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THIAGO DONIZETH DA SILVA

PAPEL DAS METALOPROTEINASES DE MATRIZ NA INFLAMAÇÃO GRANULOMATOSA INTESTINAL NA ESQUISTOSSOMOSE MANSÔNICA

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências pela Universidade Federal de Alfenas. Área de concentração: Biociências Aplicadas à Saúde.

Orientador: Prof. Dr. Rômulo Dias Novaes

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O Presidente da banca examinadora abaixo assina a aprovação da Dissertação/Tese apresentada como parte dos requisitos para a obtenção do título de Mestre em Ciências pela Universidade Federal de Alfenas. Área de concentração: Biociências Aplicada à Saúde

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Prof. Dr. Rômulo Dias Novaes

Presidente da Banca Examinadora

Instituição: Universidade Federal de Alfenas

UNIFAL-MG

Profa. Dra. Graziela Domingues de Almeida Lima

Prof. Dr. Eduardo Henrique Beber

Instituição: Universidade Federal do Espírito Santo

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“Deus não esgotou seu poder criador em Galeno. Seria um absurdo e quase uma heresia acreditar que Ele concedeu a Galeno uma genialidade sublime, com a condição de que nenhum outro mortal depois dele descobrisse algo novo... Miserável seria o nosso espírito se pudéssemos conhecer apenas o que foi descoberto antes de nós!”.

(Henri de Mondeville, 1260-1320)

RESUMO

A esquistossomose mansônica é uma doença parasitária negligenciada causada pelo helminto *Schistosoma mansoni*. Esta doença está intimamente relacionada com a pobreza e causa elevada morbidade entero-hepática, associada a uma intensa reação inflamatória granulomatosa desencadeada por ovos do parasita retidos na microcirculação do fígado e do intestino. Na enteropatia esquistossômica, a inflamação granulomatosa está associada à erosão intestinal e à translocação de ovos de *S. mansoni* para a luz intestinal, resultando na excreção fecal desses ovos. Este processo é essencial para completar o ciclo biológico envolvido na atividade do parasita e na transmissão para os organismos hospedeiros. Há evidências de que as metaloproteinases (MMP) estão envolvidas na inflamação granulomatosa e na dinâmica do colágeno nos órgãos-alvo de ovos de S. mansoni. No entanto, a relação entre MMP e o acúmulo de colágeno com a retenção intestinal e a excreção de ovos do parasita permanecem pouco compreendidas. Nesse sentido, o presente estudo investigou se a doxiciclina, um potente inibidor de MMP, é capaz de modular a inflamação granulomatosa e a remodelação morfológica intestinal até o ponto de influenciar a dinâmica entre retenção intestinal e excreção de ovos em ratos infectados por S. mansoni. No geral, nossas descobertas indicaram que o tratamento com doxiciclina subverte a dinâmica do colágeno na esquistossomose. Ao atenuar a atividade da MMP-2 e MMP-9, este medicamento é capaz de potencializar o acúmulo de colágeno na parede intestinal, fibrose tecidual e impedir a translocação de ovos de *S. mansoni*. Embora o conteúdo de colágeno não tenha apresentado correlação com a atividade de MMP, retenção intestinal e excreção fecal de ovos do parasita, essas correlações foram observadas em animais tratados com doxiciclina. Assim, nosso estudo fornece evidências de que a doxiciclina é capaz de atenuar a eliminação ambiental de ovos de S. mansoni, agravando a inflamação granulomatosa e inibindo a atividade de MMP-2 e MMP-9, eventos potencialmente associados ao acúmulo excessivo de colágeno, o que dificulta a translocação dos ovos através da parede para o lúmen intestinal. Variações na dinâmica do colágeno intestinal são relevantes, uma vez que podem representar alterações na dispersão ambiental dos ovos de S. mansoni, trazendo repercussões no ciclo de vida do parasita e na propagação da esquistossomose.

Palavras-chave: Doenças parasitárias; esquistossomose; inflamação granulomatosa; íleo; patologia experimental; Schistosoma mansoni.

ABSTRACT

Schistosomiasis mansoni is a neglected parasitic disease caused by the helminth *Schistosoma mansoni*. This disease is closely correlated with poverty and causes high enterohepatic morbidity associated with intense granulomatous inflammatory reaction triggered by parasite eggs retained in the microcirculation of the liver and intestine. In schistosomal enteropathy, granulomatous inflammation is associated with intestinal erosion and S. mansoni eggs translocation into the intestinal lumen, resulting in fecal excretion of these eggs. This process is essential to complete the biological cycle involved in parasite transmission to host organisms. There is evidence that metalloproteinases (MMP) are involved in granulomatous inflammation and collagen dynamics in the target organs of *S.mansoni* eggs. However, the relationship between MMP and collagen accumulation with the intestinal retention and excretion of parasite eggs remains poorly understood. In this sense, the present study investigated whether doxycycline, a potent MMP inhibitor, is capable of modulating granulomatous inflammation and intestinal morphological remodeling to the point of influencing the dynamics between intestinal retention and eggs excretion in *S. mansoni*-infected mice. Overall, our findings indicated that doxycycline treatment subverts collagen dynamics in schistosomiasis. By attenuating MMP-2 and MMP-9 activity, this drug is able to potentiate collagen accumulation in the intestinal wall, tissue fibrosis and hinder *S. mansoni* eggs translocation. Although the collagen content did not show correlation with MMP activity, intestinal retention and fecal excretion of parasite eggs; these correlations were observed for doxycycline-treated animals. Thus, our study provides evidence that doxycycline is able to attenuate the environmental elimination of S. mansoni eggs by aggravating granulomatous inflammation and inhibiting MMP-2 and MMP-9 activity, events potentially associated with excessive collagen accumulation, which hinders eggs translocation through the wall to the intestinal lumen. Variations in intestinal collagen dynamics are relevant since they may represent changes in the environmental dispersion of S. mansoni eggs, bringing repercussions for parasite life cycle of and schistosomiasis propagation.

Keywords: Parasitic diseases; schistosomiasis; granulomatous inflammation; ileum; experimental pathology; Schistosoma mansoni.

LISTA DE FIGURAS

LISTA DE ABREVIAÇÕES

DX	Doxycycline hyclate
ELISA	Enzyme-linked immunosorbent assay
H&E	Hematoxylin and eosin
IL-1	Interleukin 1
IL-4	Interleukin 4
MMP	Metalloproteinases
MMP-2	Matrix metalloproteinases 2
MMP-9	Matrix metalloproteinases 9
S. mansoni	*Schistosoma mansoni*
TGF-β	Transforming growth factor β
WHO	World Health Organization

SUMÁRIO

1. INTRODUÇÃO GERAL

Schistossoma mansoni destaca-se como um importante patógeno ambiental transmitido à humanos pela água (ANDERSON; ENABULELE, 2021). Esse parasito é uma espécie de helminto causador da esquistossomose, uma doença tropical negligenciada que apresenta ampla distribuição geográfica em países da África, Asia e América do Sul (LO *et al*., 2022; KOKALIARIS *et al.*, 2022). Nesses países, a presença de *S. mansoni* apresenta importante associação com as condições ambientais, especialmente temperaturas elevadas e disponibilidade de coleções hídricas necessárias para suportar o ciclo biológico desse parasito. Além disso, pobreza, práticas inadequadas de higiene e saneamento básico precário favorecem a dispersão desse parasito nas áreas endêmicas (LO *et al*., 2022; KOKALIARIS *et al.*, 2022). Estima-se que a esquistossomose afeta pelo menos 240 milhões de pessoas e é responsável por aproximadamente 10,1 milhões de mortes anuais em todo o mundo, sendo que a maior parte dos casos registrados são causados por *S. mansoni* (PINHEIRO *et al*., 2020; LO *et al*., 2022; KOKALIARIS *et al.*, 2022). No Brasil, estima-se que aproximadamente 1,6 milhões de indivíduos estão infectados por *S. mansoni*, com a maior parte dos casos concentrados em estados das regiões Nordeste, Sudeste e Centro-oeste (BARRETO; GOMES; BARBOSA, 2014; PINHEIRO *et al*., 2020; CAFIEIRO *et al.*, 2022). No entanto, essa prevalência pode chegar a pelo menos 1% da população brasileira (PINHEIRO *et al*., 2020).

Ao contrário de outros patógenos ambientais, *S. mansoni* apresenta marcante dimorfismo sexual, sendo caracterizado por vermes adultos machos e fêmeas (MCMANUS *et al*., 2018; ANDERSON; ENABULELE, 2021). O ciclo de vida desse parasito é heteroxênico, tendo o molusco (caramujo do gênero *Biomphalaria*) como hospedeiro intermediário e o ser humano como hospedeiro definitivo. Durante o seu ciclo de vida o parasito passa por diferentes formas evolutivas, como verme adulto, ovo, miracídio, esporocisto, cercaria e esquistossômulo. O ciclo tem início quando ovos de *S. mansoni* presentes nas fezes do hospedeiro alcançam coleções hídricas contendo o caramujo hospedeiro. Os ovos eclodem no ambiente aquático liberando larvas ciliadas conhecidas como miracídios, os quais atravessam partes moles do tegumento e infectam os caramujos. Após a penetração, os miracídios transformam-se em esporocistos mediante reprodução assexuada e diferenciam se em cercarias. As cercarias são liberadas na água e penetram na pele humana, infectando o

hospedeiro definitivo (COLLEY *et al*., 2014; ANDERSON; ENABULELE, 2021). Após a penetração cutânea, a cercaria perde a sua cauda transforma-se em esquistossômulo. Por sua vez, os esquistossômulos são carreados pela corrente sanguínea e passam pelo coração e pulmão até chegarem ao fígado, local onde se diferenciam em vermes adultos. Os vermes acasalam deslocam-se para a circulação portal, de modo que a fêmea inicia a postura de ovos cerca de 35 dias após a infecção. Cerda da metade dos ovos liberados pelo parasito alcançam a luz intestinal, enquanto os demais são retidos nos tecidos e órgãos do hospedeiro, especialmente fígado, baço, pulmão e intestino; desencadeando intenso processo inflamatório (ANDRADE, 2008).

A esquistossomose desenvolve-se em duas fases distintas, aguda e crônica. A fase aguda é considerada uma forma leve e frequentemente assintomática, com predominante acometimento hepatointestinal. Quando sintomática, a fase crônica se manifesta com hepatoesplenomegalia e hipertensão portal, sendo designada como forma hepatoesplênica avançada (ANDERSON; ENABULELE, 2021).

A esquistossomose aguda é caracterizada pela ativação de processo inflamatório granulomatoso, no qual o intenso recrutamento de leucócitos desencadeia a formação de numerosos granulomas periovulares em múltiplos órgãos, especialmente fígado, intestino e pulmão. Nessa fase, os granulomas são grandes, apresentam periferia pouco delimitada e o processo inflamatório periovular manifesta predominante componente exsudativo, principalmente evidenciado por intenso acúmulo de eosinófilos e frequente necrose periovular (MALTA *et al*., 2022). No início da fase crônica, granulomas exsudativos formados pela chegada recente de ovos do parasito ainda podem ser observados. No entanto, a progressão da infecção ativa mecanismos imunomoduladores que desencadeiam uma resposta inflamatória fibrogênica nas imediações de granulomas mais antigos. Nesse processo, parte das células recrutadas morre e o recrutamento celular diminui, dando lugar ao acúmulo progressivo de elementos colagenosos ao redor dos ovos de *S. mansoni* (bainha granulomatosa). Assim, os granulomas mais antigos tornam-se cada vez menores e acumulam cada vez mais colágeno. Essa dinâmica representa uma reação tecidual na tentativa de eliminar ou isolar os ovos/antígenos do parasito, com o objetivo neutralizar ou atenuar os danos inflamatórios ao organismo hospedeiro (WEINSTOCK *et al*., 1992; MALTA *et al*., 2022). Como o hospedeiro é incapaz de eliminar naturalmente a infecção, ocorre um ciclo contínuo

de inflamação e cicatrização, e a doença progride. Na fase crônica avançada, os granulomas são menores e apresentam marcante fibrose. Caso a doença não seja adequadamente tratada, essa fase pode evoluir para um quadro grave, caracterizado por fibrose hepática periportal, lesões vasculares destrutivas e obstrutivas; culminando em hipertensão portal e comprometimento do fluxo sanguíneo porta-hepático, o que é potencialmente fatal com a progressão para falência hepática (ANDRADE, 2008; COLLEY *et al*., 2014).

Não há dúvida de que as manifestações clinicas associadas ao comprometimento hepático são alvo primário de preocupações na esquistossomose (ANDRADE, 2008; MCMANUS *et al*., 2018). No entanto, essa doença é uma síndrome complexa que prejudica a estrutura e função de múltiplos órgãos, incluindo o intestino (WEINSTOCK, 1992; ANDRADE, 2008). Embora a patogênese da esquistossomose seja principalmente determinada pela inflamação granulomatosa desencadeada pelos ovos de *S. mansoni*, o impacto da inflamação nos diferentes órgãos do hospedeiro é amplamente variável (ELBAZ; ESMAT, 2013; MALTA *et al.,* 2022). Enquanto a hipertensão portal é a repercussão mais grave da inflamação granulomatosa hepática, a dispersão ambiental dos ovos de *S. mansoni* representa a principal consequência desse processo inflamatório granulomatoso intestinal (COLLEY *et al*., 2014; McMANUS *et al*., 2018). Nesse contexto, erosões da parede intestinal desencadeadas pelo processo inflamatório permitem que os ovos do parasito alcancem as fezes e sejam excretados para o meio ambiente, o que assegura a progressão do ciclo biológico *S. mansoni* (COSTAIN *et al*., 2018; SCHWARTZ *et al*., 2018; TAKAKI *et al*., 2021).

A erosão da parede intestinal resulta de um amplo remodelamento microestrutural associado à inflamação granulomatosa, o qual é mediado pela secreção de citocinas e proteases que regulam o recrutamento de leucócitos e o balanço entre síntese e degradação do colágeno (SINGH *et al*., 2006; COSTAIN *et al*., 2018; SCHWARTZ *et al*., 2018). Ao longo do desenvolvimento dos granulomas, compressão vascular e hipóxia tecidual provoca eventos necróticos regionais, os quais levam a morte de células e degradação de elementos da matriz conjuntiva da parede intestinal. Além disso, diversas proteases como as metaloproteinases (MMP) são ativadas no processo inflamatório granulomatoso, promovendo a destruição de fibras de colágeno ao mesmo tempo em que citocinas como IL-1, IL-4 e TGF-β, estimulam a colagenosenese por fibroblastos (STAVITSKY *et al*., 2004; SINGH *et al*.,

2006; COSTAIN *et al*., 2018; SCHWARTZ *et al*., 2018). Nas fases iniciais, ocorre intenso recrutamento de células e a colagenogênese supera a colagenólise, assegurando o crescimento dos granulomas com acúmulo o progressivo do colágeno necessário para isolar os ovos do parasito (SINGH *et al*., 2006; COSTAIN *et al*., 2018; SCHWARTZ *et al*., 2018).

A medida em que esses ovos são envolvidos por uma bainha granulomatosa cada vez mais espessa, o sistema imunológico fica menos exposto aos antígenos desses ovos. Nesse processo, ocorre atenuação do recrutamento celular e da colagenogênese, predominando a expressão e atividade da MMP, as quais promovem a degradação do colágeno e a involução dos granulomas esquistossomóticos. Nesse processo, a intensa colagenólise associada os eventos necróticos favorece a erosão intestinal e a translocação dos ovos de *S. mansoni* (SINGH *et al*., 2006; COSTAIN *et al*., 2018; SCHWARTZ *et al*., 2018).

Atualmente, existe evidência de que a caga parasitária e a taxa de postura de ovos pelas fêmeas de *S. mansoni* estão associados com o conteúdo de ovos do parasito na parede intestinal e com número de ovos excretados nas fezes do hospedeiro infectado (ROCHA *et al*., 1995; CHEEVER *et al.,* 1994, MATI; MELO *et al*., 2013). No entanto, ainda não se sabe se intervenções capazes de modular a atividade de MMP e o conteúdo de colágeno associado à inflamação granulomatosa intestinal podem influenciar na excreção de ovos nas fezes em hospedeiros infectados por *S. mansoni*. Nesse sentido, o presente estudo foi delineado para investigar se a doxiciclina, um antibiótico de amplo espectro com potente efeito inibitório sobre a atividade de MMP, é capaz de modular a inflamação granulomatosa e o remodelamento morfológico intestinal a ponto de influenciar na dinâmica entre retenção intestinal e excreção de ovos nas fezes em modelo experimental de infecção por *S. mansoni*. Variações nessa dinâmica são relevantes, uma vez que podem representar mudanças na dispersão ambiental de ovos desse parasito, trazendo repercussões para o ciclo de vida do parasito e para a propagação da esquistossomose.

Diante do exposto, o presente estudo teve por objetivo investigar se o tratamento com doxiciclina é capaz de modular o remodelamento morfológico do íleo e a excreção de ovos nas fezes em um modelo experimental de infecção por *S. mansoni*.

2 REVISÃO DE LITERATURA

2.1 ARTICLE: Matrix metalloproteinases 2 and 9 are involved in granulomatous inflammation, collagen accumulation and intestinal translocation of *schistosoma mansoni* eggs *in vivo*

Abstract

Schistosomiasis mansoni is a neglected parasitic disease caused by the helminth *Schistosoma mansoni*. This disease is closely correlated with poverty and causes high enterohepatic morbidity associated with intense granulomatous inflammatory reaction triggered by parasite eggs retained in the microcirculation of the liver and intestine. In schistosomal enteropathy, granulomatous inflammation is associated with intestinal erosion and S. mansoni eggs translocation into the intestinal lumen, resulting in fecal excretion of these eggs. This process is essential to complete the biological cycle involved in parasite transmission to host organisms. There is evidence that metalloproteinases (MMP) are involved in granulomatous inflammation and collagen dynamics in the target organs of *S.mansoni* eggs. However, the relationship between MMP and collagen accumulation with the intestinal retention and excretion of parasite eggs remains poorly understood. In this sense, the present study investigated whether doxycycline, a potent MMP inhibitor, is capable of modulating granulomatous inflammation and intestinal morphological remodeling to the point of influencing the dynamics between intestinal retention and eggs excretion in *S.mansoni*-infected mice. Overall, our findings indicated that doxycycline treatment subverts collagen dynamics in schistosomiasis. By attenuating MMP-2 and MMP-9 activity, this drug is able to potentiate collagen accumulation in the intestinal wall, tissue fibrosis and hinder *S. mansoni* eggs translocation. Although the collagen content did not show correlation with MMP activity, intestinal retention and fecal excretion of parasite eggs; these correlations were observed for doxycycline-treated animals. Thus, our study provides evidence that doxycycline is able to attenuate the environmental elimination of S. mansoni eggs by aggravating granulomatous inflammation and inhibiting MMP-2 and MMP-9 activity, events potentially associated with excessive collagen accumulation, which hinders eggs translocation through the wall to the intestinal lumen. Variations in intestinal collagen dynamics are relevant since they may represent changes in the environmental dispersion of S. mansoni

eggs, bringing repercussions for parasite life cycle of and schistosomiasis propagation.

Keywords: Parasitic diseases; schistosomiasis; granulomatous inflammation; small intestine; experimental pathology; *Schistosoma mansoni*.

Introduction

Schistosoma mansoni stands out as an important environmental pathogen transmitted to humans through water (Colley et al., 2014; McManus et al., 2018; Anderson & Enabulele, 2021). This parasite is a species of helminth that causes schistosomiasis, a neglected tropical disease with wide geographic distribution in African, Asian and South American countries (Lo et al., 2022; WHO, 2023). In these countries, high temperatures, water collections availability, poverty, inadequate hygiene practices and poor basic sanitation favor parasite spread (Lo et al., 2022; WHO, 2023). It is estimated that schistosomiasis affects at least 240 million people (Lo et al., 2022; WHO, 2023) and is responsible for about 10.1 million annual deaths worldwide (Pinheiro et al., 2020), with most registered cases being caused by *S. mansoni* (Lo et al., 2022; WHO, 2023).

The life cycle of *S. mansoni* is heteroxenic, with the mollusk (snail of the genus Biomphalaria) as the intermediate host and humans as the definitive host (Colley et al., 2014; McManus et al., 2018; Anderson & Enabulele, 2021). The parasite cycle begins when *S. mansoni* eggs present in the feces of the definitive host reach water collections containing snails. The eggs hatch releasing ciliated larvae (miracidia) that infect the snails. Miracidia transform into sporocysts and differentiate into cercariae, which are released into the water and penetrate the skin to infect the definitive host (Colley et al., 2014; McManus et al., 2018; Anderson & Enabulele, 2021). After cutaneous penetration, the cercariae lose their tail and transform into schistosomula, which are carried through the bloodstream and pass through the heart and lungs until they reach the liver, differentiating into adult worms. The worms mate and move to the mesenteric veins, where the female begins eggs-laying. Half of these eggs reach the intestinal lumen and are incorporated into the feces, while the rest are retained in the host's tissues and organs, triggering a marked granulomatous inflammatory process (Andrade, 2008; Colley et al., 2014).

In schistosomiasis, the intense leukocytes recruitment and activation of

fibrogenic mechanisms trigger periovular granulomas formation in multiple organs, especially liver and intestine. This process represents a tissue reaction in an attempt to isolate parasite eggs/antigens and mitigate inflammatory damage to the host (Weinstock et al., 1992; Malta et al., 2022). As the host is unable to naturally eliminate the infection, a continuous cycle of inflammation, cell death and scarring occurs, contributing to disease progression. Thus, untreated schistosomiasis can progress to intense periportal hepatic fibrosis and obstructive vascular lesions; culminating in portal hypertension, impairment of hepatic portal blood flow, tissue necrosis and liver failure, which is potentially fatal (Weinstock et al., 1992; Andrade, 2008; Colley et al., 2014). Clinical manifestations associated with hepatic impairment are the primary target of concern in schistosomiasis (Andrade, 2008; Colley et al., 2014; McManus et al., 2018). However, this disease is a complex syndrome that impairs the structure and function of multiple organs, including the intestine (Weinstock, 1992; Andrade, 2008). Although schistosomiasis pathogenesis is mainly determined by granulomatous inflammation triggered by *S. mansoni* eggs, the impact of this process on different organs is widely variable (Weinstock, 1992; Elbaz & Esmat, 2013; Malta et al., 2022).

While portal hypertension is the most serious consequence of hepatic granulomatous inflammation (Andrade, 2008; Colley et al., 2014; McManus et al., 2018), the environmental dispersal of *S. mansoni* eggs is the main consequence of intestinal inflammation. In this context, inflammatory erosions of the intestinal wall allow parasite eggs to reach the feces and be excreted into the environment, ensuring the progression of *S. mansoni* biological cycle (Costain et al., 2018; Schwartz et al., 2018; Takaki et al., 2021). Intestinal erosion results from extensive microstructural remodeling associated with granulomatous inflammation, which is mediated by cytokines and proteases that regulate leukocytes recruitment and the balance between collagen synthesis and degradation (Singh et al., 2006; Costain et al., 2018; Schwartz et al., 2018). As granulomas form and grow, vascular compression and tissue hypoxia cause regional necrotic events, which lead to cell death and degradation of the intestinal connective stroma. Accordingly, matrix metalloproteinases (MMP) are activated, promoting collagen fibers destruction at the same time that cytokines (e.g., IL-1, IL-4 and TGF-β) stimulate a reactive collagenogenesis by fibroblasts (Stavitsky et al., 2004; Singh et al., 2006; Costain et al., 2018; Schwartz et al., 2018). At the beginning of granulomas organization,

intense cell recruitment occurs and collagenogenesis overcomes collagenolysis, ensuring granulomas growth with progressive collagen accumulation to isolate the parasite eggs (Singh et al., 2006; Costain et al., 2018; Schwartz et al., 2018). As these eggs are surrounded by an increasingly thicker granulomatous sheath, the immune system is less exposed to the antigens of these eggs. In this process, cellular recruitment and collagenogenesis are attenuated, with collagen degradation and granulomas involution predominating. Thus, intense collagenolysis and necrotic events favor intestinal erosion and *S. mansoni* eggs translocation (Singh et al., 2004, 2006; Costain et al., 2018; Schwartz et al., 2018).

There is evidence that parasite load and the rate of *S. mansoni* eggs-laying are associated with the content of parasite eggs in the intestinal wall and with the number of eggs excreted in the feces of infected hosts (Rocha et al., 1995; Cheever et al., 1994, Mati & Melo et al., 2013). However, it is not yet known whether interventions capable of modulating MMP activity and collagen content associated with intestinal granulomatous inflammation can influence eggs excretion into the feces of *S. mansoni*-infected hosts. In this sense, the present study investigated whether doxycycline (a broad-spectrum antibiotic with a potent and non-specific MMP inhibitory effect) is capable of modulating granulomatous inflammation and intestinal morphological remodeling to the point of influencing the dynamics between intestinal eggs retention and excretion in an experimental model of *S. mansoni* infection. Variations in this dynamic are relevant, as they may represent changes in the environmental dispersion of parasite eggs, bringing repercussions to the parasite's life cycle and schistosomiasis spread.

Methodology

Animals and experimental groups

Seven-week-old male *Swiss* mice were used. The animals were kept in experimental facilities with controlled temperature (22 ± 2ºC), relative air humidity (60-70%) and photoperiod (12h/12h, light/dark cycles). Commercial chow and drinking water were provided *ad libitum*. Forty mice were divided into four groups with 10 animals per group: Control group (CG) = uninfected, group infected (GI) = infected with *S. mansoni*, group infected treated with praziquantel (GIP) = infected with *S. mansoni* and treated with 200 mg/kg praziquantel (Pz), group infected treated with

doxycycline (GID) = infected with *S. mansoni* and treated with 50 mg/kg doxycycline hyclate (DX). The infected animals were inoculated subcutaneously with 25 cercariae of *S. mansoni* LE lineage. After 80 days of infection, the animals were treated with DX and Pz for 60 days, administered orally by gavage. Praziquantel was administered in a single dose (200 mg/kg), according to reference therapy (Araújo et al., 2008). Doxycycline hyclate was administered daily at 50 mg/kg, which demonstrated immunomodulatory effects on hepatic granulomatous inflammation and effective MMP inhibition in an experimental model of *S. mansoni* infection (Dias et al., 2029). This study was approved by the Institution's Animal Ethics Committee (protocol #26/2018).

Euthanasia and necropsy

At the end of treatments, the animals were anesthetized with ketamine (150 mg/kg) and xylazine (16 mg/kg). After the complete abolition of respiratory movements and caudal, foot and corneal reflexes, a median thoracotomy was performed and the animals were euthanized through cardiac puncture. The terminal portion of the small intestine (ileum) was collected and washed with 0.9% saline until the luminal contents were completely removed. The ileum was used since this intestinal segment is the preferential site of oviposition for several *S. mansoni* strains in mice, including the LE strain (Mati & Melo, 2013). Ileum fragments were fixed by immersion in a 10% buffered paraformaldehyde solution (pH=7.4 to 0.1 mol) for 24 hours.

Histological processing

After histological fixation, ileum fragments were dehydrated in increasing ethanol concentrations (70% to 99.8%). They were then diaphanized in xylene and embedded in paraffin at 60 °C. For each animal, 8 semi-serial sections with 5-µm thickness were obtained using a rotating microtome, which were collected at intervals of 100 µm to avoid analyzing the same histological area. Histological sections were stained with hematoxylin and eosin (H&E) for general histopathological and morphometric analyses. They were stained using the Sirius Red method to observe collagen fibers under polarized light microscopy (Santos et al., 2021). Furthermore, the Alcian blue method was used to mark and analyze globet cells distribution in the intestinal lining epithelium and glycoconjugates deposition in the conjunctive matrix of

the lamina propria and submucosa (Dos Santos Lima et al., 2023). The sections were mounted with a coverslip and viewed using a bright-field photomicroscope (Axioscope A1, Carl Zeiss, Germany). Six images were captured in each histological section (3 with longitudinally sectioned crypts and 3 with transversely sectioned crypts) using ×20 objective lens and the Axion Vision LE image capture software (Carl Zeiss, Germany).

Intestine histomorphometry

The following microstructural parameters were analyzed in the ileum as previously reported (Sequetto et al., 2013, 2014): (i) intestinal villi length and width, (ii) crypts depth and area, (iii) intraepithelial lymphocytes number, and (iv) goblet cells number. Parameters (i), (ii) and (iii) were estimated from H&E-stained sections, while the last parameter (iv) was quantified from Alcian blue-stained sections. Crypts were analyzed from longitudinal and transversal sections. Parameters (iii) and (iv) were normalized by tissue area and expressed in cells/mm^2. All histomorphometric analyzes were performed by computational planimetry using the linear function and the counting function of the Image Pro-Plus 4.5 image analysis software (Media Cybernetics, Rockville, MD, USA) as previously reported (Novaes et al., 2016).

Schistosoma mansoni eggs quantification in the intestinal wall and feces

The number of *S. mansoni* eggs retained in the ileum wall was estimated according to a previously described method (Pellegrino & Faria, 1965; Mati & Melo, 2013). Briefly, ileum fragments were transferred to Petri dishes containing isotonic saline. They were then opened longitudinally and excess mucus and moisture were removed with absorbent paper. Then, the specimens were weighed and placed between a glass slide and a transparent plastic plate. This preparation was pressed for 1 minute with the aid of a 2kg metal weight. All eggs on each slide were counted using a bright-field microscope coupled with a ×100 objective lens (Axioscope A1, Carl Zeiss, Germany). The number of *S. mansoni* eggs (NE) retained in the intestinal wall was determined according to the following formula: NE= ∑ eggs counted / intestinal fragment mass (mg) × 1000.

Parasite eggs excreted in feces were quantified according to a previously described protocol (Hulstijn et al., 2001). Briefly, feces from all animals were individually collected twice a week in the last three weeks of the study. Feces were

processed according to the Kato-Katz method (Katz et al. 1972) and eggs were counted in two slides per animal using a bright-field microscope coupled with a ×10 objective lens.

Microscopic assay for collagen fibers

The same image capture protocol was applied to Sirius Red-stained sections observed under polarized light microscopy (Axioscope A1, Carl Zeiss, Germany). Intestinal collagen fibers content was estimated through computational analysis using a color segmentation protocol. Briefly, the polarized images were converted to the 8-bit channel and subjected to color segmentation to black and white. With the collagen fibers segmented in black, the relative histological area (%) occupied by these structures was automatically calculated using the threshold function of the image J software (Gonçalves et al., 2019; Dos Santos Lima et al., 2023).

Type I and type III collagen fibers proportion was analyzed using the Image Pro-Plus 4.5 (Media Cybernetics, Rockville, MD, USA software considering the birefringence properties of collagen fibers observed under polarized light (Novaes et al., 2014). Thus, a color matrix between yellow and red was created for type I fibers detection, while shades of green were referenced to detect type III collagen fibers. The relative distribution (%) of different collagen fibers was automatically calculated by the software as the proportion of pixels detected in the different color matrices in relation to the total number of colored pixels (Novaes et al., 2014).

Collagen biochemical assay

Ileum samples were homogenized in sodium phosphate buffer (pH 7.4) and centrifuged at 25,000 ×*g*. After centrifugation, tissue pellets were dehydrated at 80 °C for 16h and used to measure insoluble collagenous proteins. The samples were digested at 380 °C for 3h using a solution composed by 0.5 mL H_2SO_4, 5g $CuSO_4$, 0.5g selenium and 50g K_2SO_4). An aliquot of this solution (50 µL) was diluted in 50 µL of acidic water (2 mL H_2SO_4 in 75 mL of deionized water) and incubated with 1 mL of developing solution (0.5g ethylenediaminetetraacetic acid, 0.5g phenol crystal and 2.5mg sodium nitroprusside in 50mL deionized water, 0.25g NaOH, 0.19g Na_2HPO_4, 1.59g $Na_3PO_4.12H_2O$ and 0.25g NaClO in deionized water). Samples of 50 µL were analyzed in a spectrophotometer at 630 nm and compared with a blank solution (2mL H_2SO_4, 50mg $(NH_4)_2SO_4$ in 75mL deionized water). The collagenous protein content

was obtained by multiplying the nitrogen level by the correction factor 6.25 as previously described (Novaes et al., 2015).

Matrix metalloproteinases assay

Matrix metalloproteinases (MMP-1 and MMP-9) activity was analyzed in ileum samples. To this end, 70 mg samples were homogenized in 1mL of 5mM Tris-HCl buffer (pH 7.4) containing 0.15M NaCl, 10mM $CaCl_2$ and 0.02% NaN_3. After centrifugation at 10,000 ×*g* for 30min and 4ºC, the supernatant was collected for MMP activity analysis. For this, a commercial immunoenzymatic ELISA kit was used according to the manufacturer's instructions (ABCAM, Cambridge, MA, USA). The activity of each MMP was determined by the difference between the general enzymatic activity and the enzymatic activity obtained after homogenate treatment with specific MMP-2 (cis-9-Octadecenoyl-N-hydroxylamide) (Sigma-Aldrich, St. Louis, Missouri, USA) and MMP-9 (2-(N-benzyl-4-methoxyphenylsulfonamido)-5-((diethylamino)methyl)-N-hydroxy-3-methylbenzamide) inhibitors (ABCAM, Cambridge, MA, USA), as previously described (Dias et al., 2019).

Statistical analysis

The results were expressed as mean and standard deviation of the mean (mean ± S.D.) or median and interquartile range. Normality in data distribution was assessed using the D'agostino Pearson test. Student's t and Mann-Whitney U tests were respectively used to compare parametric and non-parametric data between two groups. To compare more than two groups, data variance was analyzed using one-way analysis of variance (One-way ANOVA) followed by the Student-Newman-Keuls *post-hoc* test. Non-parametric data were analyzed using the Kruskal-Wallis test for the same type of comparison. Correlations between variables were assessed using the Pearson or Spearman methods, according to data distribution. Results with P value ≤0.05 were considered statistically significant.

Results

As shown in Fig. 1, microscopic observation indicated that animals in the CG and GIP groups presented normal intestinal villi microstructure, which were well delimited, with intact lining epithelium supported by lamina propria with high cellularity, evident lacteal lymphatic vessels, and goblet cells clearly defined by

mucins histochemical staining. In these groups, intestinal villi were slightly enlarged compared to the CI and GID groups.

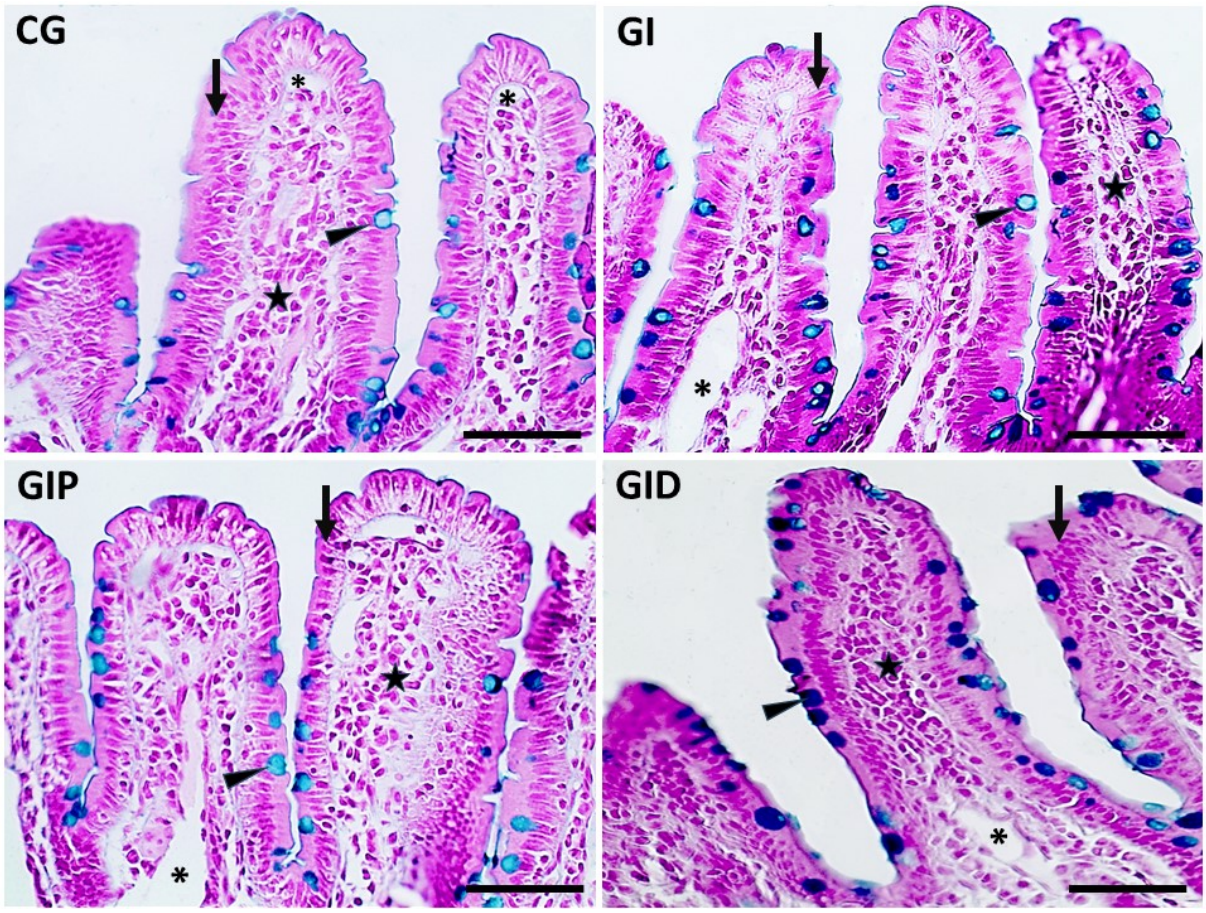


Fig 1. Microscopic images of intestinal villi observed in the ileum from *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Bright-field microscopy, Alcian blue staining, scale bars= 100 µm). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Arrow= intestinal lining epithelium, arrowhead= goblet cell, star= lamina propria, asterisk= lacteal lymphatic capillary.

As indicated in Fig. 2, the intestinal submucosa showed thin thickness and low cellularity in uninfected animals (CG). Submucosa thickening associated with connective tissue expansion was observed in all infected groups, especially GID and GI. Schistosomal granulomas were identified in these groups. Larger granulomas with greater cellularity in the granulomatous sheath were observed in the GID group followed by the GI group. The animals in the GIP group presented small granulomas with low cellularity.

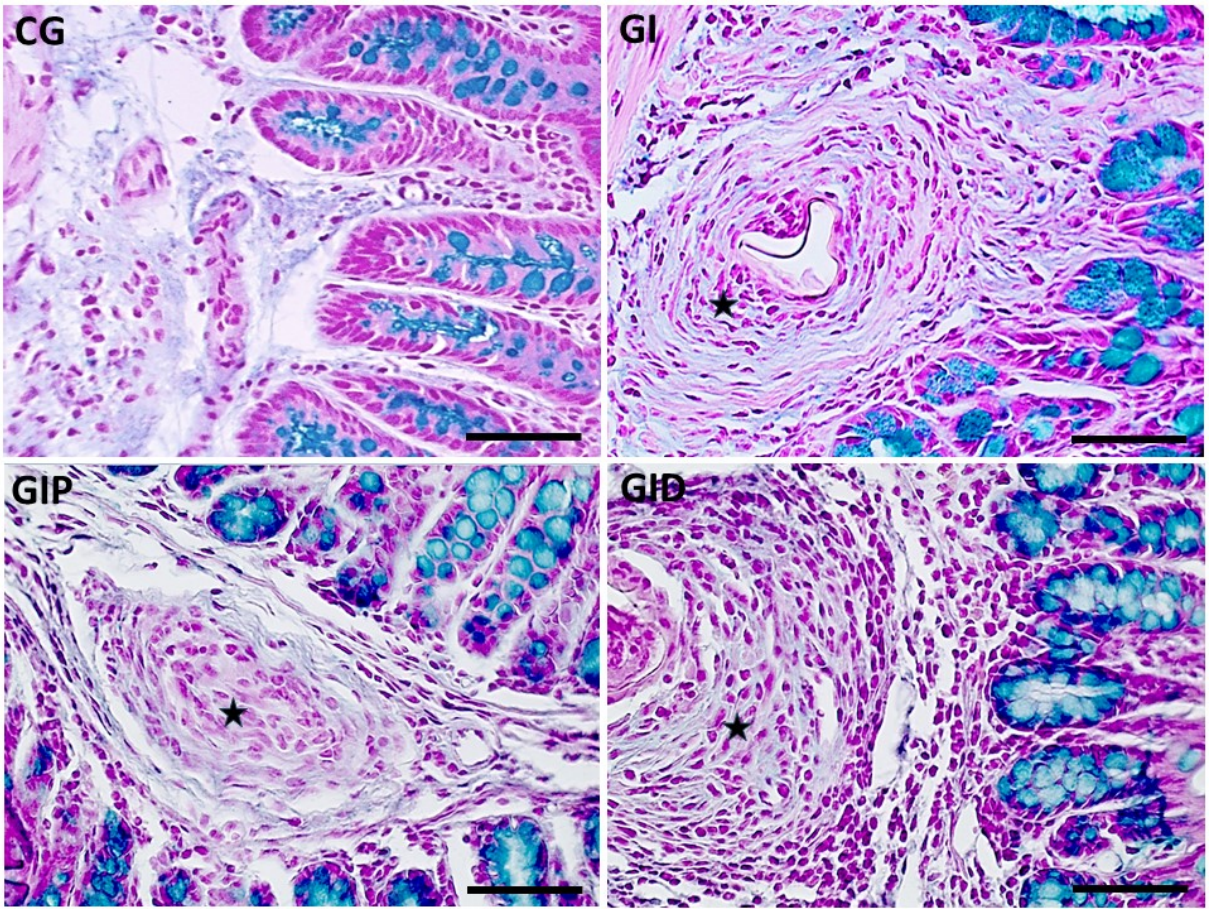


Fig 2. Microscopic images of the ileum submucosa in *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Bright-field microscopy, Alcian blue staining, scale bars = 100 μm). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Star= Schistosomal granuloma. Globet cells are stained in blue.

The results of the quantitative microstructural analysis (Fig. 3) reinforced the qualitative findings, indicating that intestinal villi length was similar in both groups ($P>0.05$). However, the average villi width was greater in the GI and GID groups compared to the CG and GIP groups ($P<0.05$). Goblet cells number was higher in the GID group compared to the GI group ($P<0.05$), and this parameter was also higher in these two groups compared to the CG and GIP groups ($P<0.05$). Intraepithelial lymphocytes number was higher in the GI group compared to the other groups ($P<0.05$), which demonstrated similar results ($P>0.05$).

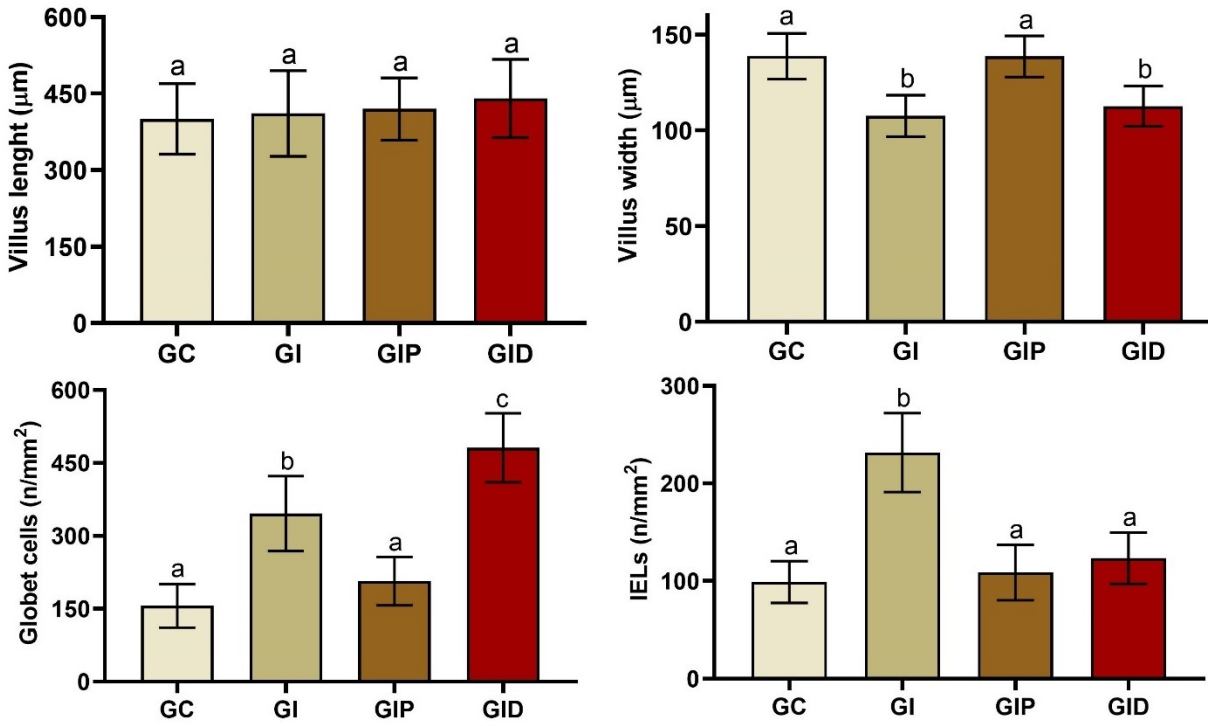


Fig 3. Microstructural parameters of the ileum from *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx). IELs: intraepithelial lymphocytes. Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Data are expressed as mean and standard deviation. Different letters in the columns (a, b, c) indicate statistical difference between groups ($P<0.05$). Groups with the same letters do not show statistical difference ($P>0.05$).

As shown in Fig. 4, well-defined intestinal crypts with intact lining epithelium and mucins filled lumen were observed in all groups. Crypts with increased cross-sectional profile were observed in the GI and GID groups. The morphometric analysis indicated that crypts depth was reduced and their width increased in the GI and GID groups compared to the CG and GIP groups ($P<0.05$). These parameters were similar between GI and GID animals ($P>0.05$).

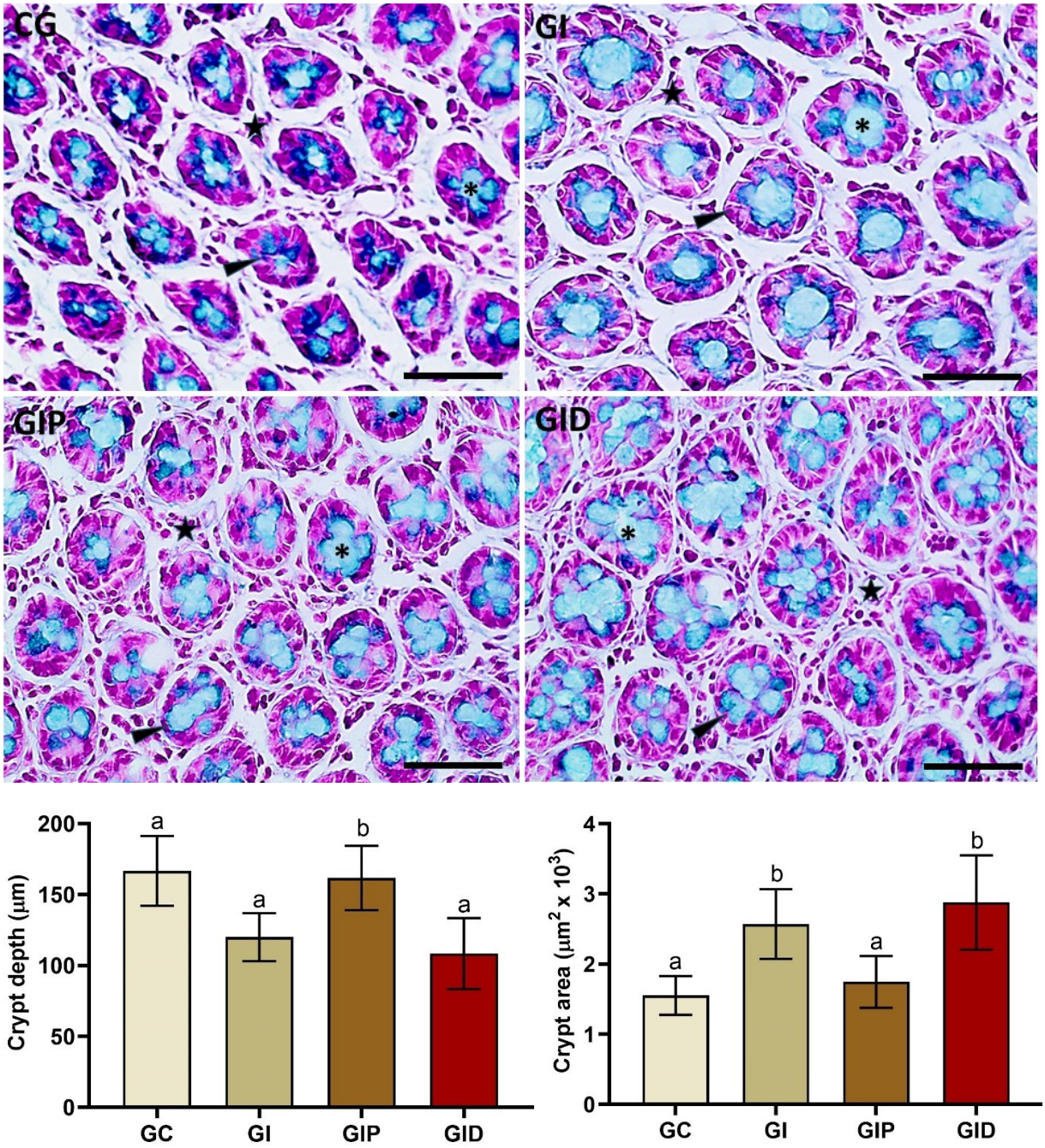


Fig 4. Microscopic images of cross-sectioned intestinal crypts, crypts depth and cross-sectional area in the ileum of *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Bright-field microscopy, Alcian blue staining, scale bars = 100 µm). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Arrowhead= intestinal crypts, asterisk= mucins, star= lamina propria. Data are expressed as mean and standard deviation in the graphics. Different letters in the columns (a, b, c) indicate statistical difference between groups ($P<0.05$). Groups with the same letters do not show statistical

difference (P>0.05).

Fig 5 shows collagen fibers distribution in the lamina propria that supports the intestinal crypts. Polarized light microscopy revealed the presence of thin collagen fibers organized in an anastomosed network manner, structuring a delicate mesh formed by type I (presenting birefringence in shades of yellow, orange and red) and type III (characterized by birefringence in shades of green) collagen fibers. Greater collagen fibers accumulation was observed in the lamina propria of GID animals.

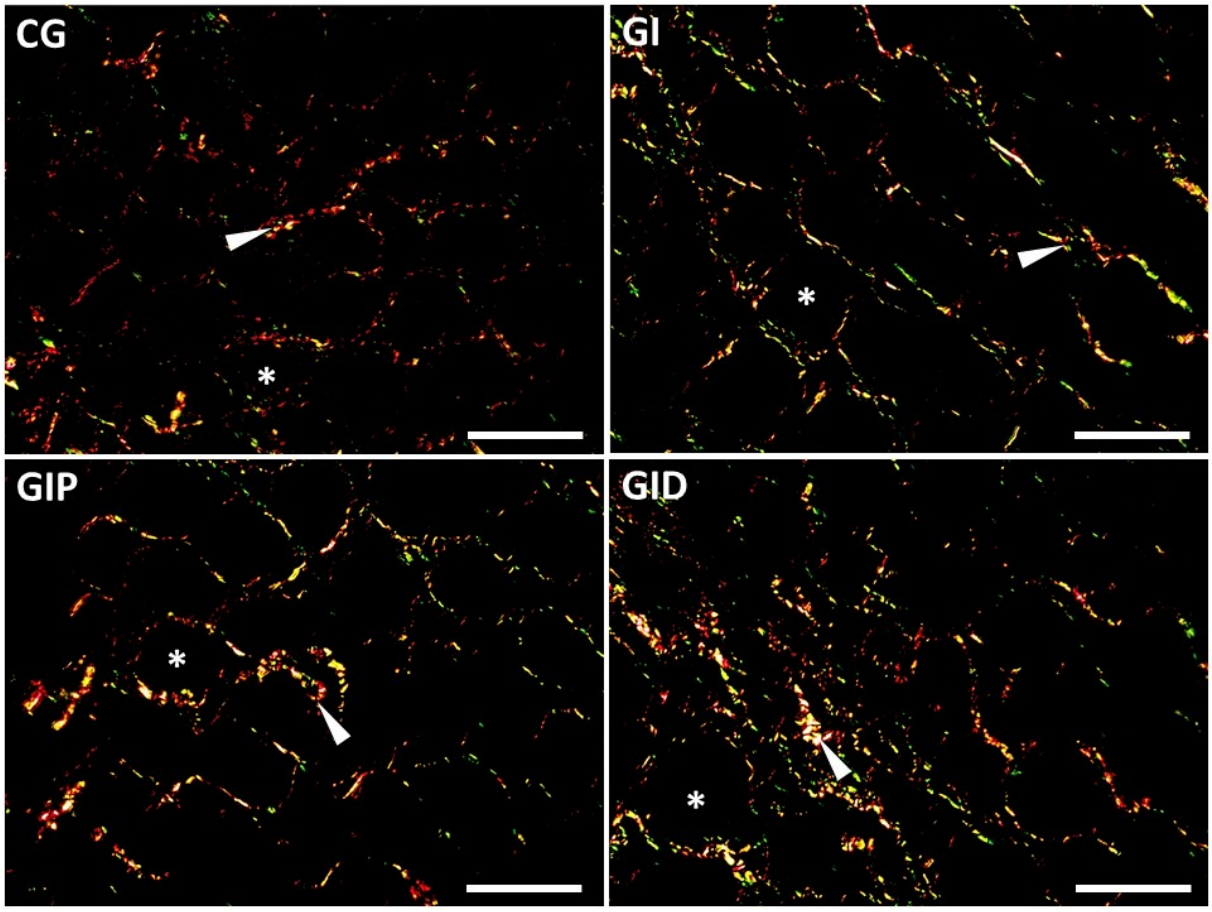


Fig 5. Microscopic images of collagen fibers in the lamina propria surrounding transversely sectioned intestinal crypts in the ileum of *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Polarized light microscopy, Sirius staining Red, scale bars = 100 μm). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Collagen fibers appear in the image as bright structures in different shades of yellow, orange, red and green. Asterisk= area occupied by intestinal crypts, arrowhead= collagen fibers in the lamina propria surrounding the intestinal crypts.

Collagen fibers accumulation and distribution in the intestinal submucosa is shown in Fig. 6. Greater collagen fibers accumulation was observed in the submucosa of all infected animals compared to the CG group. The greatest accumulation of these fibers was observed in the GI and GID groups, indicating tissue fibrosis. Thin type III collagen fibers were predominant in the GI group, while thicker type I collagen fibers were predominant in the submucosa of animals in the GID group.

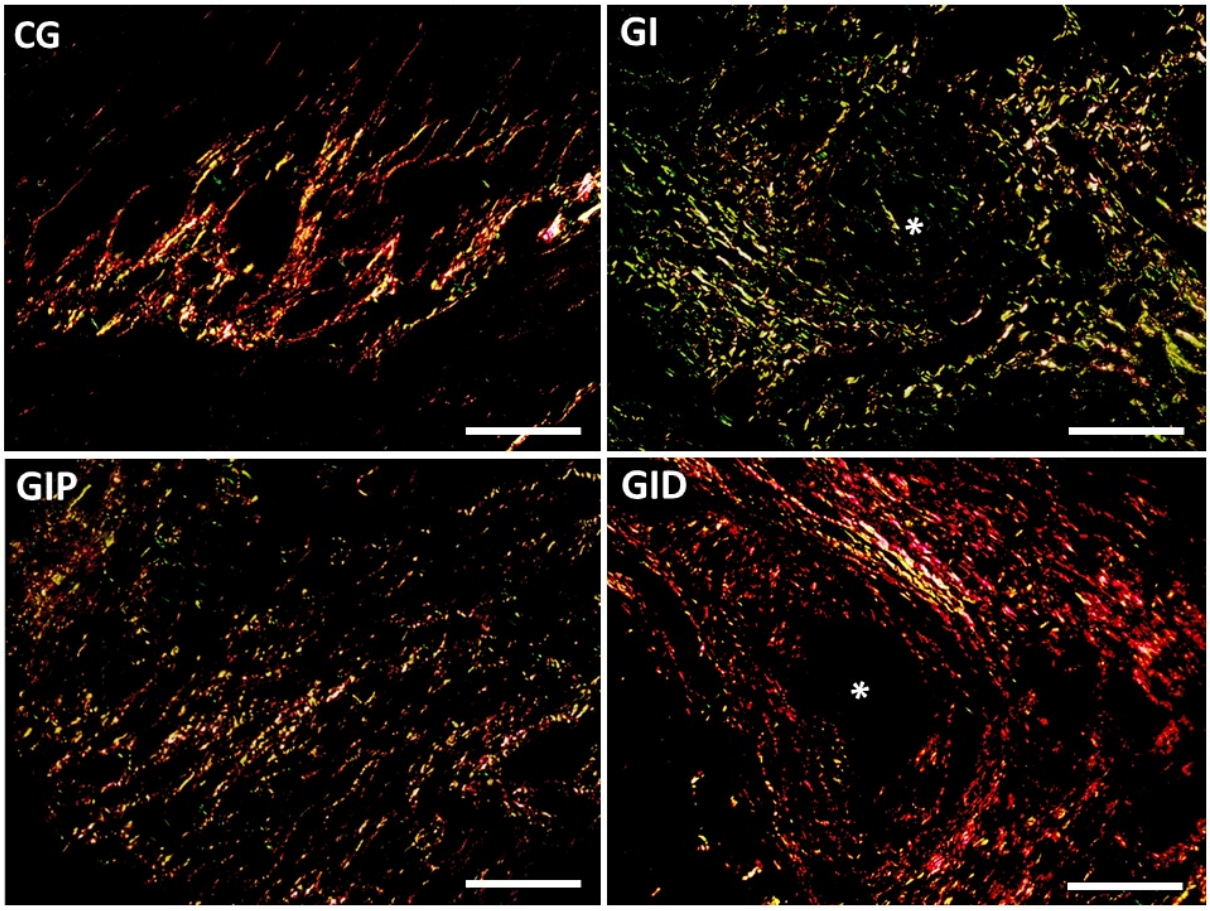


Fig 6. Microscopic images of the ileum submucosal in *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Polarized light microscopy, Sirius red staining, scale bars = 100 μm). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Asterisk= schistosomal granuloma.

Semiquantitative and quantitative collagen analyzes (Fig. 7) were consistent with the histopathological findings. The intestinal proportion of collagen fibers was similar in the CG and GIP groups ($P>0.05$). This proportion was higher in the GI and GID groups compared to the other groups ($P<0.05$). The percentage of these fibers

was also higher in the GID group compared to the GI group (P<0.05). Collagenous protein levels were elevated in all infected groups compared to the CG group (P<0.05). This parameter was higher in the GI group compared to the GIP group (P<0.05), and even higher in the GID group compared to these two groups (P<0.05). The proportion of type I collagen fibers was higher in the GID group (P<0.05), while the proportion of type III fibers was higher in the GI group (P<0.05) compared to the other groups. The relative content of type III collagen fibers was lower in the GIP group compared to the CG group (P<0.05). The colorimetric differences between these fibers are clearly demonstrated in Fig. 7.

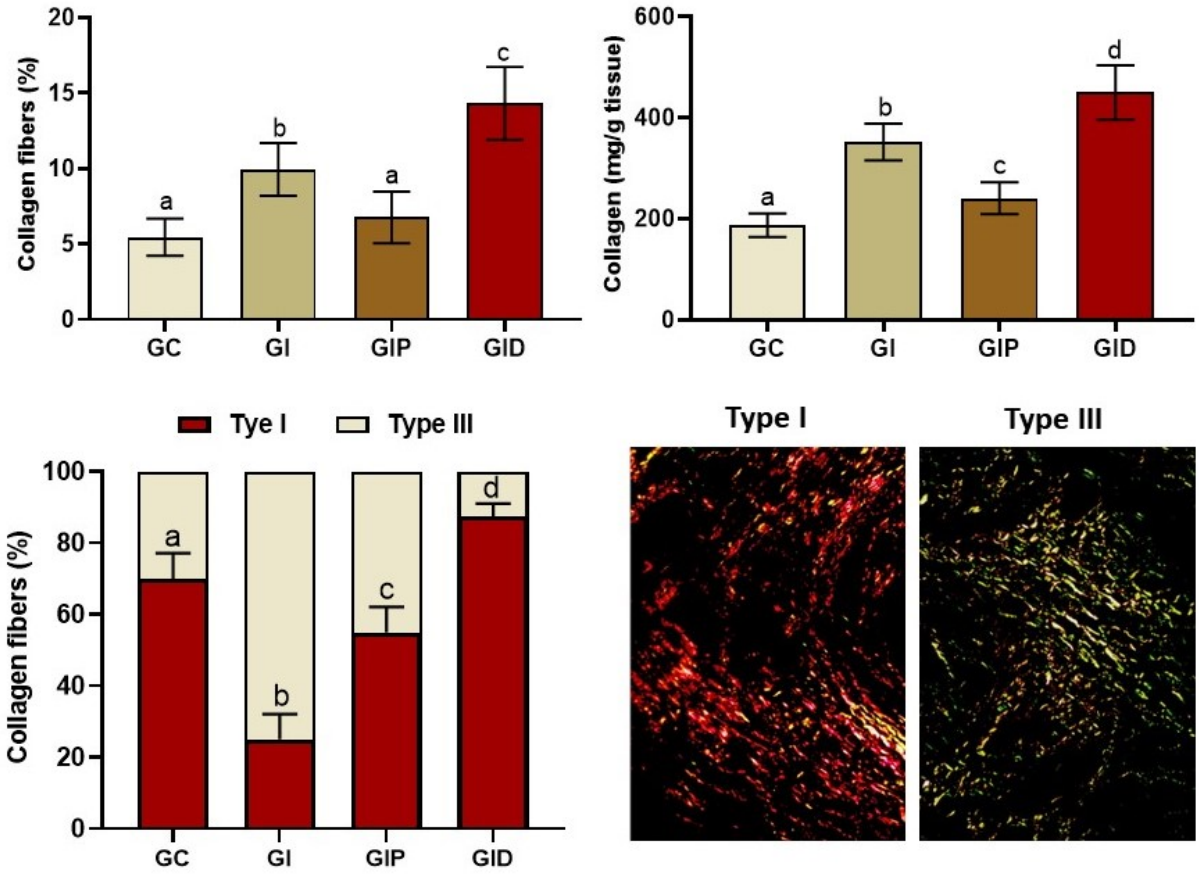


Fig 7. Collagen fibers distribution and of collagenous proteins content in the ileum of *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Data are expressed as mean and standard deviation. Different letters in the columns (a, b, c) indicate statistical difference between groups (P<0.05). Groups with the same letters do not show statistical difference (P>0.05). The histological

images (bottom right) differentiate type I (red, orange and yellow) and type III (green) collagen fibers under polarized light.

Fig 8 demonstrates that collagen fibers present predominant accumulation in the intestinal submucosa and serosa in the CG group. On the other hand, greater collagen fibers accumulation was observed in the intestinal submucosa of all infected animals. In this region, collagen deposition was closely associated with intestinal granulomatous lesions. Furthermore, animals in the GID group presented areas of the external muscular layer with marked infiltration of collagen fibers starting from the submucosa and serosa. Animals from the GID group showed a more pronounced collagen accumulation in the schistosomal granulomas sheath compared to the other infected groups.

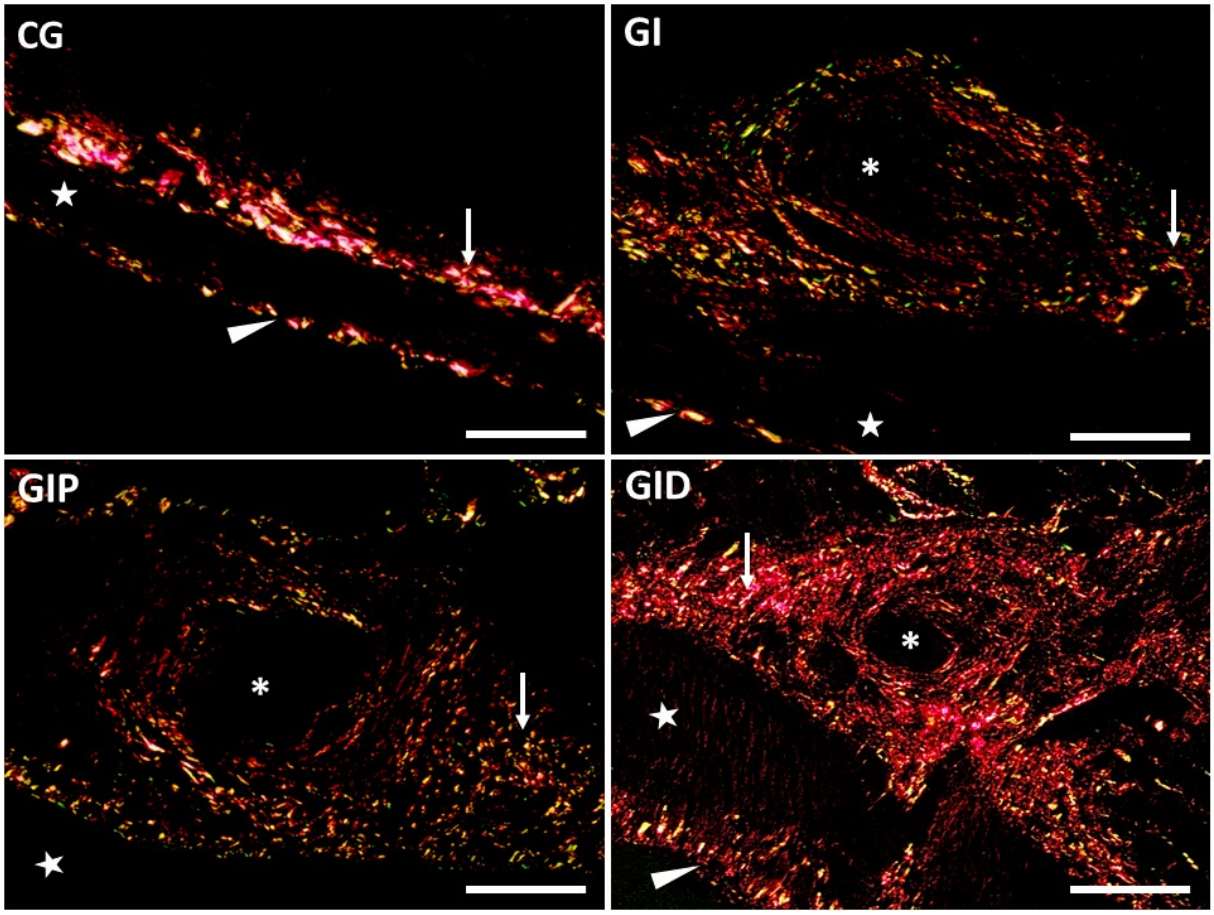


Fig 8. Microscopic images of the submucosa, muscular externa and serosa in the ileum from *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx) (Polarized light microscopy, Sirius Red staining, color bars scale= 100 μm). Groups: CG= untreated uninfected, GI=

untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Asterisk= schistosomal granuloma, arrow= submucosa, star= muscular externa, arrowhead= serosa.

The intestinal and fecal distribution of *S. mansoni* eggs is shown in Fig. 9. Parasite eggs were identified retained in the intestinal wall in all infected groups. No parasite eggs were identified in the feces of GIP animals. Eggs number was higher in the intestine of animals in the GI and GID groups compared to the GIP group ($P<0.05$). This parameter was higher, while fecal eggs excretion was lower in the GID group compared to the GI group ($P<0.05$).

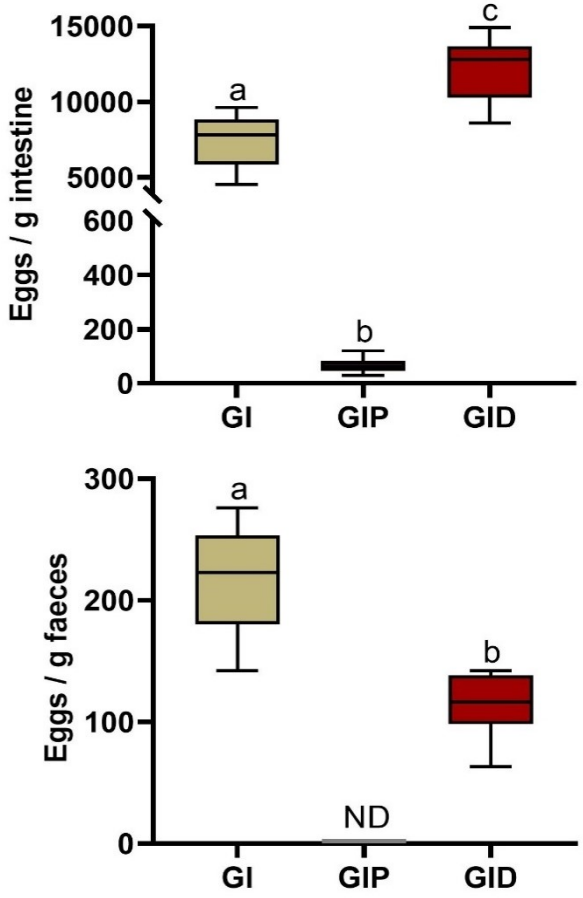
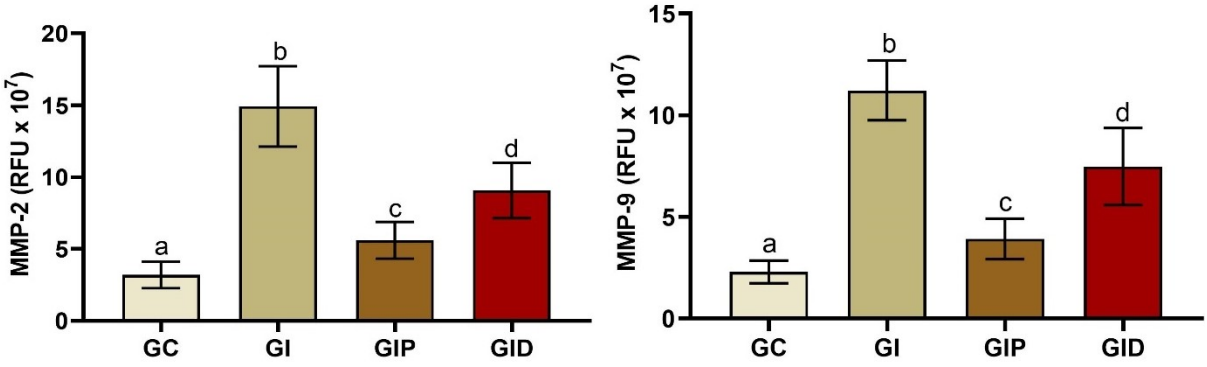


Fig 9. Number of eggs retained in the ileum wall and eggs excreted in the feces of *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx). Groups: GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. Data are expressed as median and interquartile range. Different letters in the columns (a, b, c) indicate statistical difference between groups ($P<0.05$). Groups with the same letters do not show statistical difference ($P>0.05$).

As shown in Fig 10, MMP-2 and MMP-9 activity was increased in all infected groups compared to the CG group. On the other hand, MMP-2 and MMP-9 activity was higher in the GI and GID groups compared to the other groups (P<0.05). The activity of these enzymes was higher in the GI group compared to the GID group (P<0.05). Collagenous proteins levels in the ileum wall did not correlate with MMP-2 and MMP-9 activity, eggs number retained in the intestinal wall and fecal eggs excretion in the GI group (P>0.05). However, all these parameters showed a significant and inverse correlation with collagenous proteins content in the intestinal wall (P<0.05).



Collagen (mg/g tissue) *vs.*	GI		GID	
	R	*P value*	R	*P value*
MMP-2	-0.314	0.54	-0.867	***0.02***
MMP-9	-0.586	0.22	-0.845	***0.03***
Eggs / g faeces	-0.619	0.19	-0.880	***0.02***
Eggs / g tissue	-0.488	0.32	-0.898	***0.01***

Fig 10. Matrix metalloproteinases (MMP-2 and MMP-9) and correlation between collagen levels with MMP activity, number of eggs in the ileum wall and feces in *Schistosoma mansoni*-infected mice, untreated and treated with praziquantel (Pz) and doxycycline hyclate (Dx). Groups: CG= untreated uninfected, GI= untreated infected, GIP= infected treated with 200 mg/kg Pz, GID= infected treated with 50 mg/kg Dx. The data presented in the graphs are expressed as mean and standard deviation. Different letters in the columns (a, b, c) indicate statistical difference between groups (P<0.05). Groups with the same letters do not show statistical difference (P>0.05). R= Correlation coefficient.

Discussion

In the present study, we investigated the impact of DX treatment on MMP activity, collagen levels, intestinal retention and fecal excretion of *S. mansoni* eggs in mice infected with this parasite. Our findings indicated that the animal model (animal lineage, parasite strain, inoculum size and infection time) used was efficient in inducing experimental schistosomiasis. This disease was confirmed in all inoculated animals by microscopic detection of parasite eggs in feces and/or in the ileum wall. The current model was used to induce a chronic disease, ensuring a sufficient time for *S. mansoni* to mature,lay eggs and develop granulomatous lesions in the tissues and organs of the infected host (Dias et al. al., 2019; Santos et al., 2021). Although intestinal damage was the primary focus of the present study, the selected model also proved to be effective in inducing liver and lung granulomatous lesions in mice (Dias et al., 2019; Santos et al., 2021), corroborating the systemic impact of schistosomiasis mansoni (Weinstock, 1992; Andrade, 2008). In the intestine, the pathogenic potential manifested itself through marked granulomatous reactions and extensive microstructural remodeling of the ileum, which was accompanied by an increase in MMP-2 and MMP-9 activity and extensive tissue fibrosis in *S. mansoni*-infected animals. As expected, treatment with the reference drug Pz prevented schistosomiasis progression, attenuated granulomatous inflammation severity, MMP activity, microstructural remodeling of the intestine and *S. mansoni* eggs excretion. On the other hand, DX treatment aggravated granulomatous inflammation, tissue fibrosis and parasite eggs retention in the ileum wall, processes that were correlated with MMP activity attenuation.

In experimental schistosomiasis mansoni, the ileum is the main intestinal region of interest. There is evidence that adult worms migration and intense eggs laying in the portal vascular system, as well as the abundant vascular network in the ileum region, favors eggs retention and the development of granulomatous inflammatory reactions in this region (Domingo et al., 1969; Mati & Melo, 2013; Costain et al., 2018). Thus, the greater inflammatory damage accumulation makes the ileum a important segment associated with parasite eggs translocation across the intestinal wall, providing a relevant contribution to the total load of eggs excreted in the feces of *S. mansoni*-infected mice (Domingo et al., 1969; Elbaz & Esmat, 2013; Mati & Melo, 2013). In this intestinal segment, we identified a predominant eggs accumulation in the submucosal layer, which may be associated with the presence of

a well-developed vascular network formed by larger blood vessels that allow the passage of parasite eggs. On the other hand, the mucosal vasculature is predominantly formed by smaller blood vessels (mainly capillaries) derived from branches of the submucosal vascular plexus, which naturally represents a mechanical barrier to the passage of *S. mansoni* eggs (Kloetzel et al., 1970, 1971; Bloch, 1980; Bernier-Latmani & Petrova, 2016). As the tissue inflammatory reaction develops in response to and around the parasite eggs, an inflammatory process in the mucous layer was not frequently observed, which is consistent with the limited morphological changes observed in this layer. In this sense, the main changes observed were intestinal villi thickening and increased numerical density of goblet cells in infected animals, especially in those exposed to DX. On the other hand, intraepithelial lymphocytes distribution was reduced in DX-treated animals, which may be linked to the direct antibacterial effect of this drug, which can attenuate regional immunological reactions by reducing the intestinal antigenic load (Boynton et al., 2017; Xu et al., 2022).

Interestingly, mean crypts depth was reduced and their cross-sectional area was increased in untreated and DX-treated infected animals. The mechanisms associated with this morphological remodeling are not completely understood. However, they may be related to the greater proximity of the crypts to the molecular microenvironment of the submucosal layer. In this region, an intense leukocyte infiltrate with frequent projection towards the basal lamina propria, numerous granulomas, marked conjunctival expansion and increased submucosa thickness were observed, indicating intense granulomatous inflammation mainly associated with this layer of the intestinal wall. In this sense, cells closer to this region are more susceptible to several trophic factors with a Th2 immunological profile (which predominates in chronic schistosomiasis) such as IL-4, IL-5, IL-13 and TGF-β; molecules that are known to influence several cellular processes such as energy metabolism, secretion, survival and proliferation (Costain et al., 2018; Abdel et al., 2022; Malta et al., 2022). Furthermore, there is evidence that Th2 cytokines can stimulate hyperplasia and mucus secretion in *S. mansoni*-infected animals (Scheer et al., 2014; Gologorsky et al., 2023), which is consistent with the increased goblet cells number, expansion of the cytoplasmic mucin storages and intestinal crypts dilation identified in the present study.

Along with the crypt’s changes, a greater collagen fibers accumulation in the

lamina propria that supports these structures was microscopically evident only in DX-treated animals. In addition to these animals, those infected untreated also showed a marked increase in the relative density of collagen fibers in the submucosa, with frequent infiltration into the external muscular layer. As collagenogenesis and tissue fibrosis are phenomena directly associated with granulomatous inflammation, these findings reinforce the evidence that the submucosa is a primary site of the immunological response directed to antigens released by *S. mansoni* eggs. Morphologically, this proposition is corroborated by collagen fibers accumulation around the parasite eggs, a phenomenon involved in the progressive granuloma's organization (Junqueira et al., 1986; Andrade & Grimaud, 1988; Costain et al., 2018). Biochemical analysis of collagenous proteins confirmed the increased collagen levels in untreated and DX-treated infected animals. Interestingly, the intestinal content of collagenous proteins also increased in Pz-treated animals in relation to the control group, a finding that was not evidenced from the semiquantitative morphological analysis of collagen fibers. Therefore, it is believed that the biochemical method used may be more sensitive to variations in intestinal collagen content in relation to the microscopic method, which is consistent with the use of reference biochemical assays for collagen quantification in organ samples (Junqueira et al. al., 1986; Novaes et al., 2015). However, morphological analysis was relevant as a complementary method to reveal differences in the intestinal distribution of collagen fibers. In this sense, a predominant accumulation of type I and III collagen fibers was respectively identified in the granulomatous lesions of untreated infected animals and those exposed to DX. These findings indicate variations in collagen formation dynamics, as type III fibers are initially produced and gradually replaced by thicker type I collagen fibers as the granulomas organize. Thus, the relative proportion of collagen I fibers is often higher in older granulomas (Junqueira et al., 1986; Andrade & Grimaud, 1988). These characteristics indicate that DX favored collagen I accumulation, which reinforces the hypothesis that this drug is capable of accelerating granulomas organization and tissue fibrosis in schistosomiasis mansoni (Dias et al., 2019; Santos et al., 2021).

By revealing that collagenous proteins levels also increased in Pz-treated animals, our findings indicate that despite being effective in eliminating the parasite, eggs and schistosomal granulomas destruction occurs more slowly (el-Fakahany et al, 1993; Membe Femoe et al., 2022). Thus, residual granulomas were also observed

in the submucosa of animals treated with this drug. However, these granulomas often presented a degenerated *S. mansoni* egg and a lower density of collagen fibers (mainly type I) with a looser organization compared to other infected groups. These findings are reinforced by the identification of a small number of *S. mansoni* eggs in the intestinal wall of Pz-treated animals, which was not associated with fecal excretion of these eggs, an aspect that corroborates the resolving characteristic of the infection and the granulomatous process through the effectiveness of the reference treatment (el-Fakahany et al., 1993; Costa-Silva et al., 2012; Membe Femoe et al., 2022). However, untreated infected animals and especially those treated with DX presented intense eggs retention in the intestinal wall. Surprisingly, fecal excretion of these eggs was higher in untreated animals and approximately 50% lower in DX-exposed animals. These findings indicated less m resistance to the passage of *S. mansoni* eggs, as well as evident impairment of eggs translocation through the intestinal wall in animals treated with this drug. The mechanism associated with this response is still poorly understood, although there is evidence that anti-fibrotic drugs that attenuate collagen cross-linking can facilitate faster parasite eggs passage through tissues (Giboda et al., 1994). On the contrary, pro-fibrotic drugs such as DX can attenuate eggs excretion by enhancing collagen accumulation and accelerating intestinal granulomas organization, a process that may be analogous to that observed in the liver and lungs (Dias et al., 2019; Santos et al., 2021). Thus, our findings indicated increased MMP-2 and MMP-9 activity in all infected groups. However, the activity of these collagenases was attenuated by DX compared to untreated infected animals.

Admittedly, schistosomiasis induces increased MMP production and activity, which degrade collagen in a counter-regulatory mechanism to collagenogenesis associated with granulomatous inflammation (Singh et al., 2004, 2006; Dias et al., 2019; Santos et al , 2021). However, collagenogenic activity often overwhelms the collagenolytic response, and thus fibrosis progresses in untreated schistosomiasis mansoni (Dias et al., 2019; Santos et al., 2021). This process is reversed when antiparasitic treatment is successful, so that MMP-mediated collagenolytic activity supplants collagenogenesis, promoting gradual collagen degradation and tissue fibrosis resolution (Singh et al., 2004, 2006; Dias et al., 2019; Santos et al., 2021). Interestingly, our findings indicated that DX treatment subverts collagen dynamics in schistosomiasis. Thus, by attenuating MMP-2 and MMP-9 activity, this drug is

capable of enhancing collagen accumulation in the intestinal wall, tissue fibrosis and increasing the mechanical resistance to *S. mansoni* eggs translocation. Although the collagen content in the intestinal wall did not show a correlation with the activity of these enzymes, intestinal retention and fecal excretion of parasite eggs; these correlations were observed for DX-treated animals. Thus, our study provides evidence that DX is capable of attenuating environmental elimination of *S. mansoni* eggs by aggravating granulomatous inflammation and inhibiting the MMP-2 and MMP-9 activity, events potentially associated with excessive collagen accumulation, which makes it difficult for these eggs to translocate through the wall to the intestinal lumen.

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Conflict of interest

None to declare.

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3 CONSIDERAÇÕES FINAIS

Os achados do presente estudo indicaram o tratamento com doxiciclina subverte a dinâmica do colágeno na esquistossomose. Assim, ao atenuar a atividade de MMP-2 e MMP-9, essa droga é capaz de potencializar o acúmulo de colágeno na parede intestinal fibrose tecidual e aumentar a resistência mecânica à translocação de ovos de *S. mansoni*. Embora o conteúdo de colágeno na parede intestinal não tenha apresentado correlação com a atividade dessas enzimas, retenção intestinal e excreção fecal de ovos desse parasito; essas correlações foram observadas para animais tratados com doxiciclina. Dessa forma, o nosso estudo fornece evidências de que a doxiciclina é capaz de atenuar eliminação ambiental de ovos de *S. mansoni* ao agravar a inflamação granulomatosa e inibir a atividade de MMP-2 e MMP-9, eventos potencialmente associados ao excessivo acúmulo de colágeno que dificulta a translocação desses ovos pela parede até a luz intestinal.

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APÊNDICE

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Matrix metalloproteinases inhibition reveals the association between inflammation, collagen accumulation and intestinal translocation of *Schistosoma mansoni* eggs *in vivo*

Thiago D. Silva [a], Elda Gonçalves-Santos [a], Reggiani V. Gonçalves [b], Raquel L.M. Souza [c], José Edson Caetano [d], Ivo S. Caldas [c,d], Livia F. Diniz [c,d], Marcos J. Marques [c,d], Rômulo D. Novaes [a,b,c,d,*]

[a] Programa de Pós-Graduação em Biociências Aplicadas à Saúde, Universidade Federal de Alfenas, Alfenas 37130-000, Minas Gerais, Brazil
[b] Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa 36570-900, Minas Gerais, Brazil
[c] Instituto de Ciências Biomédicas, Universidade Federal de Alfenas, Alfenas 37130-000, Minas Gerais, Brazil
[d] Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Alfenas, Alfenas 37130-000, Minas Gerais, Brazil

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Experimental pathology

ABSTRACT

Schistosomiasis mansoni is a parasitic infection that causes enterohepatic morbidity associated with severe granulomatous inflammation triggered by parasite eggs. In this disease, granulomatous inflammation leads to intestinal erosion and environmental excretion of *S. mansoni* eggs from feces, an essential process for propagating the parasite and infecting host organisms. Metalloproteinases (MMP) are involved in *S. mansoni*-induced hepatic granulomatous inflammation and fibrosis. However, the relationship between MMP and collagen accumulation with the intestinal excretion of parasite eggs remains unclear. Thus, the present study investigated whether MMP inhibition is capable of modulating granulomatous inflammation, collagen accumulation and mechanical resistance to the point of influencing the dynamics between intestinal retention and excretion of *S. mansoni* eggs in infected mice. Our findings indicated that doxycycline (a potent MMP inhibitor) aggravates intestinal inflammation and subverts collagen dynamics in schistosomiasis. By attenuating MMP-2 and MMP-9 activity, this drug is capable of enhancing fibrosis and mechanical resistance of the intestinal wall, hindering *S. mansoni* eggs translocation. Although collagen content was not correlated with MMP activity, intestinal retention and fecal excretion of parasite eggs in untreated mice; these correlations were observed for doxycycline-treated animals. Thus, our study provides evidence that doxycycline is able to attenuate fecal elimination of *S. mansoni* eggs by inhibiting MMP-2 and MMP-9 activity, events potentially associated with excessive collagen accumulation, which increases intestinal mechanical resistance and hinders eggs translocation through the intestinal wall. Variations in intestinal collagen dynamics are relevant since they may represent changes in the environmental dispersion of *S. mansoni* eggs, bringing repercussions for schistosomiasis propagation.

1. Introduction

Schistosoma mansoni is a species of helminth that causes schistosomiasis, a neglected infectious disease with wide geographic distribution in African, Asian and South American countries [24,44]. It is estimated that schistosomiasis affects at least 240 million people [24,44]and is responsible for about 10.1 million annual deaths worldwide [31], with most registered cases being caused by *S. mansoni* [24,44]. To infect the vertebrate host, *S. mansoni* cercariae penetrate the skin and transform into schistosomula, which are transported through the bloodstream until they reach the liver, differentiating into adult worms [2,8]. The worms mate and move to the mesenteric veins, where the female begins eggs-laying [2,8].

Half of *S. mansoni* eggs reach the intestinal lumen and are incorporated into the feces, while the rest are retained in the host's tissues, triggering a marked granulomatous inflammation in multiple organs, especially liver and intestine [2,8]. As the host is unable to naturally eliminate the infection, a continuous cycle of inflammation, cell death

* Corresponding author.
E-mail address: romulo.novaes@unifal-mg.edu.br (R.D. Novaes).

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