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CAROLINE FELICIO BRAGA DA SILVA

Avaliação *in vivo* do efeito de medicamento homeopático feito a partir de *Toxoplasma gondii* em camundongos infectados pelo protozoário.

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutora em Ciências da Saúde.
Área de concentração: Doenças Infecciosas e Parasitárias

Orientadora: Prof.ª Dr.ª Silvana Marques de Araújo

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FOLHA DE APROVAÇÃO

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Profª Drª Débora de Mello Gonçales Sant'Ana
Universidade Estadual de Maringá (Titular)

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Local de defesa: Sala 01, Bloco 126, *campus* da Universidade Estadual de Maringá.

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“... Confie em si mesmo, quem acredita, sempre alcança.”

Renato Russo

Avaliação *in vivo* do efeito de medicamento homeopático feito a partir de *Toxoplasma gondii* em camundongos infectados pelo protozoário

RESUMO

Introducão: A avaliação de um medicamento ultradiluído que estimule reação funcional efetiva para o sistema biológico na relação parasito/hospedeiro na toxoplasmose, além de constituir esperança para minimizar danos de uma infecção congênita (animais/humanos), contribuirá para o entendimento do mecanismo de ação de medicamentos homeopáticos. O objetivo deste estudo foi investigar o efeito de diferentes dinamizaççoes do bioterápico de *T. gondii* em camundongos pré-tratados e infectados com este protozoário. *Métodos:* Em ensaio cego, controlado, randomizado, camundongos Swiss machos, com 60 dias de idade, foram divididos em grupos: GCN – grupo controle não infectado e não tratado; GCinf - grupo controle infectado e pré-tratado com álcool de cereais 7%; BIOT-TG200 - pré-tratados com bioterápico de *T. gondii* 200dH, BIOT-TG300 - pré-tratados com bioterápico de *T. gondii* 300dH, BIOT-TG400 - pré-tratados com bioterápico de *T. gondii* 400dH e BIOT-TG500 - pré-tratados com bioterápico de *T. gondii* 500dH. Os medicamentos foram preparados de acordo com a Farmacopéia Homeopática Brasileira, com macerado de cérebro de camundongos infectados (20 *T. gondii* cysts/100μL). Pré-tratamento: 3 dias consecutivos antes da inoculação. Infecção: 20 cistos cepa ME49-*T. gondii*, por via oral. Avaliação clínica, contagem de cistos no cérebro, contagem e morfometria neuronal mientérica e avaliação de citocinas anti e pró-inflamatórias foram avaliadas aos 30 e 60 dias após infecção. *Análise estatística:* Teste anova ou teste T, com 5% de significância. *Resultados:* O BIOT-TG 300 apresentou ascite após a infecção. O 400dH diminuiu o consumo de água quando comparado ao GCinf. BIOT-TG500 expressou variação negativa do peso dos camundongos e houve mortalidade de 10% no GCinf. Para a dinamização 200dH, observou-se clínica favorável para camundongos tratados com BIOT-TG200 durante todo o estudo. O número de cistos cerebrais estava reduzido no grupo BIOT-TG200 aos 30 e 60 dpi quando comparado ao GCinf. Houve proteção neuronal mientérica no colón de camundongos tratados com BIOT-TG200 e modulação do sistema imunológico de forma diferenciada, para as citocinas pro-inflamatórias houve aumento do IFN-γ aos 30 e 60 dpi e diminuição ao longo do tempo de infecção. A citocina IL-6 estava aumentada aos 30 dpi e reduzida ao longo do tempo de infecção. O TNF-α estava aumentado aos 30 dpi e houve redução desta citocina ao longo do tempo de infecção. Para a IL-17 houve redução na evolução do tempo. Não houve variação significativa para IL-2. Para as citocinas anti-inflamatórias, a citocina IL-10 apresentou redução ao longo do tempo de infecção e não houve variação significativa para IL-4 no modelo experimental utilizado. *Conclusão:* As diferentes dinamizações apresentaram efeitos diversos podendo variar de efeitos benéficos a prejudiciais. A dinamização 200dH apresenta os melhores resultados, com efeito benéfico do medicamento altamente diluído de *Toxoplasma gondii*, promovendo sintomatologia clínica favorável, redução da carga parasitária, proteção neuronal e modulação do sistema imunológico com predomínio de resposta Th1.

Palavras-chave: *Toxoplasma gondii*; homeopatia; sistema nervoso entérico; citocinas

In vivo evaluation of the effect of homeopathic medicine of *Toxoplasma gondii* on protozoan infected mice

ABSTRACT

Introduction: The evaluation of an ultradiluted medication that stimulates effective functional reaction for the biological system in the parasite/host relationship in toxoplasmosis, in addition to provide expectancy for minimizing damages of a congenital infection (animal/human), will contribute to the understanding of the mechanism of action of homeopathic medications. The aim of this study was to investigate the effect of different dinazations of *T. gondii* biotherapic in mice pretreated and infected with this parasite. *Methods:* In a blind, controlled, randomized assay, *Swiss* male mice, were divided: GCN:uninfected and untreated control group; GCinf: infected control group pretreated with 7%grain alcohol; BIOT-TG200: pretreated with 200dH*T.gondii* medicine; BIOT-TG300: pretreated with 300dH *T.gondii* medicine; BIOT-TG400: pretreated with 400dH*T.gondii* medicine and BIOT-TG500: pretreated with 500dH *T.gondii* medicine. Medicines were prepared according to the Brazilian Homeopathic Pharmacopoeia. Clinical, parasitological, histopathological, and immunological parameters were evaluated. *Results:* BIOT-TG300 presented ascites after infection. The 400dH decreased water consumption when compared with GCinf. BIOT-TG500 expressed negative variation in the weight of mice and 10% mortality occurred in GCinf. A favorable clinical outcome was observed for mice treated with BIOT-TG200 the study. The number of brain cysts was reduced in the BIOT-TG200 group on the 30^{th} and 60^{th} dpi, compared with GCinf. There was myenteric neuronal protection in the colon of mice treated with BIOT-TG200 and for proinflammatory cytokines there was an increase of IFN-γ at 30 and 60 dpi and decrease over the course of infection. The cytokine IL-6 increased at 30 dpi and decreased over the course of infection. TNF-α increased at 30 dpi and reduced over the course of infection. For IL-17 there was a reduction in the over the course of infection. There was no significant variation in IL-2. The anti-inflammatory cytokine IL-10 decreased over the course of infection and IL-4 did not vary significantly, predominantly in Th1 response. *Conclusion:* The several dynamizations presented different effects, varying from beneficial to damaging effects. Dynamization 200dH showed beneficial effect of the highly diluted biotherapic of *T. gondii*, promoting more favorable clinical symptomatology, parasite load reduction, neuronal protection and immune system modulation with predominance of Th1 response.

Keywords: *Toxoplasma gondii*; homeopathy; enteric nervous system; cytokines

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SUMÁRIO

CAPÍTULO I

1.1 HISTÓRICO

O *Toxoplasma gondii* foi descoberto no Brasil por Alphonso Splendore (1908) ao necropsiar e analisar a morte de coelhos por paralisia. O parasito recebeu o nome de *Toxoplasma cuniculi*, na ocasião. Simultaneamente, Nicolle, Manceaux (1908) em Tunis, identificaram o parasito num roedor (*Ctenodactylus gondii*) originário do norte da África,

identificando-o como *Leishmania gondii*. Em 1909, estes autores constataram que se tratava de novo protozoário e modificaram o gênero para *Toxoplasma*. O nome deriva do grego no qual *toxon* = arco e *plasma* = forma, referindo-se ao formato de lua crescente do taquizoíto (DUBEY, BEATTIE; 1988). Levantamentos sobre casos de infecção por *T. gondii* têm sido relatados desde então. Segundo Pizzi (1997), o primeiro caso de toxoplasmose humana, menino com quadro febril e com esplenomegalia, foi descrito por CASTELLANI, em 1913. De acordo com ORÉFICE, BAHIA-OLIVEIRA (2005), o primeiro caso de toxoplasmose no Brasil foi descrito por Magarino (1927) que relatou o encontro do parasito em necrópsia de um paciente com meningoencefalite, miocardite e miosite.

Pinkerton, Weinman, em 1940, nos Estados Unidos, relataram o re-isolamento do parasito em adultos. Mas, somente após o desenvolvimento de um teste sorológico, o clássico teste do corante de Sabin & Feldman, desenvolvido em 1948, é que foi possível demonstrar a alta prevalência da toxoplasmose em todo mundo, o que contribuiu imensamente para o diagnóstico laboratorial da toxoplasmose, possibilitando a realização de inquéritos epidemiológicos (NEVES et al., 2012). Nos anos 70, completou-se o conhecimento do ciclo biológico desse parasito através da descoberta dos estágios sexuais no intestino delgado de gatos (FRENKEL et al., 1970). Miller et al. (1972) provaram que os únicos mamíferos capazes de suportar o ciclo sexuado intestinal do *Toxoplasma gondii* e excretar os oocistos são os felinos, tanto domésticos quanto selvagens.

1.2 *Toxoplasma gondii e* **TOXOPLASMOSE**

T.gondii é um patógeno intracelular obrigatório capaz de infectar e se replicar em qualquer célula nucleada de mamíferos e aves (SOUZA, 2010). O *T gondii* pertence ao reino Protista, subreino Protozoa, filo Apicomplexa, classe Sporozoea, subclasse Coccidea, ordem Eucocciidida, subordem Eimeriina, família Sarcocystidea, subfamília Toxoplasmatinae, gênero *Toxoplasma*, espécie *T.gondii* (NEVES et al. 2012). O *T. gondii* pode ser encontrado em três diferentes formas infectantes: taquizoítos, bradizoítos e oocistos (MONTOYA, LIESENFELD, 2004).

"Taquizoíto" (taqui = rápido), estágio de rápida multiplicação em qualquer célula de um hospedeiro intermediário e em células epiteliais não intestinais dos hospedeiros definitivos (DUBEY et al., 2010). O termo "taquizoíto" substitui o termo "trofozoíto" (trophicos = alimentação em grego) que era usado anteriormente. Estas formas estão presentes em grande quantidade na fase aguda da infecção no interior das células afetadas, sendo responsável pela sintomatologia característica da doença. Montoya e Liesenfeld 2004, descrevem que os

taquizoítos responsáveis pela transmissão transplacentária. Os taquizoítos medem aproximadamente 2 a 6μm e se dividem assexuadamente no interior da célula do hospedeiro por sucessivas endodiogenias, uma forma de reprodução especializada onde duas células-filhas se formam no interior da célula-mãe que se rompe e libera a progênie, a qual cresce atingindo o tamanho adulto e repete o processo. A célula do hospedeiro se rompe quando não suporta mais o aumento do número de taquizoítos. As taxas de invasão e crescimento variam dependendo da cepa de *T. gondii* e o tipo de célula hospedeira (DUBEY et al, 2010).

Os bradizoítos (*brady* = lento) são morfologicamente semelhantes aos taquizoítos, mas possuem uma replicação lenta. É a forma de resistência do *T.gondii* nos tecidos, encontrada durante a fase crônica da infecção. É mais delgado e mede cerca de 7 μm de comprimento por 1,5 μm de largura. São encontrados no interior dos cistos teciduais, cujo tamanho varia de 10-100 μm (DUBEY et al, 2010). Estes autores ainda relatam que cistos grandes podem conter até 3000 bradizoítos que se dividem lentamente por endodiogenia. Os cistos teciduais são característicos da fase crônica da toxoplasmose, mas podem ocasionalmente ser encontrados na fase inicial da infecção (começam a se formar entre o sexto e o oitavo dia de infecção). Em estados de imunossupressão, os cistos teciduais se rompem e os parasitos se proliferam rapidamente. Os cistos teciduais representam uma importante forma de transmissão da toxoplasmose já que persistem ao longo da vida nos tecidos dos animais infectados e podem ser ingeridos por carnívoros, incluindo os humanos. Esta forma é resistente à digestão péptica e sobrevivem várias horas após a exposição às enzimas digestivas (NEVES et al. 2012). Após a ingestão, a parede do cisto é rompida liberando bradizoítos viáveis, capazes de invadir o trato digestivo do hospedeiro (REY 2010).

Já o oocisto representa o estágio mais resistente de *T. gondii*, por possuir uma parede dupla bastante resistente às condições ambientais (DUBEY et al., 2010). São produzidos nas células intestinais de felídeos não imunes, e eliminados imaturos juntamente com as fezes (NEVES et al. 2012). Os oocistos medem 10 x 13 μm e possuem em seu interior dois esporocistos (6 x 8 μm), os quais contém quatro esporozoítos cada (2 x 8 μm) (REY 2010). O oocisto esporulado contém oito esporozoítos e é a forma madura e infectante do oocisto, que sob condições favoráveis, pode permanecer infectivo por mais de um ano (NEVES et al. 2012).

A toxoplasmose é uma zoonose de distribuição mundial causada por *Toxoplasma gondii*, um protozoário apicomplexo intracelular de ciclo de vida heteroxênico, capaz de infectar uma ampla variedade de vertebrados de sangue quente e até 30% da população

mundial humana (TENTER et al., 2000). O gato doméstico e outros felídeos são os hospedeiros definitivos, e muitas espécies de vertebrados, inclusive o homem, podem servir como hospedeiros intermediários. A infecção em hospedeiros definitivos ou intermediários ocorre comumente, mas os sinais clínicos são raros (DUBEY et al. 2010). A infecção por *T. gondii* é transmitida principalmente pelo consumo de carne crua ou mal cozida contendo cistos teciduais, mas também potencialmente pela ingestão de vegetais ou água contaminados com oocistos esporulados (TENTER et al., 2000).

Em humanos saudáveis, a infecção é assintomática em 70% dos casos. Em contraste, em indivíduos imunossuprimidos, tais como portadores da AIDS (Síndrome de imunodeficiência adquirida) e pacientes em uso de quimioterápicos ou imunossupressores, a toxoplasmose aguda causa uma infecção potencialmente letal (JONES E ROBERTS 2012). Além disso, a toxoplasmose congênita pode causar dano fetal grave, podendo culminar em aborto espontâneo (YDAV et al. 2014). Jones e Roberts 2012**,** salientam que quando a infecção aguda manifesta-se no primeiro trimestre de gravidez, 14% dos fetos apresentam-se infectados, no segundo 29% e no terceiro 59%. Para esse autor, 90% das mães que apresentaram infecção aguda durante a gravidez eram assintomáticas e a incidência de toxoplasmose aguda na gravidez varia de 0,06 a 1,4%.

A infecção aguda em gestantes pode estar associada a lesões fetais que podem variar de formas subclínicas, morte intra-uterina ou danos no sistema nervoso central (SNC) como calcificações cerebrais, hidrocefalia, microcefalia e coriorretinite (JONES E ROBERTS 2012). O recém-nato freqüentemente pode apresentar baixo peso, hepato e esplenomegalia, quadros de anemia, presença de plaquetopenia e danos oculares resultantes de processos inflamatórios da retina. Crianças aparentemente normais ao nascer podem subseqüentemente desenvolver injúrias associadas à toxoplasmose (YDAV et al. 2014). A retinicoroidite é a causa mais comum de uveíte posterior em várias partes do mundo, incluindo regiões da Europa e Américas do Norte e do Sul (HIGA et al, 2010). A prevalência da doença ocular em pacientes infectados pelo *T. gondii* ainda não está bem estabelecida, mas sabe-se que o envolvimento ocular é mais freqüente e mais grave em neonatos e adultos imunocomprometidos (HIGA et al, 2010; BOSCH-DRIESSEN et al., 2002).

Na fase aguda da toxoplasmose, primeiro ocorre a produção de imunoglobulina IgM, seguida da produção de imunoglobulina IgG. A infecção pode também produzir imunoglobulina IgA, no caso da transmissão ter sido por via oral. Pela técnica de imunofluorescência, os anticorpos IgM podem ser dosados 1 a 2 semanas depois do início da

infecção, alcançando um pico em 6 a 8 semanas, quando então declinam. Títulos baixos podem persistir por mais de 12 meses. O anticorpo IgG persiste por toda a vida na maioria dos pacientes (SKARIAH et al. 2010**)**. Em função da variedade fisiopatológica e clínica da infecção, as modalidades de diagnóstico devem ser diferenciadas em se tratando de uma reativação em indivíduos imunodeprimidos, de infecção congênita e neonatal ou infecção primária e infecção em indivíduos imunocompetentes (HO-YEN 2009). O diagnóstico pode ser feito pela demonstração do parasito. No entanto, a pesquisa pelo exame direto é difícil e deve, freqüentemente, ser complementada por métodos indiretos tais como inoculação em animais de laboratório, cultura celular ou técnicas sorológicas (DUBEY et al., 2010).

1.3 EPIDEMIOLOGIA

A prevalência da infecção pelo *T. gondii* varia de acordo com os hábitos alimentares, higiene, idade dos indivíduos infectados, fatores culturais, regiões geográficas, características climáticas e medidas políticas de saúde e governo (MONCADA e MONTOYA 2012). Dubey et al., 2010 destaca que a toxoplasmose ocorre em milhões de pessoas no mundo inteiro, sendo que a prevalência da infecção humana, na maioria dos países, está entre 40% e 50%, no Brasil essa taxa pode chegar até 80%, dependendo da área estudada.

Na América Latina, a prevalência de anticorpos da classe IgG na população é geralmente elevada (>60%) especialmente em El Salvador (REMINGTON et al. 2006) e Brasil (FRENCKEl 2002), enquanto no México a prevalência média é de 27,97% (GALVAN-RAMIREZ et al. 2012).

De acordo com Fonseca et al., 2016 a prevalência da infecção por *T. gondii* em gestantes no Brasil também varia de acordo com as regiões geográficas. O Paraná apresenta 67% de positividade, Mato grosso do Sul 91,4%, Goiás 67,6%, Rio Grande do Sul 59,8%, Minas Gerais 49,5%, Espirito Santo 73,5%, Maranhão 77,9%, Bahia 64,9% e Pernambuco 69,4%.

A maioria dos isolados tanto de humanos como de animais domésticos pertencem a três genótipos denominados tipo 1, 2 e 3 (BOUGHATTAS et al. 2011). Cepas do tipo 1 (mais virulentas e amplamente distribuída na América do Sul) estão relacionadas à toxoplasmose humana congênita e à virulência aguda em camundongos, cepas do tipo 2 (comuns nos Estados Unidos e na Europa) relacionam-se aos casos de reativação da infecção crônica em humanos e as cepas do tipo 3 (menos virulentas e frequentemente isoladas na América do Sul) estão associadas às infecções animais (DUBEY et al. 2002).

Em um trabalho que avaliou as dificuldades do monitoramento dado às gestantes com suspeita de toxoplasmose aguda, atendidas em serviços públicos de saúde do noroeste do

Estado do Paraná, foram observadas 290 gestantes IgM reagentes, 112 (49,3%) receberam quimioprofilático, incluindo 13 das 35 gestantes que apresentaram aumento progressivo nos índices de IgM e IgG. Em 54 (48,2%) das que receberam quimioprofilático, o início do tratamento ocorreu trinta dias após a realização da sorologia inicial. Em 27 (24%) de 112 casos a prescrição quimioprofilática não foi disponibilizada pelos serviços públicos de saúde, sendo custeada pela família da paciente ou comunidade (CASTILHO-PELLOSO et al., 2007)

Ao analisar a prevalência de sorologia positiva para toxoplasmose em 100 doadores de sangue do Banco de sangue de Cascavel/PR, sendo estes, 67 homens e 33 mulheres, verificou-se uma prevalência de 62% de IgG positivo para ***Toxoplasma gondii*** e 100% de IgM negativos. Dentre os homens a soropositividade foi de 59,7%, e entre as mulheres de 66,67% **(PILLATI et al., 2013)**

Um dos maiores surtos de toxoplasmose ocorreu no Brasil, em Santa Isabel do Ivaí-PR, onde, aparentemente, uma única gata com seus filhotes contaminaram o manancial de água da cidade, o que levou a infecção de mais de 500 pessoas com todas as consequências da doença, ou seja, formas congênitas e oculares (DUBEY et al., 2004). Em Londrina-PR, foram onfirmados 17 casos de infecção pela doença entre os funcionários da unidade do Instituto Agronômico do Paraná (Iapar) de Londrina. Com os testes positivos para o protozoário que causa a toxoplasmose, a direção do Iapar acionou os setores de Vigilância Epidemiológica, Sanitária e Ambiental para tentar detectar a origem da doença (CALSAVARA, 2015). **No Rio Grande do Sul, o** Ministério da Saúde divulgou o resultado de uma pesquisa sobre o surto de toxoplasmose que foi registrado em **São Marcos**, na Serra do Rio Grande do Sul, no início de 2015. Foram pelo menos 154 casos confirmados no município. As investigações apontaram o consumo de carne mal passada como a principal causa da contaminação (G1 portal de notícias).

1.4 TRATAMENTO

Quando a infecção fetal por *T. gondii* é confirmada, a pirimetamina e sulfadiazina devem ser administrados até o nascimento com 100mg a cada 12 horas e 1mg/kg três vezes na semana, devido a toxicidade gerada para a mãe e o feto, como: supressão da medula óssea, febre, vasculite, erupções cutâneas, náuseas, vômitos, nefropatia (KAYE 2011).

Em caso de infecção materna sem infecção fetal, espiramicina é a droga de escolha para a prevenção de transmissão vertical, a qual é um antibiótico macrolídeo que não pode atravessar a barreira transplacentária, sendo administrado 500mg a cada oito horas, podendo causar efeitos colaterais, como: náusea, vômito, diarréia e casos muito raros de colite pseudomembranosa, reações de hipersensibilidade, alterações nos testes de função hepática e

hemólise aguda (MONTOYA e REMINGTON 2008). Apesar de o tratamento conseguir controlar as formas de rápida proliferação, não existe nenhuma droga capaz de eliminar os cistos teciduais latentes em humanos e animais, que se mantêm viáveis por longo período, podendo reativar a infecção (KAYE 2011). Há relatos de transmissão congênita de *T. gondii* resultante da reativação de infecção crônica em gestantes imunocompetentes. No entanto, alguns casos foram relacionados a uma presumível redução da resposta celular durante a gestação, que pode intervir no controle dos parasitos e no curso clínico da infecção materna e/ou em casos de reagudização que consequentemente eleva o risco de transmissão vertical (ANDRADE et al. 2010).

A toxoplasmose congênita é assintomática na maioria das crianças ao nascer. No entanto, se não diagnosticada e não tratada, a maioria das crianças infectadas irão desenvolver deficiências visuais ou neurológicas na idade adulta (KIEFFER e WALLON 2013). Já os pacientes imunocompetentes habitualmente são tratados quando apresentam sintomatologia extensa e prolongadas com comprometimento ocular ou visceral significativo, sendo importante realizar o monitoramento de exames clínicos regularmente para controle da infecção (KIEFFER e WALLON 2013).

1.4 HOMEOPATIA

A homeopatia é uma especialidade médica e farmacêutica que consiste em ministrar ao doente, doses mínimas de substâncias para evitar a intoxicação e estimular a reação orgânica. É baseada no princípio dos semelhantes exposto por Hipócrates no século IV a.C. e inserida pelo médico alemão Cristiano Frederico Samuel Hahnemann, em sua nova prática no final do século XVIII, após estudos de clínica e experimentações (ALMEIDA et al., 2008).

O Ministério da Saúde do Brasil, em maio de 2006, publicou a portaria nº 971 que aprova a Política Nacional de Práticas Integrativas e Complementares no Sistema Único de Saúde. Esta política aponta para uma série de diretrizes, dentre as quais, a necessidade do desenvolvimento de estudos clínicos e de pesquisa básica envolvendo a homeopatia, os quais precisam ser incentivados no âmbito federal, estadual e municipal (BRASIL, 2006). Os bioterápicos, são produtos não quimicamente definidos (secreção produtos de origem microbiana, excreções fisiológicas e/ou patológicas que serve de matéria prima para o preparo de medicamentos (Farmacopéia homeopática Brasileira 2011).

Os medicamentos ultradiluídos têm mostrado ser efetivos em diversas infecções experimentais. Vários modelos experimentais com *T.cruzi* e *T.gondii* têm sido utilizados na avaliação da eficácia de medicamentos homeopáticos, sendo realizado nestas avaliações

parâmetros parasitológicos, moleculares, hematológicos, imunológicos entre outros (SANDRI et al., 2015; BRAGA-SILVA et al., 2015; FERRAZ et al 2011; Aleixo et al., 2012).

1.5 JUSTIFICATIVA

A busca de um medicamento eficaz, com ausência de efeitos colaterais e de baixo custo, torna-se essencial na prevenção ou diminuição de danos da toxoplasmose. As gestantes destacam-se como o grupo de maior risco nesta infecção devido à possibilidade de infecção congênita do feto com conseqüências que podem envolver além do aborto, uma série de outras seqüelas graves. As lesões oculares constituem uma das principais complicações de indivíduos infectados congenitamente ou após o nascimento. Todas estas características oneram cognitivamente, comportalmente, social e economicamente não só na vida dos indivíduos infectados, como sobrecarregam o sistema de saúde, com alto custo de atenção e acompanhamento, por tempos prolongados.

A necessidade de um tratamento preventivo para toxoplasmose reveste-se de grande importância. Os bioterápicos constitutuem em importante estratégia de prevenção, garantindo uma abordagem segura e de baixo custo para infecções. Dados do nosso grupo demonstram que dinamizações baixas (7dH, 17dH, 30dH e 60dH) do bioterápico de *T.gondii* provocaram efeitos deletérios em camundongos infectados com o protozoários. A dinamização 100dH previniu efeitos oculares porem a avaliação clínica foi desfavorável. A dinamização 200dH foi mais efetiva no controle da infecção com redução de carga parasitária e clínica favorável, no entanto não tínhamos dados sobre o efeito de dinamizações mais elevadas. Diante destes resultados, era imperativo realizar um screening de dinamizações mais altas. Os dados provenientes de nosso trabalho anterior, associados aos dados do screening proporcionaria a escolha da melhor dinamização para aprofundamento dos estudos. O encontro de um medicamento que controle a infecção experimental pelo *T. gondii*, além de constituir esperança para o tratamento da toxoplasmose, contribuirá para o entendimento da ação de medicações ultra ou altamente diluídas.

1.7 OBJETIVOS

GERAL

Avaliar *in vivo* o efeito de medicamento produzido com cistos de *Toxoplasma gondii* em camundongos infectados com este protozoário

ESPECÍFICOS

1) Avaliar parâmetros clínicos e parasitológicos de diferentes dinamizações (200dH, 300dH, 400dH e 500dH) do bioterápico de *T. gondii* em camundongos infectados com este protozoário.
2) Eleger a dinamização com melhor efeito;
3) Avaliar parâmetro parasitológico (contagem de cistos cerebrais aos 30 e 60 dias após infecção) em camundongos tratados com bioterápico eleito e infectados com *T.gondii*;
4) Avaliar parâmetros histológicos (contagem e morfometria dos neurônios mientéricos do cólon aos 30 e 60 dpi) em camundongos tratados com bioterápico eleito e infectados *T.gondii*;
5) Avaliar parâmetros imunológicos (dosagem das concentrações séricas de IL-4 e IL-10 (anti-inflamatórias) e de IFN-γ, IL-6, IL-2, TNF-α e IL-17 (pró-inflamatórias) aos 30 e 60 dpi) em camundongos tratados com bioterápico eleito e infectados *T.gondii.*

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CAPÍTULO II

Artigo 1: “Screening dynamizations of biotherapic of *Toxoplasma gondii* in mice infected with the protozoan*”*

Screening dynamizations of biotherapic of *Toxoplasma gondii* in mice infected with the protozoan

Caroline Felicio Braga-Silva[a], Ricardo Nascimento Drozino[a], Denise Lessa Aleixo[a] Silvana Marques de Araújo[a]

[a]Universidade Estadual de Maringá, Departamento de Ciências Básicas da Saúde, Maringá, Paraná, Brasil

Correspondingauthorat: Caroline Felicio Braga-Silva
e-mail: **cfeliciobraga@gmail.com**
Universidade Estadual de Maringá
Departamento de Ciências Básicas da Saúde, Bloco I90, Setor Parasitologia Básica.
Av. Colombo, 5.790
Jardim. Universitário
Maringá - Paraná - Brasil
CEP 87020-900
Tel.: 55 (44) 3011- 4918
E-mail addresses: **cfeliciobraga@gmail.com** (C. F. Braga-Silva)

Abstract

Introduction: An effective treatment for toxoplasmosis is urgent and biotherapics can be an alternative. The understanding of its action and the search for adequate dynamization is challenging. The objective of this study was screening the best dynamization of a biotherapie made by *T. gondii* considering the effect in mice infected with the protozoan. *Methods:* In a blind, controlled, randomized assay, 100 *Swiss* male mice, 60 days old were divided into groups. GCinf: infected control group pretreated with 7% grain alcohol (n=20); BIOT-TG200: pretreated with *T. gondii* medicine 200dH (n=20); BIOT-TG300: pretreated with *T. gondii* medicine 300dH (n=20); BIOT-TG400: pretreated with *T. gondii* medicine 400dH (n=20); BIOT-TG500: pretreated with *T. gondii* medicine 500dH (n=20). Medicines were prepared according to Brazilian Homeopathic Pharmacopoeia, with infected mice's brain macerated (20 *T. gondii* cysts/100µL). Pretreatment: Three consecutive days before innoculation. Infection: At 60 days old, with 20 cysts of ME49 strain-*T.gondii*, orally. Clinical evaluation and counting of cysts in the brain, were performed on the 60th day post-infection (dpi). *Statistical comparison*: Anova, 5% significance level. *Results:* Favorable clinic for BIOT-TG200 and cysts decrease ($p<0.05$), compared with Gcinf were observed. BIOT-TG300 presented ascites after infection. The 400dH decreased water consumption when compared with GCinf ($p<0.05$). BIOT-TG500 expressed negative variation in the weight of mice and 20% mortality occurred in GCinf. *Conclusion:* The several dynamizations presented different effects, varying from beneficial to damaging effects. Dynamization 200dH was the dynamization of choice for further studies. The choice of the dynamization of the homeopathic medication is a matter of crucial importance in the treatment success.

Keywords: Biotherapic; *Toxoplasma gondii*; homeopathy; toxoplasmosis.

Introduction

Biotherapics constitute an alternative for the treatment of various diseases including the parasitic ones [1]. Toxoplasmosis, zoonosis of worldwide distribution, is a serious problem of human and animal public health due to clinical complications with serious consequences for humans and to sanitary, financial and commercial losses considering the infection in animals [2].

The search for the best dynamization of homeopathic medications is a challenge. Finding a functional and effective dilution for the control or reduction of toxoplasmosis damages is of great importance and it will contribute to the understanding of the action of high dynamizations in murine toxoplasmosis. Thus, the aim of this study was to perfom a screening of the dynamizations effects of high of biotherapic of *Toxoplasma gondii* in mice infected with this protozoan.

Methods

Experimental design, *animals* ***and infection***

In a duplicate, blind, controlled, randomized by draw assay, repeated twice 100 *Swiss* male mice, 60 days old, from the sectorial vivarium of the Universidade Estadual de Maringá were used. Animals were kept in sectorial vivarium under controlled environmental conditions for seven days adaptation period until the experiment beginning. They were housed in polypropylene cages (414 x 344 x 168mm size), capped with galvanized grid with central depression for feed and water bottle deposition. The animals remained in air-conditioned biotherium (temperature between 21 and 23°C) with 12 hours dark/light cycle. They received water and food (Nuvilab Cr-1® by Nuvital®) *ad libitum*. Infection was performed orally, with 20 tissue cysts of *T. gondii* ME49 strain avirlent. The cysts were contained in macerated of infected brains of mice, according to Dubey and Beattie [3] protocol. ME49 strain is classified by analysis of SAG-2 gene as belonging to genotype II [4]. Previous studies of Braga-Silva et

al [5] demonstrate that this strain promotes chronic infection and low mortality, in the experimental model used.

T. gondii Biotherapies (BIOT-TG)

Medicines were produced with infected brain of mice macerated (20 *T.gondii* cysts/100µL-242 bradyzoites/cysts average), prepared according to the Brazilian Homeopathic Pharmacopoeia [6] in laminar flow cabinet.0.9 mL of brain macerate was and addedto 9.1 mL of ethylicgrain alcohol 70% (95,0º GLTarumã – São Pedro Turvo-SP) in water bi distilled, autoclaved. The succussionwas performed using mechanical dynamizer (Denise 10-50 Autic®). The intermediary dynamizations were prepared with 70% alcohol and the dynamizations used on mice was prepared with 7% alcohol. Microbiological tests were conduced to (Microbiology Laboratory, UEM) of the inert ingredients and the results were negative, according to regulations of the Brazilian Ministry of Health - RDC no. 67 [7].

***Experimental groups and** pretreatment*

Five groups were constituted: GCinf – infected control group pretreated with 7% grain alcohol (n=20); BIOT-TG200 – pretreated with *T. gondii* medicine 200dH (n=20); BIOT-TG300 – pretreated with *T. gondii* medicine 300dH (n=20); BIOT-TG400 – pretreated with *T. gondii* medicine 400dH (n=20); BIOT-TG500 – pretreated with *T. gondii* medicine 500dH (n=20). The animals were randomly distributed so that the mean initial weight of mice in each group were statistically equal. Animals received pretreatment for three consecutive days before infection [8]. The medicines were diluted in water (1mL/100mL of water) in autoclaved drinking fountains offered *ad libitum* in a sterile amber bottle according to Aleixo et al., [9] **for 24 consecutive hours**. Since this study was designed as a screening tool to identify the best dynamization for future studies, we used a gradient with high potencies based on the experience of our group using biotherapic and *T. cruzi* [8,9].

Biological Risk

Biological risk was determined by inoculating five healthy mice, male, 8 weeks old, with 0,1 mL of biotherapic (original suspension) intaperitoneally. Mice were assessed for 30 days for weight, temperature, fur appearance, appearance of feces and mortality. No biological effects of biotherapic were found in these healthy mice.

Clinical parameters

All animals were clinically evaluated for four consecutive days during biotherapic administration and after infection on days 1st to 4th.

Weight - expressed in grams (g), it was individually assessed in digital scale. Weight variation was obtained by the difference final less initial of each period. *Temperature* - expressed in degree centigrade (°C), it was individually measured in the anterior region of the left back thigh (smaller amount of hair) using infra-red thermometer, TD-920.0387 model. *Water and feed intake* - expressed in milliliter (mL) and gram (g), respectively. They were collectively evaluated, considering the initial amount offered to the group subtracted from the measured amount after a day of intake. The result was divided by the number of animals to estimate individual amounts. *Amount of excreta* - expressed in grams (g), it was obtained by the final weighing of the lining of cages subtracted from the initial weighting. Feces and urine were considered together. All clean lining to be used was klin dried, weighted and packed in sealed plastic bags. *Mortality* - expressed as cumulative percentage (%) at the end of the study, it was analyzed and daily noted.

Euthanasia and brain collection

On the 60th days after infection (dpi), the animals were euthanized with cranio cervical dislocation after deep anesthesia with halothane steam [10]. Brain was collected.

Count of brain cysts

Brain was homogenized with mortar and pestle and resuspended in 1.0 mL of 0.9% saline solution. From this volume, 25µLwere examined between slide and cover slip in optical microscope for fresh counting of tissue cysts. The mean number of cysts ± standard deviation per milliliter of macerate for each group was determined according to Dubey and Beattie [11] protocol.

Statistical analysis

Data were compared between groups using the *BioEstat* 5.0 software (Manaus-Brazil). Data with normal distribution were expressed as mean ± standard deviation and compared with ANOVA-Tukey, significance level 5%.

Ethics

The study was submitted and approved by the UniversidadeEstadual de Maringa Ethics Committee on Research Involving Animal Experimentation, Registration No. 109/2011. For ethical reasons - the principle of the 3Rs were used this number of mice [12]

Results

During treatment (Tab. 1), stage in which the animals were healthy, water consumption was decreased in BIOT-TG400 group, when compared with GCinf ($p<0.05$). In the analysis of feed intake and disposal of excreta, BIOT-TG200, BIOT-TG300 and BIOT-TG500 groups presented higher values when compared with GCinf ($p<0.05$). There was no significant difference for the temperature and initial and final weight variation per period between the different groups and GCinf.

Table 1. Clinical parameters evaluated in *Swiss* healthy mice, during administration of *T. gondii* medicine for the different experimental groups. Values are expressed as mean± standard deviation.

Groups	Weight variance (g)	Temperature (°C)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
Control	0.2±1.5a	34.8±0.4a	199.3±2.5a	135.0±4.5a	96.7±4.7a	0/20 (0)
BIOT-TG200	0.6±0.6a	34.6±0.9a	182.3±13.6a	166.0±6.6b	127.0±6.9 b	0/20 (0)
BIOT-TG300	0.5±0.9a	34.7±0.7a	186.7±7.4a	175.9±6.0b	126.3±7.1 b	0/20 (0)
BIOT-TG400	0.7±1.4a	34.7±0.2a	160.3±21.4b	145.4±1.9a	109.7±9.0a	0/20 (0)
BIOT-TG500	0.2±1.3a	34.7±0.7a	218.3±1.5a	172.5±1.8b	140.7±3.1 b	0/20 (0)

N= number of deaths. n= total number of animals in the group

Control– control group treated with 7% grain alcohol, BIOT-TG200 infected and treated with *T. gondii* medicine 200dH, BIOT-TG300 infected and treated with *T. gondii* medicine 300dH, BIOT-TG400 infected and treated with *T. gondii* medicine 400dH and BIOT-TG500 infected and treated with *T. gondii* medicine 500dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Immediately after infection (Tab. 2), although BIOT-TG200, BIOT-TG300 and BIOT-TG400 groups have shown to less weight than GCinf, the difference between them was not significant. For BIOT-TG500, weight negatively varied with statistical difference compared with GCinf. The feed intake was increased for BIOT-TG300 when compared with GCinf. BIOT-TG200 and BIOT-TG300 groups had higher amount of excreta compared with Gcinf. There was no significant difference in temperature and water consumption in the different groups compared with GCinf. Note that treated mice showed no mortality. Two mice in BIOT-TG300 had ascites right after infection.

Table 2. Clinical parameters evaluated in *Swiss* mice treated with *T. gondii* medicine or control group, after infection with cysts of ME49 strain of *T. gondii*. Values are expressed as mean± standard deviation.

Groups	Weight variance (g)	Temperature (ºC)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
GCinf	1.9±2.3a	34.5±0.5a	179.5±12.4 a	158.7±15.0 a	92.5±12.4a	2/20 (10)
BIOT-TG200	0.5±0.9a b	34.7±0.9a	196.3±10.3 a	173.8±6.3a	114.3±10.1b	0/20 (0)
BIOT-TG300	1.4±1.3a	34.7±0.7a	211.3±6.9a	180.8±3.2b	116.0±12.2b	0/20 (0)
BIOT-TG400	0.8±1.6a b	34.0±0.8a	174.8±14.9 a	148.5±4.3a	88.8±5.3a	0/20 (0)
BIOT-TG500	-0.6±1.6b	34.3±1.1a	198.8±23.4 a	151.8±7.6a	97.3±2.9a	0/20 (0)

N= number of deaths. n= total number of animals in the group

GCinf– infected control group treated with 7% grain alcohol, BIOT-TG200 infected and treated with *T. gondii* medicine 200dH, BIOT-TG300 infected and treated with *T. gondii* medicine 300dH, BIOT-TG400 infected and treated with *T. gondii* medicine 400dH and BIOT-TG500 infected and treated with *T. gondii* medicine 500dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Regarding parasitological evaluation (Fig.1), BIOT-TG200 group had number significantly lower of cysts than all the other groups ($p<0.05$).

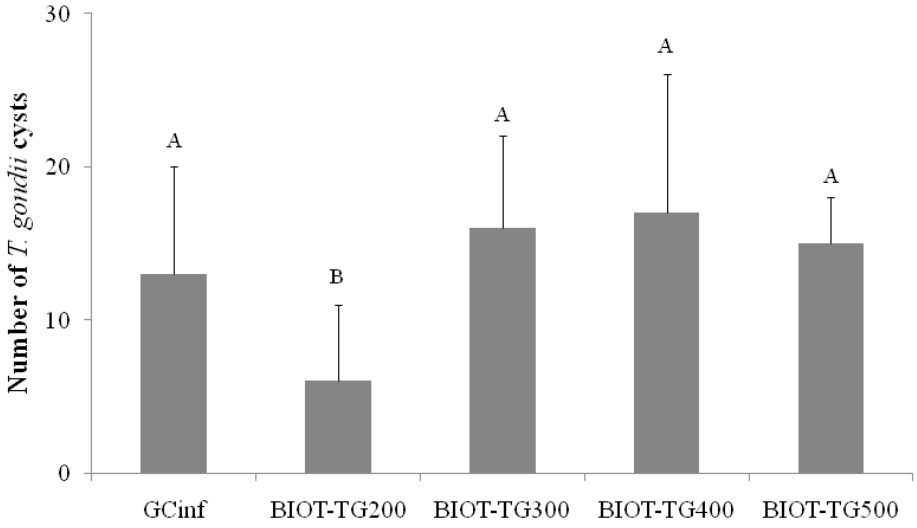

Figure 1. Number of *T. gondii* cysts quantified in 25 µL of brain of mice macerate for the different groups on 60 days after of infection: GCinf– infected control group treated with 7% grain alcohol, BIOT-TG200 infected and treated with *T. gondii* medicine 200dH, BIOT-TG300 infected and treated with *T. gondii*medicine 300dH, BIOT-TG400 infected and treated with *T. gondii* medicine 400dH and BIOT-TG500 infected and treated with *T. gondii* medicine 500dH.Values are expressed as mean±standarddeviation. Different capital letters indicate significant differences between the groups. 5% significance level.

Discussion

The treatment with 200dH was beneficial for infected mice that showed higher feed intake and increased excreta quantity demonstrating improved health status, since this behavior is related with greater metabolic utilization [9]. Treated animals also displayed better clinical development and reduction of the parasite load demonstrating better homeostasis in treated mice.

When deepening our studies, specifically analyzing the effects of dynamization 200dH [13] in mice treated with medication diluted in water, we noticed reduction of cysts in brain, myenteric neuronal protection in colon, immunomodulation of the evaluated cytokines with

for proinflammatory cytokines increased by IFN-γ at 30 and 60 dpi and decrease over time of infection. The cytokine IL-6 increased and reduced at 30 dpi along infection. TNF-α increased to 30 dpi and reduced the course of infection. For IL-17 there was a reduction in the evolution time. There was no significant variation in IL-2. Whereas the anti-inflammatory cytokine, the cytokine IL-10 decreased over the infection and IL-4 did not significantly varied in the experimental model used, response with Th1 predominance.

In the pre-infection clinical assessment performed during the administration of the biotherapics, dynamizations 200dH, 300dH and 500dH expressed favorable clinical conditions when compared with GCinf. It might be associated with the characteristic stimulation of biotherapics, which, when administered before the exposure to an infectious agent, prepares the organism for the infection with protective effect [14]. Dynamization 400dH was less effective at this stage, since the mice consumed less amount of water, which is one of the clinical signs of toxoplasmosis in animals [15]. This result might tell us that the mice treated with BIOT-TG400 presented unwanted results to the treatment, indicating inadequate potency. Homeopathic medicines generally have a sinusoidal behavior with an oscillatory potency-effect-curve [16]. Similar behavior of parasite load was observed, demonstrating the effects of the sinusoidal curve related to the potency/effect of *T.gondii* biotherapic. After infection, BIOT-TG500 presented weight loss, an important clinical sign of severe animal toxoplasmosis. This dynamization was not effective in controlling the infection, again indicating the inadequacy of potency. BIOT-TG200 e BIOT-TG300 groups showed greater effectiveness at this phase of infection. However, the mice treated with dynamization 300dH had ascites after infection, showing negative response to this dynamization, understood as inadequate potency in the evaluated context.

The groups of animals treated with biotherapic no showed mortality. The animals GCinf group showed losses in 30 and 45 dai. Mortality is an important clinical sign in murine

toxoplasmosis and in general, [15] and the fact that biotherapic prevent this event constitutes a promising result even that was not observed statistical difference in this assessment. According to Backer [17], recent statement of the American Statistical Association (ASA) advises researchers to avoid drawing scientific conclusions or making policy decisions based only on *P* values. A *P* value cannot determine the importance of a finding, for example, a drug can have a statistically significant effect on glucose levels in the patient's blood without having therapeutic effect [17]. Thus, the fact that mortality occurred in the untreated control group (GCinf) reveals an important result, which coincides with the most serious complication of both human and animal toxoplasmosis. It is an important criterion in judging the action of biotherapics.

The parasitological evaluation showed that mice treated with BIOT-TG200 had decreased number of cysts in the brain. This finding is in agreement with our previous studies [5,13]. The reduction of parasite load in toxoplasmosis is an extremely important factor, since *T. gondii* cysts can cause inflammation surrounding them with consequent complications such as blindness (in case of retina) and encephalitis, the latter a lethal disease in 20% of HIV-positive patients [18]. Besides tropism for the central nervous system (CNS), in congenital form, the parasite infects the placenta and later the fetus, which may present severe injuries such as hydrocephalus, cerebral calcifications and retinochoroiditis. Thus, congenital transmission is important both for public human and animal health and can occur when uninfected females acquire toxoplasmosis during pregnancy [19]. Moreover, in recent years, possible roles of toxoplasmosis in the etiology of certain mental, behavioral, and personality profiles disorders have been discussed in humans and animals [20,21].

The studied dynamizations (200dH, 300dH, 400dH and 500dH) of the biotherapic prepared from *T. gondii* cysts (strain ME49), offered prior to infection of mice by the parasite showed different effects.

The deepening of this research originated with the studies of Braga-Silva et al., [5] who, when examining one gradient of dynamizations between 7dH and 200dH in mice infected and pre-treated by gavage with biotherapic of *T. gondii*, found high sensitivity of the biological system for dynamization 200dH. This dynamization expressed positive results against the infection with a significant reduction in parasite load, TGF-β immunomodulation and decrease of ocular alterations. The data of the present study repeat the previous, confirming and reinforcing the finding of Braga-Silva et al., [5].

In conclusion, dynamization 200dH proved effective in reducing parasitism and damage, controlling toxoplasmosis in mice, while dynamizations 300dH, 400dH and 500dH, despite of the effects demonstrated, were not consistent with the benefits, considering the parameters evaluated in this study, indicating being inadequate dynamizations for toxoplasmosis in mice. The choice of the dynamization of the homeopathic medication is a matter of crucial importance for the treatment success.

Conflicts of interests

There is no conflict of interest.

Acknowledgments

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Artigo 2: “Biotherapic of *Toxoplasma gondii* reduces parasite load, improves experimental infection, protects myenteric neurons and modulates the immune response in mice with toxoplasmosis”

Biotherapic of *Toxoplasma gondii* reduces parasite load, improves experimental infection, protects myenteric neurons and modulates the immune response in mice with toxoplasmosis

Caroline Felicio Braga-Silva[a], Camila Santa Rosa Suhett[c], Ricardo Nascimento Drozino[a], Neide Martins Moreira[b], Débora de Mello Gonçales Sant'Ana[c], Silvana Marques de Araújo[a]

[a] Universidade Estadual de Maringá, Department of Basic Health Sciences, Maringá, Paraná, Brasil

[b] Universidade Estadual do Oeste do Paraná, Center of Education, Languages and Health, Foz do Iguaçu, Paraná, Brasil

[c] Universidade Estadual de Maringá, Department of Morphophysiology Sciences, Maringá, Paraná, Brasil

Corresponding author at: Caroline Felicio Braga-Silva

e-mail: **cfeliciobraga@gmail.com**

Universidade Estadual de Maringá

Departamento de Ciências Básicas da Saúde, Bloco I90, Setor Parasitologia Básica.

Av. Colombo, 5.790

Jardim. Universitário

Maringá - Paraná - Brasil

CEP 87020-900

Tel.: 55 (44) 3011- 4918

E-mail addresses: **cfeliciobraga@gmail.com** (C. F. Braga-Silva)

Abstract

Introduction: The evaluation of an ultradiluted medication in toxoplasmosis, will contribute to the understanding of the mechanism of action of homeopathic medications. This study evaluated the effect of the dynamization 200dH of *T. gondii* biotherapic in mice. *Methods:* In a blind, controlled, randomized assay, 45 *Swiss* male mice, were divided: GCN:uninfected and untreated control group(n=15);GCinf: infected control group pretreated with 7%grain alcohol(n=15);BIOT-TG200: pretreated with 200dH*T.gondii* medicine(n=15). Medicines were prepared according to the Brazilian Homeopathic Pharmacopoeia. Clinical, parasitological,

histopathological, and immunological parameters were evaluated on the 30th and 60th day post-infection(dpi).*Results:* A favorable clinical outcome was observed for mice treated with BIOT-TG200 the study. The number of brain cysts was reduced in the BIOT-TG200 group on the 30th($p<0.01$) and 60th dpi($p<0.05$), compared with GCinf. There was myenteric neuronal protection in the colon of mice treated with BIOT-TG200 and for proinflammatory cytokines there was an increase of IFN-γ at 30 and 60 dpi and decrease over the course of infection. The cytokine IL-6 increased at 30 dpi and decreased over the course of infection. TNF-α increased at 30 dpi and reduced over the course of infection. For IL-17 there was a reduction in the over the course of infection. There was no significant variation in IL-2. The anti-inflammatory cytokine IL-10 decreased over the course of infection and IL-4 did not vary significantly, predominantly in Th1 response.*Conclusion:* There was beneficial effect of the highly diluted biotherapic of *T. gondii*, promoting more favorable clinical symptomatology, parasite load reduction, neuronal protection and immune system modulation.

Keywords: Toxoplasma gondii; homeopathy; enteric nervous system; cytokines

Introduction

Toxoplasma gondii is protozoan distributed worldwide, with high serologic prevalence, affecting more than 60% of the population in certain countries, varying by region, according to socio-cultural habits, geographical and climatic factors [1]. It is well known that in women during the prenatal period, *T. gondii* infection can cause congenital toxoplasmosis, characterized by the Sabin's Tetrad: chorioretinitis, hydrocephalus or microcephaly, cerebral calcifications and neurological alterations [2]. Moreover, in recent years, possible roles of toxoplasmosis in the etiology of certain mental disorders, behavioral disorders, and human and animal personality profiles have been discussed [3,4]. In veterinary medicine, pathological damages and economic losses related to toxoplasmosis in commercialized species are significant and always merit great attention from entrepreneurs and researchers [5].

Furthermore, in *T. gondii* infection, alterations in intestinal functions such as diarrhea and inflammations compromising motility, secretion and absorption in the intestine may occur. These alterations mostly involve structural and/or functional changes in the enteric nervous system (ENS) [6].

All these alterations caused by *T. gondii* can affect neuronal function and structure, since this protozoan directly infects neurons and interferes in their survival and function, both in the brain and in the ENS. Thus, congenital infection can cause intestinal disorders that may persist throughout the host's life, leading to functional, behavioral and even psychological damages, which are not fully understood yet.

On the lack of knowledge of the scope of the consequences of congenital *T. gondii* infection and the absence of effective treatment, regardless of the phase of disease, homeopathy is one of the therapy possibilities. Conceptually, homeopathy may be defined as a science consisting of administering minimal doses of medication to the patient in order to stimulate organic reaction and prevent intoxication [7]. Among the medications which can be used are those prepared with the etiological agent itself [8], which can minimize future deleterious effects when administered before infection.

However, little is known about the effects of these medications against the experimental infection by *T. gondii* in the murine model [9,10]. The finding of a medication that stimulates an effective functional reaction in the host's biological system in the parasite/host relationship, besides providing expectancy for fighting toxoplasmosis, will contribute to the understanding of the mechanism of action of homeopathic medications. Thus, the aim of this study was to investigate the effects of dynamization 200dH of *T. gondii* biotherapic in mice pretreated and infected with this protozoan over 30 and 60 days. For this purpose, we analyzed clinical, parasitological, myenteric neurons, and immunological parameters, with assessment of the modulation direction of serum concentrations of anti-inflammatory cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10); and proinflammatory cytokines interferon-γ (IFN-γ), interleukin 6 (IL-6), interleukin 2 (IL-2), tumor necrosis factor (TNF-α) and interleukin 17 (IL-17).

Methods

Experimental design, Animals and Infection

The experiment was conducted in duplicate as a blind, controlled, randomized-by-draw assay. In each repetition, 45 *Swiss* male mice, 60 days old, from the central animal facility of the Universidade Estadual de Maringá were used. The animals were kept in a sectorial vivarium under controlled environmental conditions for a seven day adaptation period until the beginning of the experiment. They were housed in polypropylene cages (414 x 344 x 168mm in size), capped with galvanized grid with central depression for feed and water bottle deposition. The animals remained in air-conditioned biotherium (temperature between 21 and 23°C) with 12-hour dark/light cycle. They received water and food (Nuvilab Cr-1® by Nuvital®) *ad libitum*. Infection was performed orally, with 20 tissue cysts of *T. gondii* ME49 strain. The cysts were obtained from macerated ~~of~~ infected brains of mice, according to Dubey and Beattie [11] protocol. ME49 strain is classified by analysis of SAG-2 gene as belonging to genotype II [12].

T. gondii Biotherapies (BIOT-TG 200)

Medicines were produced with macerated infected mice's brains (20 *T. gondii* cysts/100µL-242 bradyzoites/cysts average), prepared according to the Brazilian Homeopathic Pharmacopoeia [13] in laminar flow cabinet. 0.9 mL of brain macerate was added to 9.1 mL of ethylic grain alcohol 70% (95,0º GL Tarumã – São Pedro Turvo-SP) in autoclaved bi-distilled water, with 100 succussions between each dilution. The succussion was performed using mechanical dynamizer (Denise 10-50 Autic®). The intermediary dynamizations were prepared with 70% alcohol and the dynamizations used on mice was prepared with 7% alcohol. Microbiological tests (Microbiology Laboratory, UEM) of the inert ingredients were negative, according to regulations ~~of~~ from the Brazilian Ministry of Health - RDC no. 67 [14]. The presence of total coliform and fecal coliform/*E. coli* was investigated.

The choice of this potency began with the studies of Braga-Silva et al. [9] who, when analyzing a gradient of potencies between 7dH and 200dH in mice infected and pretreated with *T. gondii* biotherapic, verified high sensitivity in the biological system for 200dH. This potency expressed compensatory results against the infection, with a significant decrease in parasite load, TGF-β immunomodulation and reduction of ocular alterations.

***Experimental groups and** pretreatment*

Three groups were incorporated: GCN - uninfected and untreated control group (n= 15); GCinf – infected control group pretreated with 7% grain alcohol (n = 15); BIOT-TG200 – pretreated with 200dH *T. gondii* medicine (n=15). The animals were randomly distributed so that the mean initial weight of mice in each group was statistically equal. Animals received pretreatment for three consecutive days before infection [15]. Thus, the choice of using a biotherapic (isopathy) made from the parasite and administered as a pretreatment took into account the idea of a later practical application, using the biotherapic especially in females during the gestation period. The expected effect of the biotherapic could be related to a specific vaccine with stimulation of the immune system [16]. The medicines were diluted in water (1mL/100mL of water) in autoclaved drinking fountains offered *ad libitum* in a sterile amber bottle according to Aleixo et al., [17] **for 24 consecutive hours**.

Biological Risk

Biological risk was determined by inoculating five healthy mice, male, 8 weeks old, with 0.1 mL of biotherapic (original suspension) intraperitoneally, to assess the possible biological response to the medicine. For 30 days, mice were assessed for weight, temperature, fur appearance, appearance of feces and mortality. No biological effects from the biotherapic were found in these healthy mice.

Clinical parameters

All animals were clinically evaluated for four consecutive days during biotherapic administration and after 11-15, 25-30 and 55-60 days of infection. The following parameters were evaluated:

Weight - expressed in grams (g), it was assessed individually on a BEL® balance. *Temperature* - expressed in degrees centigrade (°C), it was individually measured in the anterior region of the left rear thigh (smaller amount of hair) using Icel thermometer, model TD-920.0387. *Water and feed intake* - expressed in milliliters (mL) and grams (g), respectively. They were evaluated collectively, considering the measured amount after a day of intake subtracted from the initial amount offered to the group. The amount obtained was divided by the number of animals to estimate individual amounts. *Amount of excreta* - expressed in grams (g), it was obtained by subtracting the initial weight of the lining of the cages from the weight of the lining of the cages obtained after a day of use. Feces and urine were considered together. All clean lining to be used was kiln dried, weighed and packed in sealed plastic bags. *Mortality* - expressed as cumulative percentage (%) at the end of the study, it was analyzed and recorded daily.

Euthanasia and organ collection

On the 30th and 60th days after infection (dpi), five and ten animals, respectively, from each group, were euthanized with craniocervical dislocation after deep anesthesia with halothane steam [18]. Serum was collected by cardiac puncture and stored according to the existing guidelines/specified Cytometric Bead Array Kit (BD). Brain was collected. After laparotomy, colon was removed, with the ileocecal fold as anatomical reference.

Count of brain cysts

Brain was removed from cranium, homogenized with mortar and pestle and resuspended in 1.0 mL of 0.9% saline solution. From this volume, 25µL were examined between slide and cover slip under optical microscope for fresh counting of tissue cysts. The

mean number of cysts ± standard deviation per milliliter of macerate for each group was determined according to Dubey and Beattie [11] protocol.

Obtaining ~~of~~ the total colon preparations

The total colon of each animal was removed and evaluated regarding the presence of macroscopic lesions, and immediately washed with 0.9% saline solution, filled and immersed in fixative solution of acetic formalin for 48h. In sequence, each colon was dissected and the total preparations of the myenteric plexus were stained with Giemsa staining solution, using a protocol based on visualization of neurons by methylene blue [19].

Quantitative and Morphometric analysis of the myenteric plexus

We quantified the neurons present in 120 microscopic fields distributed throughout the intestinal circumference (optical light photonic microscope (Olympus BX40) with 40 times magnification objective). The area of colon, expressed in cm^2, was calculated with the width and length of each organ collected according to Sant'ana et al [20] protocol.

For morphometric analysis, we measured the areas (μm^2) of the cell body and nucleus of 300 myenteric plexus neurons of each animal, using images captured in trinocular photonic microscope (MOTIC B5), coupled to an image analysis system *Motic Images Plus*, version 2.0. The measurements were performed with the Image-Pro Plus 4.5® software. The area of cytoplasm was calculated through the difference between these areas according to Sant'ana et al [20] protocol.

Dosage of Cytokines

The serum concentrations of IL-4 and IL-10 (anti-inflammatory cytokines) and IFN-γ, IL-6, IL-2, TNF-α and IL-17 (pro-inflammatory cytokines) were measured. To evaluate the serum concentrations of cytokines, the Cytometric Bead Array Kit (BD) was used in flow cytometer (BD FACSCalibur™). The results were expressed in pg/mL.

Statistical analysis

Data were compared between groups using the *BioEstat* 5.0 software (Manaus-Brazil). Data with normal distribution were expressed as mean ± standard deviation and compared using ANOVA-Tukey or T test. We used significance level of 5%.

Ethics

The study was submitted and approved by the Universidade Estadual de Maringa Ethics Committee on Research Involving Animal Experimentation, Registration No. 109/2011. For ethical reasons - the principle of the 3Rs was used with this number of mice [21].

Results

Clinical parameters

All the groups started the experiment with the same clinical condition. During the pre-treatment (Table 1) period in which the animals were not yet infected, a significant decrease was observed in body weight in the BIOT-TG200 group, compared with the GCN. During this phase, no differences between the groups were observed for the other parameters and the animals of the BIOT-TG200 demonstrated clinical behavior which was always more similar to GCN.

In the clinical evaluation, 15 days after infection - dpi (Table 2), classic signs of toxoplasmosis were observed in GCinf, such as decrease in feed intake, decrease in water intake, constipation and mortality. At this stage, weight and body temperature in GCinf and BIOT-TG200 remained with no statistical difference between them and lower than GCN ($p<0.05$). The animals in BIOT-TG200 group presented water consumption equal to GCN and increased their water intake ($p<0.05$) compared with GCinf. Animals treated with BIOT-TG200 increased ($p<0.05$) feed consumption compared with GCinf, although less than GCN ($p<0.05$). The groups were different regarding the amount of excreta eliminated, and the difference was due to the lower values observed in GCinf group compared with GCN

($p<0.05$). Animals treated with BIOT-TG200 excreted as much as the healthy group, GCN. There was no statistical difference in mortality, although there were deaths in the infected groups.

Thirty days after infection (Table 3), GCinf presented higher morbidity of toxoplasmosis, evidenced by reduction in body weight ($p<0.01$) and hypothermia ($p<0.05$), compared with BIOT-TG200 and GCN groups. Feed intake was lower in BIOT TG-200 and GCinf than in GCN ($p<0.05$). There was no difference between the groups regarding water consumption and the amount of excreta. GCinf group presented typical signs of the disease, with tendency toward lower water intake, anorexia and constipation.

On 60th dpi (Table 4), GCinf and BIOT-TG200 had no significant difference between each other in body weight, however, these groups presented decreased weight compared with GCN ($p<0.05$). Animals in the infected groups (GCinf and BIOT-TG200) consumed less feed than the uninfected animals (GCN); it is noteworthy that the treatment increased the feed intake in the infected group in relation to the untreated group ($p<0.01$). Once again, the animals pretreated with biotherapic presented clinical performance similar to GCN. The groups presented no difference between each other regarding temperature, water consumption and amount of excreta.

Count of brain cysts

In parasitological evaluation (Fig. 1), the number of brain cysts was reduced in BIOT-TG200 group on the 30th ($p<0.01$) and 60th dpi ($p<0.05$), compared with GCinf. In comparative analysis of the time of infection (30x60dpi), the number of cysts was significantly increased on the 60th dpi for each group (BIOT-TG200 ($p<0.05$) and GCinf ($p<0.05$)).

Quantitative and Morphometric analysis of the myenteric plexus

In quantitative analysis of neurons in the colon (Figs. 2 and 3), GCinf had neuronal reduction on 30th dpi ($p<0.05$), compared with BIOT-TG200; however, these groups did not differ from GCN. On 60th dpi there was no difference between BIOT-TG200 and GCN, which presented a higher number of neurons ($p<0.05$) than GCinf. The aging process (30x60dpi) caused neuronal reduction in the groups ($p<0.01$).

With regard to the morphometric analysis of the colon (Table 5), on the 30th dpi, there was no significant difference between the infected groups (BIOT-TG200 and GCinf); however, these groups differ from GCN ($p<0.01$). The following percentage alterations stood out in this period: GCinf had an increase in size of 9% in the cell body, 7% in the cytoplasm and 11% in the nucleus, when compared with GCN. The BIOT-TG200 group had an increase of 8% in the cell body, 8% in the cytoplasm and 7% in the nucleus, compared with GCN.

On the 60th dpi, BIOT-TG200 and GCN presented no statistical difference between each other regarding the plastic alterations in cytoplasm. Both groups had measurements significantly higher than GCinf ($p<0.01$). For the cell body and nucleus, BIOT-TG200 and GCinf were equal to each other and significantly lower than GCN ($p<0.01$). In this period, we observed generalized tendency toward hypertrophy in the neurons and the alterations in BIOT-TG200 were more similar to GCN. In percentage, we observed, in GCinf, a reduction in size of 15% in cell body, 11% in cytoplasm and 19% in nucleus, when compared with GCN. BIOT-TG200 presented a reduction in size of 10% in cell body, 6% in cytoplasm and 14% in nucleus, compared with GCN.

In comparative analysis along the time of infection (30x60dpi), all the organelles increased significantly on the 60th dpi in the different groups ($p<0.01$). In percentage analysis, GCN had an increase in size of 28% in the cell body, 24% in the cytoplasm and 32% in the nucleus. In GCinf, the increase was 9% in the cell body, 9% in the cytoplasm and 8% in

the nucleus. For Biot-TG200, the increase was 14% in the cell body, 13% in cytoplasm and 16% in the nucleus.

Dosage of Cytokines

Regarding immunological parameters, in assessing the serum concentration of anti-inflammatory cytokines (Fig.4 A and B), there was no significant difference between groups in each of the periods of infection evaluated for IL-4 (Fig. 4 A). However, in assessing the course of infection (30x60dpi), there was significant decrease in the levels of this cytokine in GCinf ($p<0.05$), while BIOT-TG200 and GCN groups presented no significant difference. For IL-10 (Fig.4 B), BIOT-TG200 and GCN groups presented no statistical difference between them and were different from GCinf, which had increased serum concentrations of IL-10 on the 30th dpi ($p<0.05$). On the 60th dpi there was no significant difference between the groups. The concentrations of this cytokine decreased ($p<0.05$) over time (30x60dpi) in the infected groups (BIOT-TG200 and GCinf), while in GCN there was no significant variation.

In evaluation of pro-inflammatory cytokines (Fig.5 A, B, C and D), infected animals had higher serum concentration of IFN-γ (Fig.5 A) on 30th dpi and 60th dpi, compared with GCN ($p<0.05$). Over time (30x60dpi) BIOT-TG200 and GCinf groups presented a significant decrease in this cytokine ($p<0.05$), contrary to GCN, in which there was no significant variation.

IL-6 (Fig.5 B) increased in BIOT-TG200 in comparison with GCN ($p<0.05$) on the 30th dpi, with no significant variation in GCinf. On the 60th dpi, BIOT-TG200 and GCN presented no statistical difference between each other and were different from GCinf, which presented the highest serum concentration during this period ($p<0.05$). During the course of the infection (30x60dpi), there was no significant difference for GCN, while the infected groups (BIOT-TG200 and GCinf) reduced their levels ($p<0.05$).

On the 30th dpi, animals treated with biotherapic had higher concentrations of TNF-α (Fig.5 C), compared with GCN ($p<0.05$), with no difference to GCinf. On the 60th dpi, there was no significant difference between the groups for this cytokine. In the course of the infection (30x60dpi), BIOT-TG200 and GCinf presented a decrease in this cytokine ($p<0.05$), while in GCN there was no significant difference.

The cytokine IL-2 (Fig.5 D) presented no significant difference between the groups in the different periods of infection or between the times for each evaluated group. For cytokine IL-17 (fig. 6), there was no significant difference between the groups for each period of infection assessed. In assessing the course of the infection (30x60dpi), animals treated with BIOT-TG200 presented a significant decrease in the levels of this cytokine ($p<0.05$), while GCN and GCinf had no significant difference.

Discussion

Pretreatment with biotherapic prepared with ultradiluted *T. gondii* cysts (ME49 strain) in dynamization 200dH (BIOT-TG200 group), in mice experimentally infected with this protozoan, stimulated a favorable biological response to the host over the course of toxoplasmosis infection.

In the pre-infection clinical evaluation performed during the administration of biotherapic, a period in which all animals were equally healthy, a significant reduction in body weight was observed in BIOT-TG200 group compared to GCN. The consumption of feed and water, and elimination of excreta was not different between the groups, however, the animals in BIOT-TG200 group showed behavior more similar to what was observed in the uninfected control group (GCN). These results are indicative of the homeopathic medication stimulation in order to prepare the organism for infection. Velkers et al. [22] describe that isopathy is used as a method of host protection against infection. Furthermore, biotherapics are considered therapeutic resources, used in order to stimulate biological activity and thereby

inhibit causal pathogen activity, providing activation of the immune defenses in the organism [23]. A large number of dynamized biotherapics, prepared with parts or by-products of infection or pathogen, are used in the so called "isopathic prophylaxis" [24].

In the clinical evaluation shortly after the infection (11-15 dpi), the infected animals did not differ in weight and body temperature; however, the parameters related to intake/elimination in animals treated with BIOT-TG200 demonstrated favorable results for the host. Although the BIOT-TG200 medication has promoted clinical and parasitological benefits in the group as a whole, it could not prevent the death of one of the infected and treated animals (no statistical difference between the groups). Tenter et al. [25] reported that the formation of cysts may develop early, around 6-7 days after infection, persisting throughout the life of the host, and is related to the clinical severity typical of the disease, and may progress with irreversible damage to some animals (individuality of response), even causing death in the murine model used. In the group of untreated animals (GCinf), the consequences were statistically exacerbated. In the clinical evaluation performed 30 days after infection, despite the lower feed intake, animals treated with biotherapic 200dH demonstrated behavior more similar to GCN, while GCinf presented pronounced clinical manifestations of toxoplasmosis, related mainly to weight loss and hypothermia.

The counting of brain cysts in this period was significantly lower in the animals treated with biotherapic 200dH than in GCinf, expressing great benefit of the biotherapic to the host. Several studies prove the capacity of ultra/highly diluted medications (dilution beyond Avogadro's number) in improving/healing various diseases [9;17;26;27]. According to Almeida [28], the choice of the potency of medication in homeopathic treatments is performed considering that low potencies (6 to 12cH) are used for more organic or lesional cases, medium potencies (18 to 30cH), for less serious and functional cases, and high potencies (above 200cH), for predominantly mental cases. The potency 200dH corresponds to

a medication in 100cH, being therefore considered as a higher potency medication [29]. In this sense, we noticed that this biotherapic in this high potency caused functional and immunological alterations, with a differentiated balance of pro and anti-inflammatory cytokines, which controlled the parasite load and provided better clinical conditions in the treated animals.

During clinical evaluation, 60 days after infection, we observed balance of infection in the hosts, which can be related to the time that the infection achieved chronicity characteristic of the infection caused by the ME49 strain in Swiss mice, as it was also observed in GCinf. The infected animals had behavior with no statistical differences, especially the animals treated with biotherapic, which consumed more feed. This difference was considered beneficial as the increased food intake is compatible in veterinary medicine with healthier condition [30]. This result is also considered as a benefit, considering that decrease in food intake is a typical symptom of toxoplasmosis according to Galvão et al., [31]. Also during this time of infection, a higher number of cysts (parasite load) were found in the infected control group, compared with the group treated with biotherapic, showing beneficial effects in the treated group compared to the untreated group. When analyzing the influence of the time of infection (30x60dpi) on the number of cysts, we observed that the more chronic the infection, the higher the number of cysts found. However, when comparing the infected groups on 30th and 60th dpi, we noticed a lower number of cysts in the treated group (BIOT-TG200) when compared with the untreated group (GCinf). This lower number of cysts observed in animals treated with biotherapic is certainly related to the action of the ultradiluted medication, which alters the biological response in this complex parasite/host relationship. In this study, we demonstrated that the differentiated modulation of different cytokines is one of the factors related to the benefits observed. Bellavite et al., [32] claim that homeopathic medicines are able to stimulate self-regulation of the living systems in which they act. This self-regulation

observed in the treated group, in addition to the control of parasite load, provided better clinical conditions and neuronal protection in the colon, which was not observed in GCinf.

Cysts are common in the central nervous system, in skeletal and cardiac muscle, but can also be found in visceral organs, as reported by Tenter et al., [25] producing consequences. Several studies demonstrated the quantitative and morphological alterations caused by *Toxoplasma gondii* in the ENS, reporting alterations in the intestinal wall of infected animals, possibly due to the fact that the gastrointestinal tract is the route of entry of *T. gondii*. These studies also suggest that digestion and absorption might be compromised [33; 34; 35; 36; 37; 38; 39; 40]. In fact, when quantitatively analyzing the colon neurons of all the mice involved in the experiment, again, we observed beneficial effects of biotherapic in neuronal protection in the animals treated with BIOT-TG200, since both on 30th dpi and 60th dpi, the group treated with *T. gondii* biotherapic presented a higher number of neurons in the colon of the mice when compared with GCinf. The animal's aging (30x60dpi) caused neuronal reduction of the colon in the different groups involved in this study.

Regarding the morphometric alterations evaluated in this study, we observed a different dynamic for the infection of *T. gondii* in mice. The measurements taken on the 30th dpi on the colon of mice in the infected groups in this study were increased, compared with GCN. Sugauara et al. [34] describe that acute infections caused by strain of genotype II did not induce morphometric alterations in the neurons of the descending colon of rats. The authors attribute this fact to the lack of time to cause proliferative inflammatory response.

On the 60th dpi, the measurements for the cytoplasm of neurons of the animals in GCN and BIOT-TG200 presented no significant difference between each other and were increased when compared with GCinf. Once again, we suggest the benefit of homeopathic medication by maintaining myenteric neuronal plasticity, attempting to compensate for possible disorders caused by infection. Despite presenting no significant difference from

GCinf, the other parameters, like cell body and nucleus, demonstrated protection of these organelles with tendency toward normality in animals treated with BIOT-TG200. We believe that this cytoplasmic regulation may be related to a compensation mechanism in the ENS in order to keep the colon of the animals treated with ultradiluted medication in suitable conditions for digestion and absorption of nutrients, maintaining the homeostasis of intestinal motility, which corroborates the data of improved clinical outlooks as observed in the group treated with biotherapic.

The natural aging of the host provided hypertrophy in the remaining neurons, both in healthy and infected animals (30x60dpi). In percentage terms, the increase was greater in GCN, followed by those observed in BIOT-TG200 and finally, GCinf. Sugauara et al. [34] observed hypertrophy in the neurons of the terminal ileum, while no morphometric alteration was observed in the descending colon of mice infected with the ME49 strain. In studies performed on the jejunum of rats infected with *T. gondii* oocysts, neuronal atrophy on the 30th dpi and tendency to hypertrophy on the 90th dpi were observed [35]. Soares et al. [41] observed induction to hypertrophy in colon neurons of rats infected with *T. gondii* on the 30th dpi. The results show that the data can be very varied and should be analyzed in the context of each experiment, as **Sant'Ana** et al. [37] demonstrated that these plastic alterations depend on various factors, such as strain of the parasite, infectious stage (tachyzoites, bradyzoites, sporozoites), inoculation route (intraperitoneal or oral) of the parasite, infection phase (acute or chronic), digestive tract region and group of nerve cells evaluated, taking into consideration that the host species should always be considered. Moreover, the chronic neuroinflammation after infection with *T. gondii* in immunocompetent hosts may potentially cause behavioral and neurological disorders, which can lead to neurodegeneration, neurotransmitter abnormalities, and/or trigger alterations in morphology and functionality of neurons [4].

The effective control of parasite replication and disorders in the parasitophorous vacuole is dependent on extrinsic and intrinsic cellular mechanisms also mediated by cytokines [42;43]. Cytokines play an important role in the pathogenesis of toxoplasmosis [44;45]. One of the most important functions of the innate immune response to *T. gondii* is the ability to detect the pathogen and to produce the cytokine IL-12, which stimulates natural killer (NK) and T cells to produce pro-inflammatory cytokine IFN-γ [46]. Although literature indicates IFN- γ as an important factor in controlling and eliminating *T. gondii* in the brain [47;48], this study demonstrated that both on 30th and 60th dpi, the infected groups maintained serum concentrations of this cytokine equal to each other and increased in comparison with **GCN**. When comparing the time of infection (30x60dpi), the levels of this cytokine decreased in the infected groups. This modulation may be associated with increased IL-6 and TNF-α on 30dpi characterizing host resistance to parasite.

TNF-α, pro-inflammatory cytokine, is directly involved in regulating the growth of tachyzoite. The balance between IFN-γ and TNF-α is a key factor in triggering effector functions against *T. gondii* during both acute and chronic phases of infection [49]. Likewise, IL-6, known for controlling the parasite load and inflammatory activity in the brain, is also a mediator responsible for the production of acute phase proteins in toxoplasmosis and increasing ~~of~~ the cytotoxic activity of NK cells [50;51]. In this experiment, on the 30th dpi, animals treated with biotherapic presented higher concentrations of TNF-α and IL-6 than GCN, unlike GCinf. Over the course of the infection (30x60dpi), these cytokines were reduced in BIOT-TG200 and GCinf ($p<0.05$), while in GCN there was no significant difference. This result may be related to better performance in controlling parasite multiplication with positive consequences in clinical outlook and neuronal protection in the group treated with biotherapic, demonstrating benefits to the host by altering the parasite/host relationship.

In toxoplasma infection, cytokines of Th2 profile can promote parasite multiplication; however, they can also be recruited to control harmful pro-inflammatory immune response [52]. In our results, over the course of the infection in IL-4 (30x60dpi), there was significant reduction in the levels of this cytokine in GCinf ($p < 0.05$), while the groups treated with BIOT-TG200 and GCN showed no significant difference. This decrease may be connected to a harmful immune response seen in GCinf, since the treated animals exhibit similar behavior to the healthy group (GCN). IL-10 was increased in ***GCinf*** on the 30th dpi, while GCN and mice treated with BIOT-TG200 remained the same at lower concentrations. The chronicity (30x60dpi) caused a reduction ($p<0.05$) of this cytokine in the infected groups (BIOT-TG200 and GCinf), while in GCN, there was no significant variation. Therefore, we believe that the increase of this cytokine in ***GCinf*** may be related to the greater number of parasites found in this group in addition to the adverse clinical performance. On the other hand, the fact that the BIOT-TG200 group was significantly equal to GCN may indicate that the inflammatory process was controlled with treatment using biotherapic, leading to equal performance to that observed in GCN, which is the healthy, uninfected group.

Th17 cells, another subset of effector T cells, produce the cytokines IL-17, IL-21 and IL-22 and contribute to the inflammatory response in parasite infection [53]. According to Afzali et al., [54], IL-17 is a marker for the severity of toxoplasmosis and its presence in ocular and cerebral toxoplasmosis is usually associated with the induction of autoimmune responses in the brain and eye. In this article, cytokine IL-17 presented no significant difference between the groups on 30th and 60th dpi, however the chronicity of infection significantly reduced the levels of this cytokine in the BIOT-TG200 group. We suggest that this reduction is indicative of benefits for the treated mice, since the absence and/or reduction of this cytokine is related to better pathogenesis of toxoplasmosis.

Pretreatment with biotherapic provided immune modulation in mice infected with *T. gondii*, with alterations in the expression of cytokines with profile Th1, Th2 and Th17, with predominance of Th1, with positive repercussions in the complex parasite/host relationship, triggering specific reactions toward the compensatory cure for the parameters evaluated in this study.

Conclusion

Mice treated with *T. gondii* biotherapic 200 dH before infection develop an adaptive response, promoting more favorable clinical symptomatology throughout the experimental period, parasite load reduction, neuronal protection, immune system modulation in a differentiated manner, and characterized response of Th1 related host resistance to parasite. For proinflammatory cytokines there was an increase of IFN-γ on 30 and 60 dpi and reducing the course of infection. The cytokine IL-6 was increased ~~to~~ at 30 dpi and reduced throughout the course of infection. TNF-α increased at 30 dpi and reduced over the course of infection. IL-17 decreased over the course of time. There was no significant variation in IL-2. Whereas the anti-inflammatory cytokine IL-10 decreased over the course of infection. There was no significant variation in IL-4 in the experimental model used. These results demonstrate that the biotherapic provides effective clinical/functional benefits to the host by differentiation in the course of parasite infection.

Conflicts of interests

There is no conflict of interest.

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Tables

Table 1. Clinical parameters evaluated in *Swiss* healthy 57-59 days old mice, during administration of *T. gondii* medicine for the different experimental groups. Values are expressed as mean± standard deviation.

Groups	Weight (g)	Temperature (ºC)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
GCN	43.8±0.7a	31.8±3.9a	130.3±7.6a	131.7±13.7 a	122±12.4a	0/15 (0)
GCinf	41.9±0.5a b	31.1±4.4a	117±14.4a	118.2±15.6a	116.5±4.7a	0/15 (0)
BIOT-TG200	40.9±0.8b	31.7±3.8a	130.3±16.2 a	124.3±21.9 a	120.6±11.1a	0/15 (0)

N= number of deaths. n= total number of animals in the group

GCN - uninfected and untreated control group; GCinf - infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Table 2. Clinical parameters evaluated in *Swiss* mice treated or untreated with *T. gondii* medicine, with 11-15 days of infection with cysts of ME49 strain of *T. gondii*. Values are expressed as mean± standard deviation.

Groups	Weight (g)	Temperature (ºC)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
GCN	46±0.4a	34.3±0.2a	140±18.7a	118.1±6.8a	107.6±8.6a	0/15 (0)a
GCinf	42.1±0.3 b	33.2±0.5b	120±13.5ab	90.2±8.6b	91.9±9.9b	2/15(13.2)a
BIOT-TG200	42.1±1.0 b	33.2±0.3b	145±20.4ac	102.6±14.5c	106.1±18a b	1/15 (6.6)a

N= number of deaths. n= total number of animals in the group

GCN - uninfected and untreated control group; GCinf - infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Table 3. Clinical parameters evaluated in *Swiss* mice treated or untreated with *T. gondii* medicine, with 25-30 days of infection with *T. gondii* ME49 strain cysts. Values are expressed as mean± standard deviation.

N= number of deaths. n= total number of animals in the group

Groups	Weight (g)	Temperature (°C)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
GCN	48.1±0.5a	33.3±0.6a	153.8±22.9a	119±11.1a	110.8±16.1a	0/15 (0)
GCinf	36.2±0.1 b	28.6±0.1b	133.8±26.9a	81.8±3.6b	96±14.5a	0/13 (0)
BIOT-TG200	39.1±0.5c	30.3±0.6c	151.3±35.7a	87.5±5.7b	109.6±21.5 a	0/14 (0)

GCN - uninfected and untreated control group; GCinf - infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Table 4. Clinical parameters evaluated in *Swiss* mice treated or untreated with *T. gondii* medicine, with 55-60 days of infection with *T. gondii* ME49 strain cysts. Values are expressed as mean± standard deviation.
N= number of deaths. n= total number of animals in the group

Groups	Weight (g)	Temperature (°C)	Water intake (mL)	Feed intake (g)	Excreta (g)	Mortality N/n (%)
GCN	48±0.4a	33±0.7a	100±7.1a	83.2±2.3a	78.6±12.8 a	0/10 (0)
GCinf	44.7±0.4 b	34±0.6a	116.3±13.1a	60±4.0b	73.4±11.8a	0/8 (0)
BIOT-TG200	44.5±0.7 b	33.8±0.4a	118.8±12.5a	66±2.5c	72.3±6.5a	0/9 (0)

GCN - uninfected and untreated control group; GCinf - infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Different letters in the same column indicate significant difference between groups ($p<0.05$).

Table 5. Mean ± standard deviation of the cell body, cytoplasm and nucleus of myenteric neurons in colon of mice on 30th and 60th dpi per *T. gondii* **cysts. Uninfected and untreated control group (GCN),**

infected and treated with 7% grain alcohol control group (GCinf), and treated with 200dH (BIOT-TG200) group. Values are expressed as mean ± standard deviation.

	30 days			60 days		
Groups	Cell body	Cytoplasm	Nucleus	Cell body	Cytoplasm	Nucleus
GCN	125.9±52.7Aa	65.2±32.3Aa	60.7±27.6Aa	175.1±88.9Ba	86.2±56.6Ba	88.9±42.7Ba
GCinf	138.7±64.3Ab	70.5±45.0Ab	68.2±33.5Ab	152.3±75.2Bb	77.8±53.0Bb	74.5±34.7Bb
BIOT-TG200	136.6±64.6Ab	71.0±47.9Ab	65.6±30.9Ab	159.2±87.1Bb	81.3±56.1Ba	77.9±41.1Bb

* Values followed by different lowercase letters in the same column present statistically significant difference between groups ($p<0.01$). Different capital letters in the same line and in the same organelle indicates statistically significant difference between the times for the same group ($p<0.01$). Test: ANOVA-Tukey. Area (μm^2).

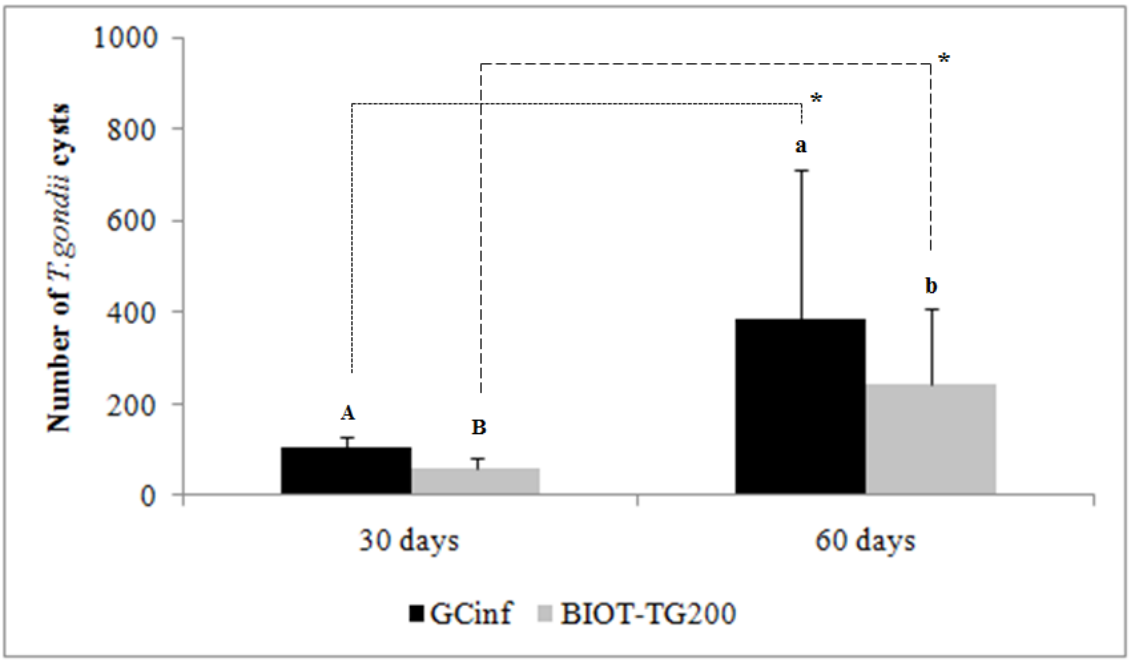

Figure 1. Number of *T. gondii* cysts quantified in 1mL of brain of mice macerate for the different groups on 30 and 60 days after of infection: GCinf - infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Values are expressed as mean±standard deviation. Different capital letters indicate significant differences between the groups on 30th dpi. Different lowercase letters indicate significant differences between the groups on 60th dpi. (*) indicates significant difference comparing values between 30 and 60 days of infection for each group. 5% significance level.

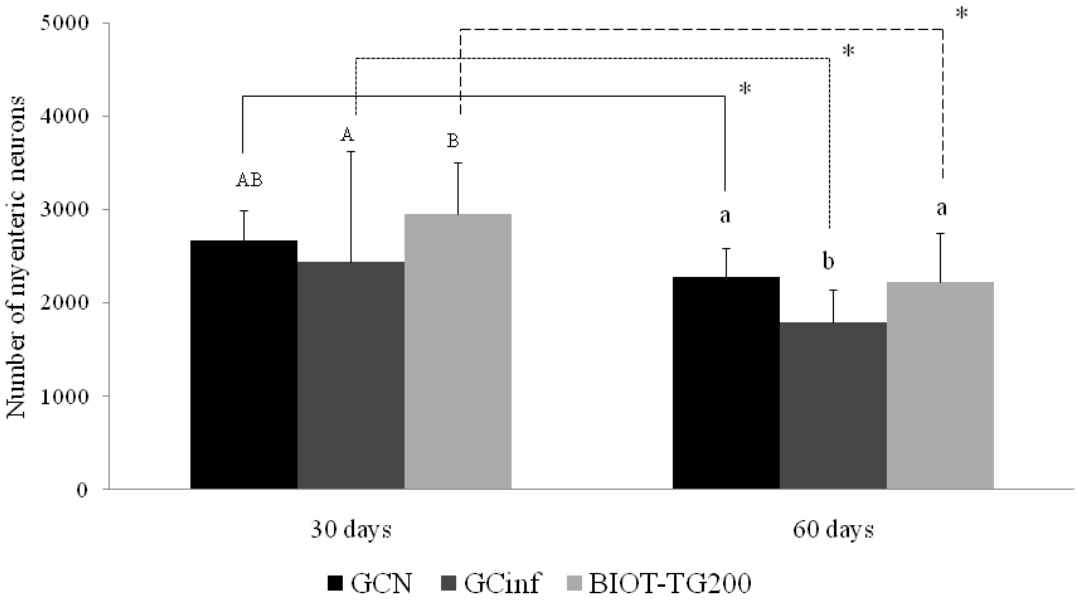

Figure 2. Density of the total population of myenteric neurons in the colon of mice on 30th and 60th dpi per *T. gondii* cysts. Uninfected and untreated control group (GCN), infected and treated with 7% grain alcohol control group (GCinf), and infected and treated with 200dH group (BIOT-TG200). Values are expressed as mean ± standard deviation. Different capital letters indicate significant differences between the groups on 30th dpi. Different lowercase letters indicate significant differences between the groups on 60 dpi. (*) indicates significant difference comparing values between 30 and 60 days of infection for each group. 5% significance level.

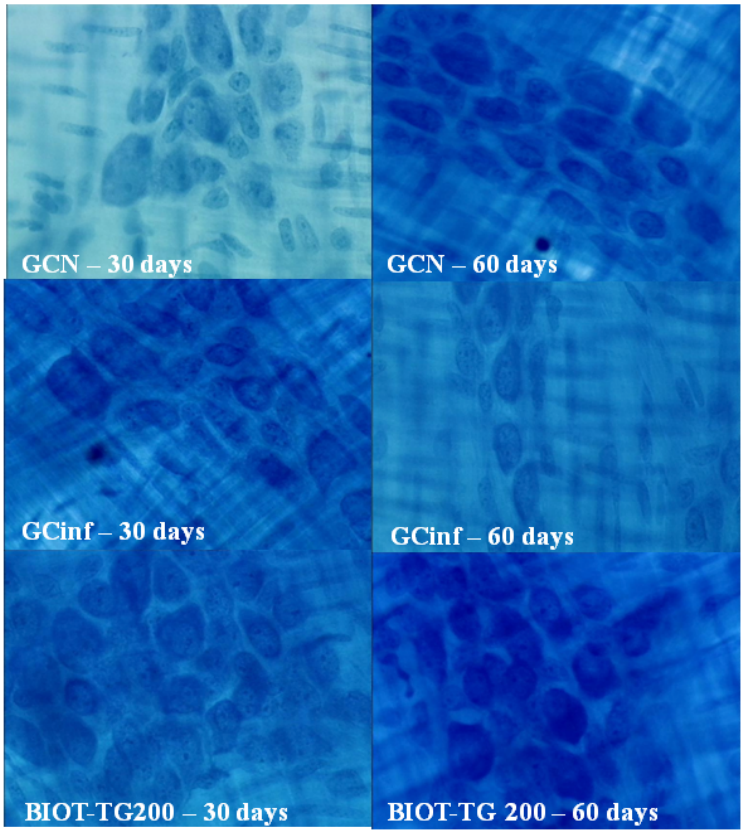

Figure 3. Photomicrograph of the neurons of the myenteric plexus in the colon of mice on 30th and 60th dpi per *T. gondii* cysts. Uninfected and untreated control group (GCN), infected and treated with 7% grain alcohol control group (GCinf), and infected and treated with 200dH group (BIOT-TG200). Giemsa 40X.

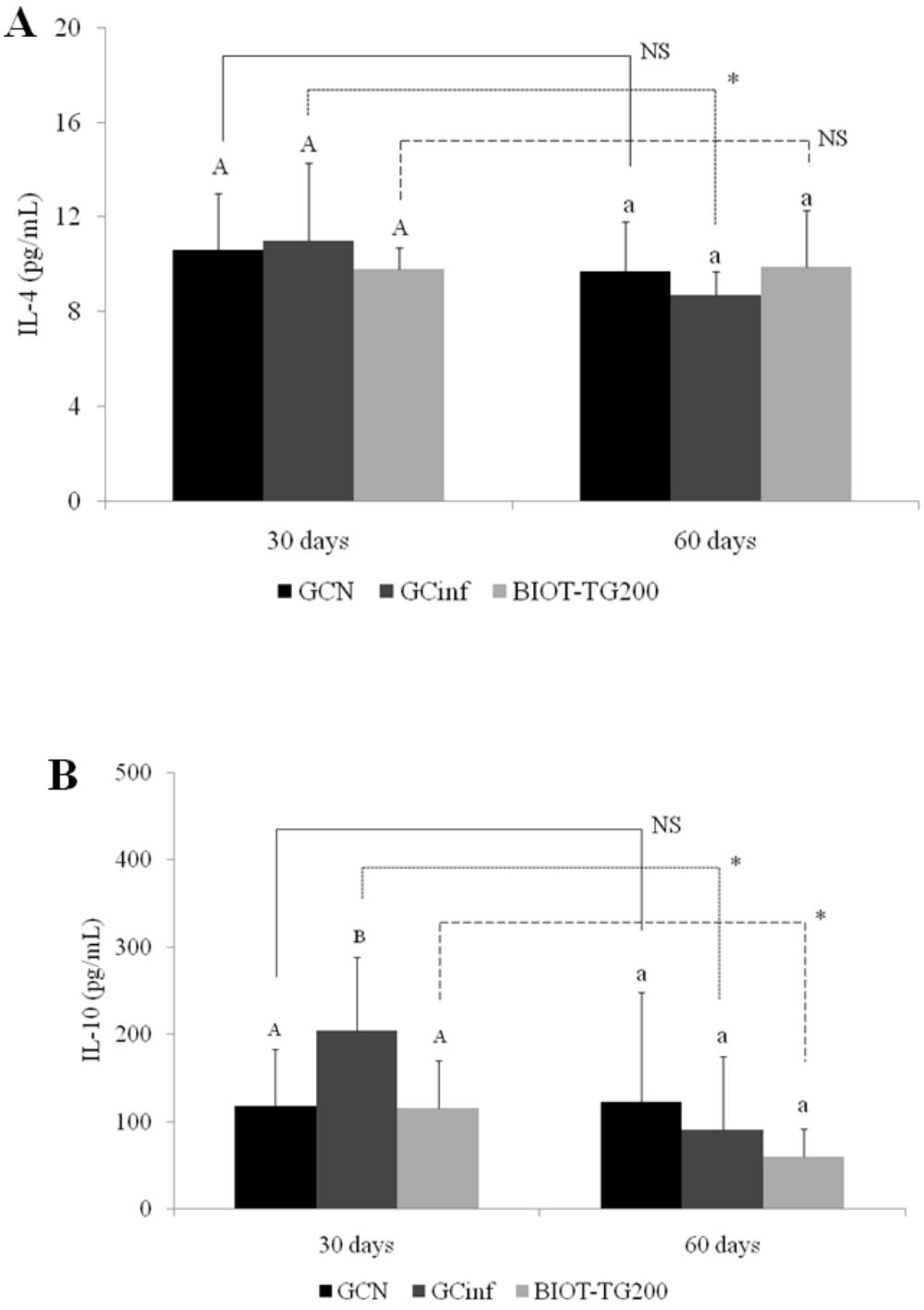

Figure 4. Serum concentration of IL-4 (A) and IL-10 (B) in different experimental groups on 30 and 60 days after of infection. GCN – uninfected and untreated control group; Gcinf – infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Values are expressed as mean±standard deviation. Different capital letters indicate significant differences between the groups on 30th dpi. Different lowercase letters indicate significant differences between the groups on 60th dpi. (*) indicates significant difference comparing values between 30 and 60 days of infection for each group. NS: Not significant. 5% significance level.

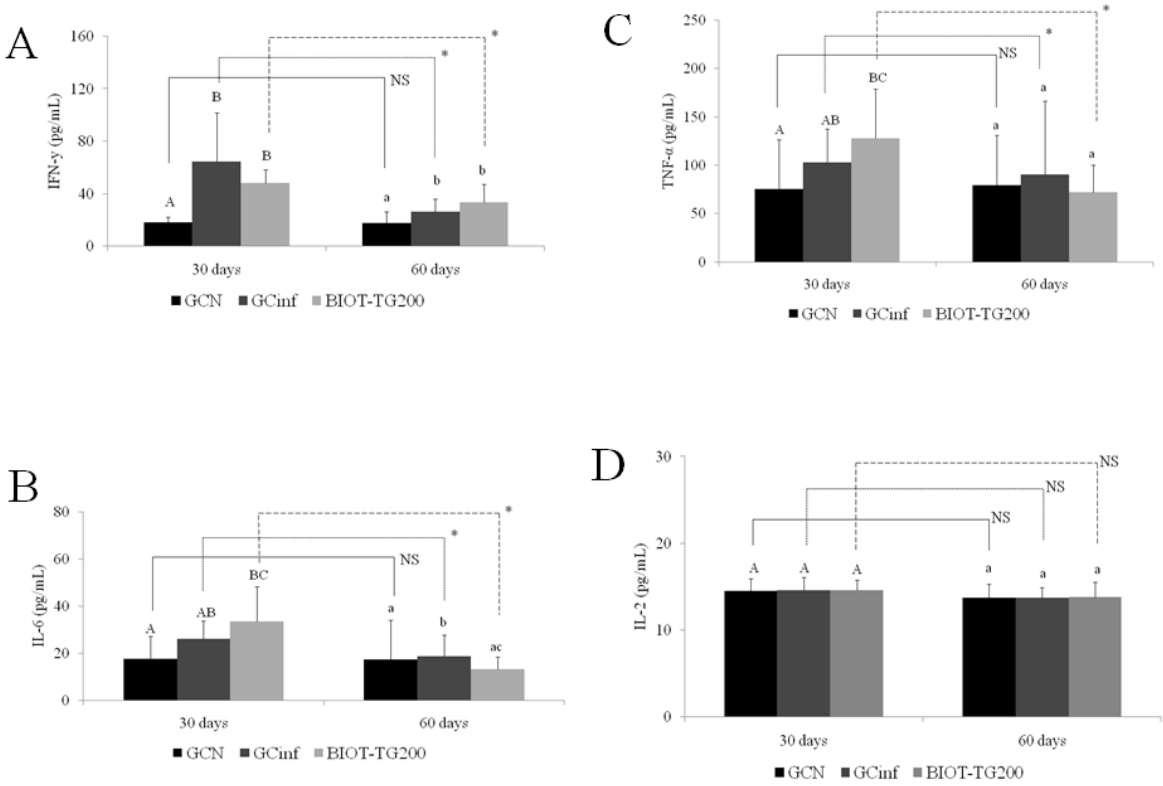

Figure 5. Serum concentration of IFN-γ (A), IL-6 (B), TNF-α (C) and IL-2 (D) in different experimental groups on 30 and 60 days after of infection. GCN – uninfected and untreated control group; Gcinf – infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Values are expressed as mean±standard deviation. Different capital letters indicate significant differences between the groups on the 30th dpi. Different lowercase letters indicate significant differences between the groups on the 60th dpi. (*) indicates significant difference comparing values between 30 and 60 days of infection for each group. NS: Not significant. 5% significance level.

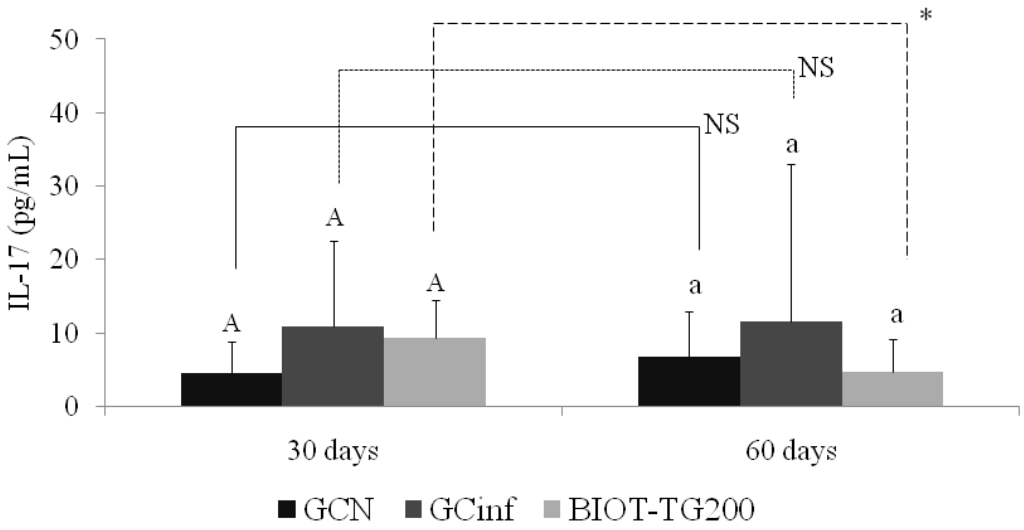

Figure 6. Serum concentration of IL-17 in different experimental groups on 30 and 60 days after of infection. GCN – uninfected and untreated control group; Gcinf – infected control group treated with 7% grain alcohol and BIOT-TG200 infected and treated with *T. gondii* medicine 200dH. Values are expressed as mean±standard deviation. Different capital letters indicate significant difference between the groups on 30th dpi. Different lowercase letters indicate significant difference between the groups on 60 dpi. (*) indicates significant difference comparing values between 30 and 60 days of infection for each group. NS: Not significant. 5% significance level.

CAPÍTULO III

CONCLUSÕES

Deste estudo realizado para comparar o efeito de diferentes dinamizações de medicamento produzido com cistos de *Toxoplasma gondii* em camundongos infectados com este protozoário, concluí-se que:

1) Todos os animais tratados com bioterápico apresentaram efeitos verificados visual e estatisticamente;

2) BIOT-TG 200, considerando todos os parâmetros avaliados (clínicos, parasitológicos, histológicos e imunológicos) apresenta melhor desempenho no tratamento pré-infecção da toxoplasmose em camundongos sendo esta a dinamização de escolha para aprofundamento dos estudos;

3) BIOT-TG200 apresentou clínica favorável em camundongos durante todo o estudo, além de redução no número de cistos cerebrais aos 30 e 60 dpi, houve proteção neuronal mientérica no colón de camundongos e modulação do sistema imunológico de forma diferenciada, para as citocinas pro-inflamatórias houve aumento do IFN-γ aos 30 e 60 dpi e diminuição ao longo do tempo de infecção. A citocina IL-6 estava aumentada aos 30 dpi e reduzida ao longo do tempo de infecção. O TNF-α estava aumentado aos 30 dpi e houve redução desta citocina ao longo do tempo de infecção. Para a IL-17 houve redução na evolução do tempo. Não houve variação significativa para IL-2. Para as citocinas anti-inflamatórias, a citocina IL-10 apresentou redução ao longo do tempo de infecção e não houve variação significativa para IL-4.

4) Analisando os parâmetros clínicos e parasitológicos considerados, todos os animais tratados com as dinamizações 300dH, 400dH e 500dH (bioterápicos BIOT-TG300, BIOT-TG400 e BIOT-TG500) apresentaram efeitos prejudiciais em camundongos e o número de cistos no cérebro variou como uma curva senóide dependendo da dinamização.

PERSPECTIVAS FUTURAS

Como perspectivas de futuros trabalhos com a dinamizaçãode escolha 200dH, descrevemos:

- A utilização de diferentes esquemas terapêuticos;

- Aprofundamento dos estudos em análises imunológicas para evidenciação de células em apoptose e avaliação de outras citocinas;

-Avaliação comportamental dos camundongos infectados e tratados;

-Testes com hospedeiros prenhes, caprinos, porcinos e outros, contribuirão para o esclarecimento do mecanismo de ação de substâncias ultradiluídas e a dinâmica da relação hospedeiro-parasito x bioterápicos.