats seropositive for *Toxoplasma gondii* and *Neospora caninum*

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ABSTRACT

The aim of this study was to assess and analyze the levels of nitric oxide (NO) and advanced oxidation protein products (AOPP) in serum of goats naturally infected by *Toxoplasma gondii*, *Neospora caninum*, or concomitantly infected by these two parasites. Thus, it was measured NO_x and AOPP levels in twenty ($n = 20$) sera samples of goats seronegative for *T. gondii* and *N. caninum* [negative control group (A)]; while the positive groups were composed by sera of infected animals, twelve ($n = 12$) seropositive for *N. caninum* [group B]; eighteen ($n = 18$) positive for *T. gondii* [group C]; and thirteen ($n = 13$) seropositive for *N. caninum* and *T. gondii* [group D]. As results, it was observed that animals seropositive for *N. caninum* and *T. gondii* (Groups B to D) showed higher serum levels of NO_x ($P < 0.001$; $F = 9.5$), when compared with seronegative animals. Additionally, it was observed a positive correlation between NO_x levels and antibodies titrations for *N. caninum* ($P < 0.01$; $r = 0.68$) and *T. gondii* ($P < 0.05$; $r = 0.56$). AOPP levels were increase in groups C and D ($P > 0.05$). Interestingly, group B did not show increase in AOPP, what led us to hypothesize that the major protein damage is linked to *T. gondii* infection. Therefore, our results showed an increased in NO_x levels, which was probably related to the immune response, since it is an important inflammatory mediator; and AOPP were increased in groups where there was seropositivity for *T. gondii*, but not for the group composed only by animals seropositive for *N. caninum*, allowing us to suggest higher protein damage in toxoplasmosis.

1. Introduction

Toxoplasma gondii and *Neospora caninum* are closely related protozoan parasites with a worldwide distribution [1,2]. *T. gondii* can affect many animal species, including humans, and can cause disease varying from subclinical to fatal infection depending on the host immune status and species, consisting in a major cause of abortion in sheep and goats [3]. Contrary to *T. gondii*, *N. caninum* is one of the most causes of abortion and neonatal mortality in cattle [4], but it was already reported causing severe disease also in sheep and goats [5].

Both protozoan diseases usually are able to trigger different nonspecific host response mechanisms [6]. One of these nonspecific mechanisms is represented by regulation of nitric oxide (NO) expression [7]. It is known that endogenous and exogenous NO is able to generate antiparasitic effects on Protozoa and Metazoa; however, NO production requires a significative and precise control in order to limit cytotoxic damage to the host's own cells [6]. Besides its antiparasitic effects, NO also can be subverted in some immunologic functions in benefit of the parasites, e.g., suppressing leukocytes adherence [8–11], and inhibiting (or promoting a downregulation) in cytokines production by macrophages [12,13]. Currently, some parameters have been used for determining oxidative stress, such as advanced oxidation protein products (AOPP). This marker identifies oxidative damage to proteins [14].

Considering the importance of *T. gondii* and *N. caninum* in animal's production and the puzzling functions of nitric oxide (NO) and the action of advanced oxidation protein products (AOPP) as

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a biomarker of protein oxidation, the objective of this study was to assess and analyze the levels of NO and AOPP in serum of seropositive goats for *Toxoplasma gondii* or *Neospora caninum*, or concomitantly infected by these two protozoans.

2. Material and methods

2.1. Samples

Serum samples of goats ($n = 654$) from the western and mountainous mesoregions the Santa Catarina state (SC/Brazil) were used for the assessment of two infectious diseases (*T. gondii*, and *N. caninum*) through serological tests. The samples were collected in the period from August 2012 to March 2013 according to the distribution of goat flocks in SC, according to recommendations from epidemiological research [15]. This study quantified the municipalities with the highest number of animals in both regions. Collected blood samples, began the processing of the same for serological tests for *T. gondii* and *N. caninun*. In this study prioritized only serological tests for these two parasites.

The assessment of IgG anti-*Neospora* and anti-*Toxoplasma* was carried out by indirect immunofluorescence assay (IFA). Briefly, tachyzoites of *N. caninum* (NC-1 strain) were used as antigens, grown in *Vero* cells. 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 goat, conjugated with fluorescein were used as the secondary antibody in this reaction [16]. A sample was considered as positive when fluorescence occurred on the entire tachyzoite surface and negative when fluorescence was absent, or only apical [17]. Seropositive and negative samples were used as the controls. The sera were tested for antibodies at a dilution of 1:50 (*N. caninum*) and 1:64 (*T. gondii*). Positive samples were tested up to the maximum dilution, at which was possible to detect antibodies against the protozoa. Based on serological testing it was possible to form the experimental design as described below.

2.2. Experimental design

Our experimental design was composed by: twenty ($n = 20$) sera samples of goats seronegative for *T. gondii* and *N. caninum* [group A]; twelve ($n = 12$) sera of goats seropositive for *N. caninum* [group B]; eighteen ($n = 18$) sera of goats positive for *T. gondii* [group C]; and thirteen ($n = 13$) sera of goats seropositive for *N. caninum* and *T. gondii* [group D]. Goats seropositive for *N. caninum* had titrations ranging from 1:50 to 1:400; while animals positive for *T. gondii* showed titrations ranging from 1:64 to 1:256.

To reduce the influence of external and internal factors were included in the study only animals with good body condition, females, crossbred, and older than 1 year. As the animals were from different properties, diets also were different, with some animals received only forage while others received forage and concentrate supplementation. Therefore, the influence of diet on biochemical variables studied was included in the statistical analysis.

2.3. Nitric oxide assessment

Nitric oxide levels in serum of goats were analyzed indirectly, by the quantification of nitrite/nitrate (NO_x) [18]. Therefore, NO_x was measured by the modified Griess method using the Cobas Mira automated analyzer (Roche Diagnostics, Basel, Switzerland). Results were expressed in μmol/L.

2.4. Protein oxidation

Protein oxidation was assessed through measurement of AOPP concentrations by a method previously described [14]. Briefly, 200 μL of serum diluted 1/5 in PBS was placed on a 96-well microtiter plate, and 20 μL of acetic acid was added. AOPP were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions that in the presence of potassium iodide absorb at 340 nm. Results were expressed as micromoles per liter [14].

2.5. Statistical analysis

The data were evaluated by the Student's *t*-test. Values with probability less than 5% were considered statistically different. The effects of antibodies level to *T. gondii* and *N. caninum* (groups B and C) on NO_x levels were analyzed by linear correlation. The effect of diet on NO_x and AOPP levels were analyzed into an unvariable logistic regression model (chi-square test).

3. Results and discussion

Results of NO and AOPP assessment are shown in Table 1. Seropositive animals for *N. caninum* and *T. gondii* (Groups B to D) had higher levels of NO_x in serum ($P < 0.001$) when compared with seronegative animals (group A). Additionally, it was observed a positive correlation between NO_x levels and antibodies titrations for *N. caninum* ($P < 0.01$; $r = 0.68$) and *T. gondii* ($P < 0.05$; $r = 0.56$) (Fig. 1).

Table 1
Mean and standard error of the levels of nitrite/nitrate (NO_x) and advanced oxidation protein products (AOPP) in the serum of seronegative goats (group A – control), seropositive for *Neospora caninum* (group B), seropositive for *Toxoplasma gondii* (group C), and seropositive for *N. caninum* and *T. gondii* (group D – mixed infection).

Groups	NO_x (μmol/L)	AOPP (μmol/L)
A	65.2 ± 7.1^a	17.6 ± 4.9^a
B	176.8 ± 13.1^b	20.2 ± 6.4^a
C	182.9 ± 29.4^b	31.5 ± 6.8^b
D	214.8 ± 32.2^b	47.6 ± 11.3^c
P	<0.001	<0.001

Note: Means with equal letters, on the same line, do not differ statistically among themselves to 5% of significance ($P > 0.05$).

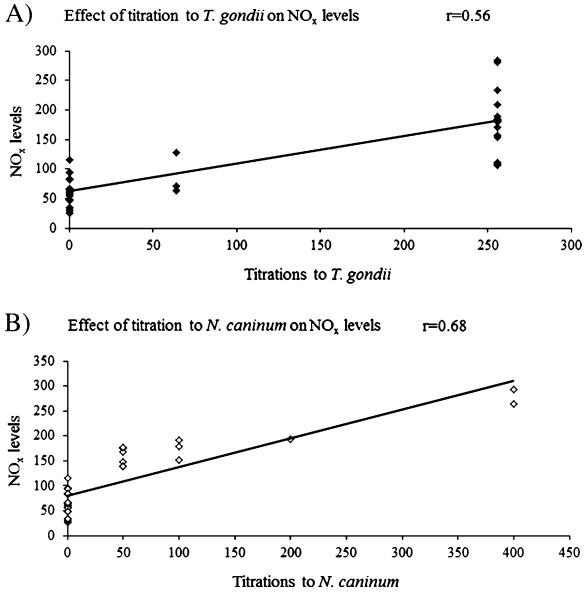

Fig. 1. Relationship between antibody levels to *Toxoplasma gondii* (A) and *Neospora caninum* (B) on NO_x levels (nitrite/nitrate) in goats naturally infected by these parasites. *Note*: The negative control group was correlated with the groups infected with *T. gondii* and *N. caninum*.