THE INFLUENCE OF SALT INTAKE ON THE COURSE OF EXPERIMENTAL TOXOPLASMOSIS IN OUTBRED OR INBRED MOUSE STRAINS

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ABSTRACT

Many environmental factors contribute to an effective immune response against Toxoplasma gondii (Tg) infection, among which diet is important in triggering the immune response of the host to infection. Emerging reports suggest that salt intake undermines the regulatory mechanisms mediated by innate and adaptive immune cells. Unfortunately, the impact of an Intermediate Salt Diet (ISD) on the pathogenesis and immune response to toxoplasmosis remains unclear. The purpose of this study was to evaluate the susceptibility profile to an ISD (NaCl 2%) of two mouse strains (outbred Swiss and inbred C57BL6) infected by the ME49 strain of Tg. Our data confirm an antagonistic susceptibility to oral Tg infection among the two mouse strains. Sodium intake induced the highest mortality in C57BL6 compared to Swiss mice in the infected groups. A simultaneous ISD with the infection did not induce significant differences in body weight in either mouse strains. Both mouse strains showed an antagonistic response to a sodium intake diet on the number of parasite brain cysts. An increased number of brain cysts in C57BL6 ISD-Tg animals were noted while Swiss ISD-Tg animals presented a decrease in the number of brain cysts compared to NSD-Tg (Normal Salt Diet) for both mouse strains. Furthermore, sodium intake caused a significant reduction in the specific humoral immune response against Tg in inbred C57BL6 mice. Thus, our data reveal that an ISD affects the humoral immune response in the murine model and influences the course of Tg infection.

KEY WORDS: Toxoplasma gondii; sodium intake; infection.
INTRODUCTION

Toxoplasma gondii (Tg) is an obligate intracellular parasite that can infect any nucleated cell of warm-blooded vertebrates, belonging to the Apicomplexa group (Oliveira, 2016c). It is widely distributed in nature and can infect a range of hosts, including more than one-third of the world population, causing toxoplasmosis (Tenter et al., 2000). Toxoplasmosis is a common benign infection in immune competent individuals but may cause serious clinical manifestations in immunosuppressed patients and vertically infected children (Costa et al., 2008; Weiss & Dubey 2009; Wang et al., 2014).

The clinical manifestations of toxoplasmosis may be heterogeneous depending largely on the immune status of the host, which is defined by both genetic and environmental determinants. Some comorbidities among seropositive patients include neoplasms, chronic kidney disease, schizophrenia, and diabetes mellitus. In turn, diabetes has comorbidity with hypertension, being common, intertwined conditions that share a significant overlap in underlying risk factors, i.e., lifestyle determinants as well as sharing their complications (Long & Dagogo, 2011).

Amar and colleagues (2007) showed that diet could alter the immune response to infections. In this case, their data indicate that obesity interferes with the immune system. Jantsch et al. (2015) found that an increased sodium chloride concentration augments LPS-mediated and IL-1α or IL-1β + TNF-induced macrophage activation. Additionally, they demonstrated that increasing the sodium chloride concentration in the cell culture medium boosted nitric oxide (NO) production in Escherichia coli-infected macrophages and promoted E. coli removal and, similarly boosted Leishmania major elimination in lipopolysaccharide (LPS)-treated macrophages. Furthermore, salt overconsumption can cause the development of autoimmune diseases by inducing pathological Th17 (Kleinewietfeld et al., 2013; Wu et al., 2013), which is also important for the toxoplasmosis pathogenesis (Miller et al., 2009). Despite advances in the understanding of the effects of salt consumption in several situations, knowledge of its influence on the clinical course of some diseases remains scarce.

To date, available knowledge is still limited concerning the prominent role of salt overconsumption as an unconventional risk factor upon the clinical course and medical comorbidities in parasitic infections, such as toxoplasmosis. In this sense, the purpose of this study was to reveal the susceptibility of two mouse strains submitted to an intermediate sodium intake diet and infected by Tg.
MATERIALS AND METHODS

Animals and parasite strain

Swiss-Webster (n = 11) and C57BL6 mice (n = 10) (6 - 8 weeks of age) were used for all the tests and received water and food ad libitum. The choice of two strains of mice was due to the differences in mortality after infection with Tg (Oliveira et al., 2016a). Thus, it was possible to observe different patterns of infection evolution in outbred (Swiss) animals, generally more resistant, and inbred (C57BL6), which are more susceptible to the parasite.

Experiments were performed using the Guidelines for Ethical Conduct in The Care and Use of Animals from the Federal University of Rio Grande do Norte (CEUA-UFRN, Protocol number 46/2013).

The Tg (ME-49 strain) was maintained in Swiss female mice, and tissue cysts were obtained from the macerated brain of infected animals.

Intermediate salt diet

The intermediate salt diet (ISD) was developed according to Oliver et al. (1997, 1998), with modifications. Briefly, male C57BL6 or Swiss received water and food ad libitum (NSD) or normal chow and water containing 2% NaCl (ISD) ad libitum throughout the experimental period.

Infection and Anti-Toxoplasma gondii IgG ELISA

The animals were weighed on days 0 and 2 after the beginning of the ISD. On the second day, they were orally infected with 10 (C57BL6) and 25 cysts (Swiss) of the ME49 strain of Tg (Oliveira et al., 2016b). The weight alterations were monitored weekly. The animals also had blood samples collected at 14 and 30 days after infection for quantification of serum IgG antibodies. After the 30 day observation period, the animals were humanely euthanized and the brains were macerated for cyst quantification by light microscopy (Oliveira et al., 2016b).

Serum IgG concentrations were quantified only in the groups of C57BL6 mice, since these presented a more homogeneous immune pattern, from a pool of each group by Enzyme-Linked Immunosorbent Assay (ELISA) (Alvarado-Esquivel et al., 2011; Oliveira et al., 2016b). Briefly, 96-flat-bottom-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with wells containing Tg lysate antigen (TLA) in 50 mM pH 9.6 sodium carbonate buffer at a final concentration of 1 μg/mL and volume of 100 μL were incubated overnight at 4°C. The plates were then washed 4 times with
pH 7.4 PBS containing 0.05% Tween 20 (PBS-T). Next, nonspecific sites were blocked with a 2% milk solution (Molico®) for 1h at 37 °C. Subsequently, serum dilutions of 1:200 in PBS were added to triplicate wells and the plates were again incubated. Anti-mouse IgG conjugated with peroxidase enzyme (Life Technologies) was applied (100 μL/well), for 1 h at 37°C. After that, the plates were incubated for 10 min at room temperature with 50 μL/well of chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen Life Technologies, Gaithersburg, MA, USA). The reaction was interrupted by adding 30 μL/well of 4 N H₂SO₄ and absorbance was read at 450 nm.

**Statistical analysis**

Shapiro-Wilk’s and Levene’s tests were used to analyze data normality and homogeneity of variance, respectively. Parametric tests were used accordingly for data distribution and homogeneity of variance. Two-way analysis of variance (ANOVA) with repeated measures-with treatment and strain as between-subject factors and post-infection day as within-subject factor were applied to body weight alteration, number of brain cysts and for antibody production to assess effects between treatment and mice lineage throughout the experiment. Both tests were followed by Sidak’s post hoc test to highlight differences between strain and treatment groups. Results were expressed as mean ± SEM and p < 0.05 was considered to reflect significant differences. Exact p-values were expressed for each factor and factor interactions for the two-way ANOVA while differences highlighted by the Sidak’s post hoc test assumed p < 0.05. Statistical analysis and graphic design were performed using SPSS and GraphPad 5.0 software, respectively (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

The alterations in body weight during the experimental protocol are presented in Figures 1A and 1B. There were only slight alterations between the two mouse strains in which the sodium diet induced lower body weight change, in C57BL6 mice compared to the same group of Swiss mice. The body weight alterations were verified on the 7th day of the experiment and from then on both NSD Swiss and C57BL6 mice showed a progressive increase in body weight throughout the experiment. NSD-Tg C57BL6 animals presented a pronounced loss of 20% in body weight compared to a slight variance in body weight displayed by Swiss mice (p < 0.05) throughout the experimental protocol. Meanwhile, the ISD Swiss group showed similar body weight when compared with the NSD Swiss group but a progressive gain in body weight compared to the ISD C57BL6 (p < 0.05) group on the 28th day of treatment. Moreover, both the ISD-Tg Swiss and the C57BL6 mouse strain showed a
decrease in body weight from the 14th day of infection and a slight decrement until the 30th day of the experiment.

As shown in Figure 1C and 1D, following oral infection with Tg cysts (ME49 strain), C57BL6 mice exhibited a higher mortality rate compared to the Swiss mice. The Kaplan-Meier survival curve for the C57BL6 mice shows some mortality among all the infected mice although Tg-infected animals submitted to the salt diet displayed a higher mortality rate than the others (Figure 1D). Ten percent of the NSD-Tg mice died as early as day 9 post-infection and reached 90% survival rate up to day 30. Infected ISD-Tg mice, for instance, succumbed to infection as early as day 9 post-infection, and numbered 60% survivors on day 30 after infection (p < 0.05). In contrast, the Swiss mice in all groups displayed high resistance to infection, reaching 100% survival rate by day 30 post-infection (Figure 1C), and did not show any symptoms after infection.

*Figure 1. Average body weight loss (A, B) and survival rates (C, D) of Swiss (A, C) or C57BL6 (B, D) mice infected with ME49 strain of *T. gondii* and submitted to sodium intake. Data is presented as the mean ± S.E.M percentage of the original body weight. The percentage of body weight (bars indicate SEM) was monitored during the experimental protocol. The statistical significance of differences (p < 0.05) in mortality between groups was determined using the Kaplan-Meier method, and analyzed with a Log-rank (Mantel-Cox) test. *p < 0.05 for NSD vs all groups, #p < 0.05 for NSD vs ISD-Tg and NSD-Tg. ISD-Tg: mice infected under intermediate sodium intake; NSD-Tg: mice infected under normal water intake; ISD: non-infected mice under intermediate sodium intake; NSD: mice non-infected under normal water intake.*
The cyst quantification (Figure 2A) between both NSD-Tg and ISD-Tg revealed an opposite phenomenon concerning the number of brain cysts within the two mouse strains, as noted in the Swiss and C57BL6 mice. Significant differences were detected in the numbers of brain cysts in the Swiss mice compared to the C57BL6 mice. As noted, the NSD-Tg Swiss mice presented a greater quantity compared to the NSD-Tg C57BL6 mice (p = 0.032) while under ISD conditions a decrease was noted in the number of cysts in the Swiss mice whereas a greater quantity occurred in the C57BL6 mice (p = 0.048). Under ISD conditions, the number of brain cysts was inverted in both mouse strains. A smaller quantity of cysts was noted in the ISD-Tg Swiss mice and an increased number of cysts in the ISD-Tg C57BL6 mice as compared to NSD conditions (p = 0.048).

As shown in Figure 2B, the quantification of Tg specific antibodies demonstrated that the experimentally infected C57BL6 mice presented a similar pattern of progressive increase in humoral immune response throughout the experimental protocol. All the infected groups showed higher antibody titers compared to the non-infected animal groups after the 14th day post-infection (p < 0.05). On day 30, the lowest titer was detected in the ISD-Tg animals compared to the NSD-Tg animals (p < 0.01).

Figure 2. (A) Number of brain cysts in Swiss and C57BL6 mice infected with ME49 strain of *T. gondii* and submitted to sodium intake and (B) Time-course analysis of the specific anti-*T. gondii* IgG antibody production in C57BL6 mice infected with ME49 strain of *T. gondii* and submitted to sodium intake. Data is presented as the mean ± S.E.M. *p < 0.05 for NSD-Tg Swiss vs NSD-Tg C57BL6; *p < 0.05 for ISD-Tg vs NSD-Tg; & p < 0.05 for ISD-Tg C57BL6 vs ISD-Tg Swiss; #p < 0.05 for NSD-Tg vs all groups; ISD-Tg: mice infected under intermediate sodium intake; NSD-Tg: mice infected under normal water intake; ISD: non-infected mice under intermediate sodium intake; NSD: non-infected mice under normal water intake.
DISCUSSION

An important tool for understanding the life cycle characteristics of a parasite or clinical course of a disease is the constant search for new situations that may affect this cycle and hence its evolution. This study, based on the hypothesis and experimental data presented, proved that the dietary intake of sodium chloride influences the course of *in vivo* toxoplasmosis.

The severity of murine toxoplasmosis depends on many factors including dosage, mouse strain, inoculation route, and life stage of the parasite. The comparison between resistant and susceptible mouse strains allowed us to characterize protective and harmful response patterns. It is noteworthy that C57BL/6 are considered more susceptible to Tg infection than inbred BALB/c or outbred Swiss Webster mice when inoculated with the type II (ME-49) strain of the parasite (Araujo et al., 1976; Suzuki et al., 1995, 2000). Several oral cyst mouse infection models have been described to study the immunity and pathogenesis of orally induced toxoplasmosis (McLeod et al., 1989; Liesenfeld, 2002; Dubey et al., 2012), but not under sodium intake conditions.

Earlier reports provided valuable evidence about the importance of sodium chloride in the pathogenesis of various diseases in animal models (Beebe et al., 1976; Dobrian et al., 2003; Koleganova et al., 2011). *In vivo* and *in vitro* Jantsch et al. (2015) showed that salt boosts classical macrophage activation and increases leishmanicidal activity through an increase in NO production. In this model, the high-salt diet promoted skin Na⁺ storage and ameliorated cutaneous leishmaniasis. In mice, pathological changes induced by the sodium consumption appeared to be related to the differences characteristic of each strain (Hartner et al., 2003). Some recent studies have also documented the negative impact of sodium consumption on the balance between inflammatory innate and adaptive immune responses, enhancing pro-inflammatory responses and impairing regulatory mechanisms (Kleinewietfeld et al., 2013; Wu et al., 2013; Hernandez et al., 2015).

Here we performed the morbidity analysis by quantifying body weight alterations for both mouse strains. Our data revealed morbidity effects on body weight change, where the weight loss was especially significant in the NSD C57BL6-infected mice and is explained by the greater vulnerability of the lineage (Rai et al 2009). Due to increased fluid retention, by lymphatic capillary hyperplasia (Machnik et al., 2009), and other reasons, higher weight in healthy animals with an ISD rather than a NSD would be more likely. However, this effect was not observed. The C57BL6 lineage characteristics could explain the greater morbidity of these animals when infected, which was evidenced by a greater weight loss in Tg-infected ISD animals.
Mortality occurred after infection only in the C57BL6 mice. As previously mentioned (Araujo et al., 1976; Suzuki et al., 1995, 2000), this strain presents high susceptibility to Tg, which would justify the larger number of dead animals. However, mortality was even higher in the group receiving the ISD than the NSD diet, which may be explained by a change in the immune response of this group. Binger et al. (2015) reported that excessive and chronic sodium chloride intake causes the activation of pro-inflammatory cells such as Th17, and M (LPS) macrophages are boosted whereas cells such as M (IL-4+IL-13) macrophages are disfavored. Finally, they hypothesized that the overall balance between effector and regulatory arms of the immune system is disturbed by a high sodium concentration. Eddie & Medzhitov (2015) reported that macrophage inflammasomes can sense hypertonic stress caused by a high salt diet, promoting local inflammation and induction of Th17 response.

Thus, it is possible that the increased intake of sodium chloride and the immune imbalance (Kearney et al., 2005; Eddie & Medzhitov, 2015) combined with the high prevalence of Tg (Pappas et al., 2009) could increase the number of cases of severe clinical or fatal toxoplasmosis (Mendes et al., 2014; Way & Seinfeld, 2017). The usually fatal disease is also associated with atypical clonal strains of the parasite (Oliveira et al., 2017), which present well-defined characteristics in murine models, but are still inconclusive in humans (Boothroyd & Grig, 2002; Dolliwa et al., 2013).

The number of cerebral cysts shows an interesting and pronounced difference in susceptibility between mouse strains, as observed by other researchers (Rettigner et al., 2004). Despite remarkable evidence of the pro-inflammatory immune response, the clinical outcomes of sodium intake appear according to the genetic background of the animals (Kaushik et al., 1999; Duleu et al., 2004). Hence, high salt intake can trigger an increase in the innate immune response and help prevent infections (Jantsch et al., 2015) but may also impair the development and functionality of Th2-cytokine-driven alternative macrophage activation, which is required for tissue repair, inflammation resolution as well as effective Tg infection control. This control usually results in the formation of parasite cysts. Differences in the homeostasis of the different groups of animals with functional consequences in the macrophages caused by the higher sodium content in their diet may explain the reversal in the cyst pattern that was noted in the animals with ISD. Ueno et al. (2014) showed that peripheral blood monocytes infected with Tg could function as ‘Trojan horses’ for parasites spread in the bloodstream. Alterations in this mechanism induced by sodium chloride could explain the differences in the cerebral parasite load.

As shown earlier, the number of brain cysts depicts an intrinsic susceptibility profile in mouse strains (Torrentera et al., 2002). In addition, the metabolic interactions that occur in infected animals under a high sodium intake need to be better understood. Navarro et al. (1992) have long shown the
resistance profile to high concentrations of sodium chloride by Tg. Similarly, Pott et al. (2013) recently found an infective muscle tissue cyst up to 8 days after the addition of NaCl 2%. However, so far no research could identify the mechanisms associated with infection and high sodium intake related to the formation of cysts.

In relation to the humoral immune response, Rai et al. (2009) found a marked difference between inbred and outbred murine hosts. In this study these presented a coordinated humoral response, reaching similar magnitude and kinetics when analyzed longitudinally in individual inbred hosts. Contrarily, outbred hosts fail to achieve the protective immune memory in all individuals. In this regard, when the antibody production in infected animals under ISD or NSD conditions was evaluated, according to the strain differences in mice, we prioritized the specificity of the humoral response in inbred mice. Therefore this study observed a significant reduction of IgG antibodies in the infected animals that were submitted to an ISD. This reduction is important because it would limit the immune response of these animals (Couper et al., 2005). IgG antibody levels increased significantly and high titers persisted throughout the course of the infection. This increased humoral immune response requires the specific participation of a functionally distinct subset of CD4+ T cells known as T follicular helper (TFh) cells (Johnson et al., 2002; Langhorne et al., 1998). New data also reveals that IL-10-producing B regulatory cells are crucial in the course of Tg infection to induce chronic infection with cyst formation (Jeong et al., 2016). Notably, the humoral immune response has an important role in controlling cellular invasion, avoids bloodstream dissemination of Tg tachyzoites and promotes resistance to parasite infection (Couper et al., 2005). Notably, the absence of B cells or antibody secretion results in lethal infection by protozoan parasites. Liu et al. (2012) report evidence that malarial infections result in a decrease in the proportion of Dendritic Cells (DCs) that express the B-cell survival factor (BAFF) resulting in a decreased ability of these DCs to support memory B-cell differentiation into antibody secreting cells and/or the survival of these cells. In addition, Radwanska et al. (2008) relate that ongoing trypanosome infections result in a rapid loss of B cell responsiveness and prevent the induction of protective memory responses. Thus, a possible reduction in the number of B-cells caused by sodium intake could be the cause of the higher mortality rate in C57BL6 mice.

In this sense, Neuhofer (2010) report that the NFAT5 (Nuclear factor of activated T cells 5) signaling in macrophages is a major determinant of extracellular volume and hence blood homeostasis under high salt intake conditions. Additionally, Kino et al. (2009) noted that Brx-mediated induction of the NFAT5 expression on osmotic stress participates in the elevation of intracellular osmolarity and plays an essential role in the production of various cytokines as an adaptive response to the hyperosmolar environment. These authors also conclude that BAFF may be a NFAT5-responsive gene located downstream of the Brx-mediated signaling cascade, likely contributing to
both the reduced production of immunoglobulin and the alteration of B cell differentiation in the spleen observed in brx<sup>+/−</sup> mice. So, Brx-mediated induction of the NFAT5 expression plays an essential role in the production of antibodies and cytokines as an adaptive response to the hyperosmolar environment. Taken together, it is feasible that the alterations described above are involved in the reduced antibody production after infection with Tg under ISD conditions. However, these data need to be better clarified.

Finally, our study introduces a new direction to the influence of environmental factors on the clinical course of toxoplasmosis. This data reinforces the imperative need to carry out more investigations focusing on the metabolic and immune response profile of the hosts. Furthermore, health authorities should be encouraged to develop epidemiological studies in Tg infected human beings under high or moderate sodium intake to understand how this association appears clinically.

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