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Effects of sulfamethoxazole-trimethoprim associated to resveratrol on its free form and complexed with 2-hydroxypropyl-β-cyclodextrin on cytokines levels of mice infected by *Toxoplasma gondii*

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ABSTRACT

The aim of this study was to investigate the effects of resveratrol on its free form and complexed with 2-hydroxypropyl-β-cyclodextrin (HPβCD) when associated with sulfamethoxazole-trimethoprim (ST) on cytokines levels of mice (n = 60) experimentally infected by *Toxoplasma gondii*. Groups A and E were used as controls (untreated): negative and positive, respectively. The onset of treatment started 20 days post-infection (PI), and it lasted for 10 consecutive days. ST was administered orally in doses of 0.5 mg kg^{-1} for groups B and F, while 100 mg kg^{-1} was the dose for resveratrol in its free form (groups C - G), inclusion complex (groups D and H), and on free and inclusion complex together (groups I - J). On day 31 PI, blood samples were collected in order to evaluate the cytokine profile. The mice that received drug combination (I and J) showed a significant ($P < 0.05$) reduction in the number of cysts in the brain compared to other infected groups (E – H). The results showed that mice from the Group E had increased ($P < 0.001$) levels of pro-inflammatory cytokines, while IL-10 levels were reduced when compared to the Group A. Additionally, there were increased levels of IL-4 and IFN-γ in animals of groups C and D, respectively ($P < 0.05$). Animals of the Group B showed reduced levels of IL-1, IL-4, IL-6, TNF-α, and IFN-γ ($P < 0.05$). Mice infected and treated (groups F – J) showed increased levels of pro-inflammatory cytokines along with a reduction of IL-10. Treatment with the combination of drugs (the Group J) led to a protective effect, i.e. reduction in pro-inflammatory cytokines. Therefore, resveratrol associated with ST was able to modulate seric cytokine profile and moderate the tissue inflammatory process caused by *T. gondii* infection, as well as to reduce parasite multiplication.

1. Introduction

Toxoplasma gondii is a coccidian parasite that infects almost any warm-blooded animal [1], which includes the largest and most important group of obligate parasites [2]. Mammals may become infected by *T. gondii* by the ingestion of sporulated oocysts shed in the faeces of felids, which are the definitive hosts [3]. For humans, other forms of infection are epidemiologically important such as ingestion of raw or undercooked meat from infected animals. Thus, it is estimated that about one third of the world population is seropositive for toxoplasmosis [4]. The treatment of choice for toxoplasmosis in humans has been sulfadiazine, a drug that has

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some toxicity and causes undesirable side effects [5,6]. Synergism between antiprotozoal associated to antioxidant in the treatment of toxoplasmosis was observed, resulting on increased therapeutic efficacy and a reduction in cell injury [7].

Resveratrol is a polyphenol naturally found in grapes, fruit, wine and peanuts [8,9]. It has several biological properties, as mainly antioxidant and anti-inflammatory activities and antiplatelet effects [10–12]. The inclusion complexes containing resveratrol can be used, since they have better therapeutic action when interacting with tissue-specific, better absorption and controlled release [13], maintaining, or enhancing the same properties of the free resveratrol.

Exacerbated inflammatory response after *T. gondii* infection has been widely described, and this process is able to increase the cellular and tissue lesions. According to the literature, *T. gondii* infection stimulates the production of high levels of cytokines, such as IL-12 and IFN-γ through cells of the innate immune system [14]. In a recent study, sulfadiazine and selenium were used in association and it was able to modulate the cytokine release in mice infected by *T. gondii* [15]. Similarly, resveratrol also has immunomodulatory and anti-inflammatory properties described [16]; thus, the objective of this study was to assess the effects of resveratrol in its free form and complexed with HPβCD in association with ST on cytokine modulation to treat mice experimentally infected by *T. gondii*.

2. Materials and methods

2.1. T. gondii strain and preparation of inoculum

T. gondii (strain VEG) kept in liquid nitrogen was inoculated in one mouse (BALB/c) and thirty days later, its brain was collected and homogenized in saline solution. Cysts containing bradyzoites were collected and inoculated orally in other five mice. This procedure was performed in order to reactivate the parasite virulence. Twenty-five days post-infection (PI), the mice were euthanized for brain collection, cysts were counted and the inoculums were prepared for further experimental procedures.

2.2. Experimental design

Sixty male (BALB/c) mice with a mean age of 60 days and weighing 25 ± 5 g were kept in boxes with five animals each, under a 12 h light/dark cycle under controlled temperature and humidity (25 °C, 70% respectively). The animals went through an adaptation period of 10 days and were fed with commercial feed and drinking water *ad libitum*. Firstly, 60 animals were divided into two major groups: group A–D (n = 24) consisted of healthy uninfected mice, and groups E–J (n = 36) of animals infected orally with 50 cysts containing bradyzoites of a cystogenic strain (VEG) of *T. gondii*. After 20 days post-infection (PI), these two groups were divided into 10 subgroups, and began treatment for 10 consecutive days.

Animal grouping was set randomly, as follows: animals of groups A to D were not infected; thus, animals of the group A (n = 6) did not receive any kind of treatment, representing the negative control, while mice of group B (n = 6) received sulfamethoxazole-trimethoprim (ST) orally; Groups C (n = 6) and D (n = 6) were orally treated with resveratrol on its free form (C) and resveratrol-HPβCD complex (D). On the other hand, mice from groups E to J were infected by *T. gondii*, as described above. Thus, animals of the group E (n = 6) were untreated, representing the positive control. The other groups, besides the infection, received different types of treatment; mice of group F (n = 6) were treated with ST, while animals of group G (n = 6) were treated with resveratrol on its free form, and mice of the group H (n = 6) received resveratrol-HPβCD complex; animals of group I (n = 6) were treated with ST in association with free form of resveratrol; Finally, mice of the group J (n = 6) were treated with ST in combination with resveratrol-HPβCD complex.

Treatments with ST, free resveratrol and resveratrol-HPβCD complex were administered orally for 10 consecutive days at a dose of 0.5 mg kg^{-1} (ST) and 100 mg kg^{-1} (free and complex inclusion of resveratrol), individually (groups B, C, D, F, G and H) or in association (groups I and J). The periods of treatment and doses were defined in a previous pilot study.

2.3. Sampling, cyst number in brain, and histological analysis

Thirty-one days after the infection, the animals were anesthetized and euthanized according to the standard protocol approved by the Welfare Committee. Blood was collected from all the mice, and centrifuged in order to obtain the serum. Sera samples were stored at −20 °C for further cytokine assessment. For cyst counts, the left hemisphere of each animal was resuspended in salt solution and passed ten times through a 5 mL syringe with a 22 gauge needle. Then, the solution was centrifuged at 3.000 rpm for 10 min at 4 °C and the total number of cysts on each brain was determined by microscopic reading of successive aliquots of 500 μL. Additionally, fragments of liver were collected and stored in formalin 10%, in order to perform histology. Sagittal sections were prepared with an interval of 3 mm between the regions, for slide mounting and staining through hematoxylin and eosin.

2.4. Seric cytokine levels

Quantification of the following cytokines was performed: tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and interleukins (IL-1, IL-4, IL-6, and IL-10) by ELISA assay using commercial kits (Quantikine Immunoassay kits - R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.5. Statistical analysis

Normality test was performed and the results that were not normally distributed were transformed to logarithms. Then, data were submitted to analysis of variance (ANOVA) followed by Duncan test ($P < 0.05$).

3. Results

During the course of the disease, mice infected by *T. gondii* showed clinical signs such as apathy and bristling coat, especially after 20 days PI. The clinical signs remained even after post-treatment in all infected groups. The mice that received the drug combination (I and J) showed a significant ($P < 0.05$) reduction in the number of cysts in the brain compared to other infected groups (E − H), i.e. it was observed in the groups E (1.71 ± 0.51 cyst/μL), F (1.09 ± 0.55 cyst/μL), G (1.78 ± 0.18 cyst/μL), H (1.65 ± 0.54 cyst/μL), I (0.80 ± 0.09 cyst/μL), and J (0.49 ± 0.13 cyst/μL). Histological analysis showed different levels of liver damage and in the number of parasitic cysts in infected animals, e.g.: animals of group E showed random foci of necrosis and high granulomatous inflammatory infiltrate. On the other hand, mice from groups F, G and H showed moderate random multifocal lymphoplasmacytic inflammatory infiltration in the parenchyma and portal space. Regarding groups I and J, it was possible to observe a milder inflammatory infiltrate, in other words liver from animals of groups I and J had less and smaller areas of foci inflammatory infiltrates when compared to the group E (Fig. 1).

The assessment of cytokines (IL-1, IL-4, IL-6, TNF-α, INF-γ, and