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ORIGINAL PAPER

Anticoccidial activities of Chitosan on *Eimeria papillata*-infected mice

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Abstract *Eimeria* spp. multiply within the intestinal tract causing severe inflammatory responses. Chitosan (CS), meanwhile, has been shown to exhibit anti-inflammatory activities in different experimental models. Here, we investigated the effect of CS on the outcome of inflammation caused by *Eimeria papillata* in the mouse intestine. Investigations were undertaken into the oocyst output in feces and developmental stages and goblet cells in intestinal tissue. Assays for lipid peroxidation, nitric oxide (NO), and myeloperoxidase (MPO) were also performed. T cells in intestinal tissue were counted using immunohistochemistry while total IgA in serum or intestinal wash was assayed using ELISA. In addition, mRNA expression of tumor necrosis factor alpha (TNF-α), transforming growth factor β (TGF-β), interleukin (IL)-10, and IL-4 were detected using real-time PCR. The data indicated a reduction in both oocyst output and in the number of parasite developmental stages following CS treatment, while the goblet cell hypoplasia in infected mice was also inhibited. CS decreased lipid peroxidation, NO, and MPO but did not alter the T cell count or IgA levels in comparison to the infected group. The expression of TNF-α and TGF-β decreased but IL-10 and IL-4 increased after CS treatment in comparison to the non-treated infected group. In conclusion, CS showed anti-inflammatory and protective effects against *E. papillata* infection.

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Introduction

Coccidiosis is a worldwide protozoan disease caused by parasites of the genus *Eimeria*. Eimerians can develop and multiply within the intestinal tract causing destruction of intestinal mucosa that can induce a severe inflammatory response (Metwaly et al. 2012; Dkhil et al. 2013). This tissue destruction is also associated with diminished food intake and nutrient absorption, reduced body-weight gain, dehydration, blood loss, and enhanced susceptibility to other diseases (Amer et al. 2015). Coccidiosis therefore causes massive economic losses in the meat and milk production industries (McDougald 2003). *Eimeria papillata* infecting mice represents an appropriate model to study animal coccidiosis through its development within cells of the murine small intestine (Al-Quraishy et al. 2011). Intestinal coccidiosis in mice leads to serious inflammatory responses manifested by increased production of nitric oxide (NO), increased catalase activity, and increased lipid peroxidation, as indicated by the elevated malondialdehyde level (Dkhil 2013; Wunderlich et al. 2014). Also, during the course of the infection, intraepithelial cells increase the production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) but decrease transforming growth factor β (TGF-β) production (Inagaki-Ohara et al. 2006).

Chitosan (CS) exhibits anti-inflammatory activities in different experimental models (Qiao et al. 2011; Choi et al. 2012; Li et al. 2014). CS has in addition antitumor (Fernandes et al. 2012), antifungal (Hussain et al. 2012), antimicrobial (Tavaria et al. 2012), and free radical scavenging activities (Kim et al. 2012). CS has in recent years been recommended

as a healthy food supplement in Asian countries due to these properties (Nam et al. 2007). CS has well-defined properties, including good bioavailability and biocompatibility, low cost, and an ability to open intracellular tight junctions in intestinal epithelial cells (Van der Lubben et al. 2003). Moreover, functionalized forms of CS have attracted considerable interest due to improved mucoadhesivity, permeability, and stability (Islam et al. 2012). CS elicits the release of interleukin (IL)-10, IL-4, and TGF-β at mucosal tissues and the activation of $CD3^+$ T cells in spleen (Porporatto et al. 2005).

Due to all the previously mentioned properties, the present study aimed to investigate the anticoccidial and anti-inflammatory effects of CS in mice following oral infection with *E. papillata*.

Materials and methods

Animals

Adult male white albino mice weighed 20–25 g and aged 10 weeks were used for the experiments. Animals were housed under controlled temperature (21 °C) and lighting, with 12 h of light and 12 h of dark, and had free access to water and a standard mouse chow diet. Mice were handled in accordance with the protocols approved by the Zoology Department, Faculty of Science, Beni-Suef University.

Infection by *E. papillata*

E. papillata oocysts in PBS were kindly provided by Professor Al-Quraishy, Zoology Department, College of Science, King Saud University, Saudi Arabia. Prior to infection, the initial stock of oocysts was used to infect some mice in order to activate their virulence. The feces of these mice were collected and the new oocysts were sporulated to be ready for used in the experiment.

The number of the newly collected oocysts was adjusted and each mouse was given 1000 sporulated oocysts in 100 μl of water by oral gavage. To follow up the oocyst shedding numbers, fecal samples were collected and oocysts per gram were estimated using the modified McMaster technique (Schito et al. 1996).

Experimental design

The mice were divided into three groups with eight animals in each group. The first group, with uninfected mice and gavaged only with saline, served as a control group. The second and third groups were infected with *E. papillata*. The second group was treated with saline and served as the control infected group while the third group was treated with 0.5 ml of 1 % CS (250 mg/kg) in saline once a day for 5 days.

On day 5 p. i., mice were euthanized and blood was collected. Sera were separated and kept at −20 °C. Parts of jejunum were fixed in 10 % neutral buffered formalin for histological and immunohistochemical analysis. Others were weighed, kept in phosphate-buffered saline, and stored at −80 °C for homogenate preparation or trizol for RNA extraction.

Parasitic developmental stages and goblet cells

Sections stained with hematoxylin–eosin were used for parasite detection or with Alcian blue for determination of the goblet cells (Dkhil 2013). For each animal, the number of parasitic developmental stages and goblet cells in the jejunum was counted in at least ten well-orientated villous crypt units (VCUs) and results were expressed as the mean number.

Biochemical analyses

Parts of the jejunum were homogenized to give 50 % (*w*/*v*) homogenate in ice-cold medium containing 50 mM Tris–HCl and 300 mM sucrose (Tsakiris et al. 2004). The homogenate was centrifuged at 500×*g* for 10 min at 4 °C. The supernatant was used for the biochemical determinations. Lipid peroxidation in jejunum homogenate was determined (Ohkawa et al. 1979) by using 1 ml of 10 % trichloroacetic acid and 1 ml of 0.67 % thiobarbituric acid followed by heating in a boiling water bath for 30 min. Thiobarbituric acid-reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents formed. The assay of NO in jejunum homogenate was done (Berkels et al. 2004).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the established method (Sun et al. 2007; Kim et al. 2012). Briefly, tissue was homogenized in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.4) and centrifuged at 10,000×*g* at 4 °C for 30 min. The remaining pellet was resuspended in 0.5 ml of 50 mM potassium buffer at pH 6.0 with 0.5 % hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 12,000×*g* at 4 °C for 10 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer containing 50 mM phosphate buffer, 530 mM orthodianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as unit/milligram tissue.

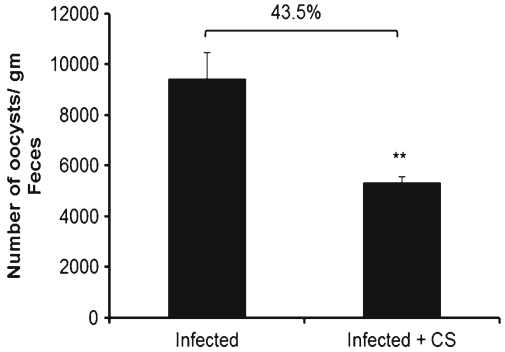

Fig. 1 The effect of CS on the oocyst output shows a reduction by 43.5 % relative to the infected mice group at day 5 p.i. All values are mean ± SEM. *Double asterisks* indicates a statistical significance at $p < 0.001$

Immunohistochemistry

Paraffin-embedded intestinal tissues were cut at 5 μm sections. The slides were then incubated in 3 % H_2O_2 for 10 min at room temperature to block the endogenous peroxidase activity. Slides were treated with 1.5 % normal serum obtained from the same species in which the secondary antibody was developed for 30 min to block non-specific staining. Subsequently, slides were incubated with monoclonal antibodies against mouse CD3 (1:100; AbD Serotec, New York, USA) for overnight at 4 °C. Then slides were treated with a biotin-conjugated secondary antibody for 10 min followed by incubation with peroxidase-conjugated streptavidin for 10 min at room temperature according to the instructions of IHC Detection Kit (Dako, Glostrup, Denmark). All the above steps were followed by washing in Tris buffer (pH 7.4) for three times. Immunolabeling was detected by incubation with 0.06 % diaminobenzidine (Sigma) dissolved in tap water containing 0.01 % H_2O_2 for 3–5 min, followed by washing and staining with Mayer's hematoxylin.

Anti-mouse IgA α chain-specific ELISA

Total IgA concentration was determined by sandwich ELISA (Lüllau et al. 1996). Goat anti-mouse IgA α chain-specific antiserum (Sigma) diluted 1:500 in 50 mM bicarbonate buffer (pH 9.6) was used to coat wells (100 μl/well) of Immulon (Dynatech) 96-well plates overnight at 4 °C, which were subsequently blocked (250 μl/well) with 1 % BSA (Sigma) in PBS, 0.1 % Tween 20 (Sigma) at 37 °C for 30 min. Between all antibody incubation steps except the last one, the plates were washed three times with PBS, 0.01 % Tween 20. IgA samples and mouse IgA standards (Sigma; range 10–200 ng) were diluted in blocking buffer and 100 μl was applied, in duplicate, to the wells. After

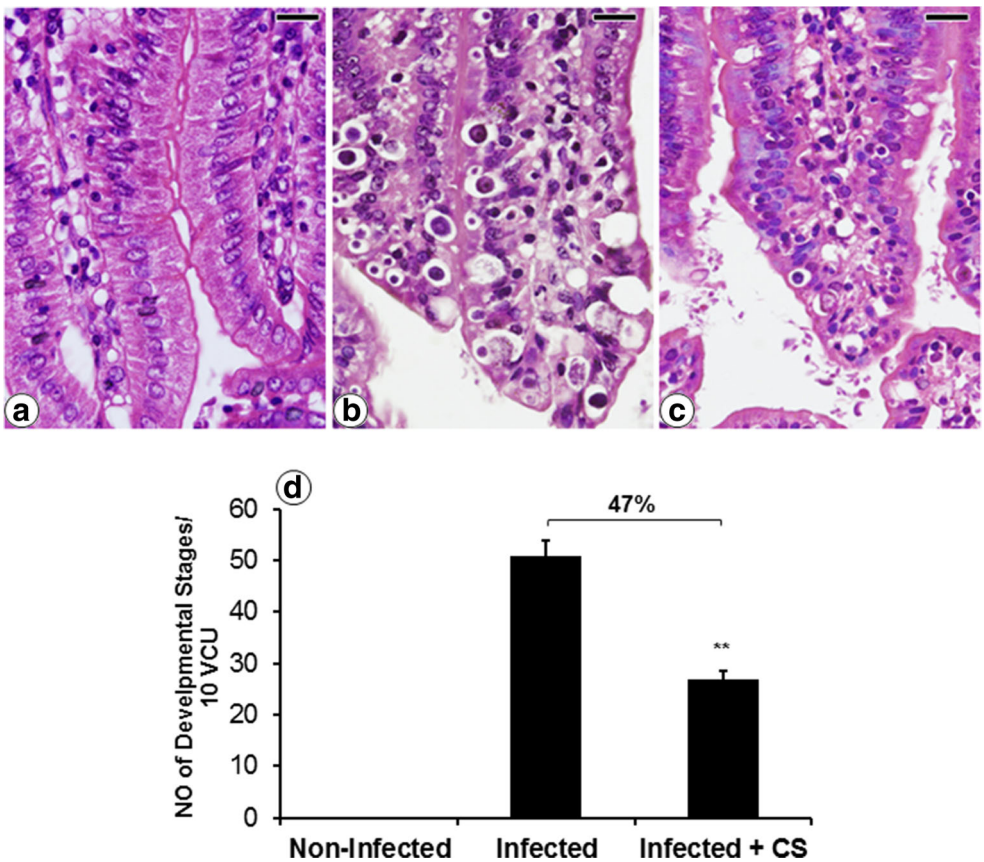

Fig. 2 The developmental stages of *E. papillata* in infected and infected + CS groups at day 5 p.i. **a** Non-infected group shows no parasitic infections. **b** Infected group shows the different developmental stages, **c** CS treatment shows decreased occurrence of developmental stages; **d** number of developmental stages per 10 villous-crypt units shows a significant ($p < 0.01$) reduction by 47 % of stages in infected + CS compared to infected. *Double asterisks* indicates a statistical significance at $p < 0.01$. *Scale bar* represents 10 μm

incubation for 2 h at 37 °C, IgA was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA antibodies (Amersham) followed by development with ortho-phenyldiamine/H_2O_2. The reaction was stopped by the addition of 0.01 % sodium azide in citrate buffer (pH 5.0). The absorbance was read at 492 and 629 nm, with the latter serving as reference reading.

Quantitative RT-PCR of mRNA

On day 5 p.i., pieces of jejunum were aseptically removed, rapidly frozen, and stored in liquid nitrogen until use. Total RNA was isolated using SV Total RNA Isolation system (Promega, Madison, WI, USA). Contaminating genomic DNA was digested with the DNA-free™ kit (Applied Biosystems, Darmstadt, Germany), before cDNA was synthesized using Reverse Transcription kit (Stratagene, USA). Real-time PCR (RT-PCR) was performed in a TaqMan7500 (Applied Biosystems) using the QuantiTect™ SYBR® Green PCR kit (Qiagen) according to the manufacturer's instructions. Qiagen (Hilden, Germany) delivered the primers for TNF-α, TGF-β, IL-10 and IL-4, and 18S rRNA. Initial incubation was done at 50 °C for 2 min, followed by Taq polymerase activated at 95 °C for 10 min, 1 cycle followed at 95 °C for 10 min, at 60 °C for 35 s, and for 30 s at 72 °C. All PCR reactions yielded only a single product of the expected size as detected by melting point analysis and gel electrophoresis. Quantitative evaluation was performed with Taqman7500 system software (Applied Biosystems). Expression of genes was normalized to that of 18S rRNA (Delic et al. 2010).

Statistical analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Dunnett's *t* test.

Results

The oocyst output was calculated per gram of feces on day 5 p.i. (Fig. 1). CS treatment reduced ($p < 0.01$) the oocyst output by 43.5 % compared to the infected group. Inspection of hematoxylin and eosin-stained sections using light

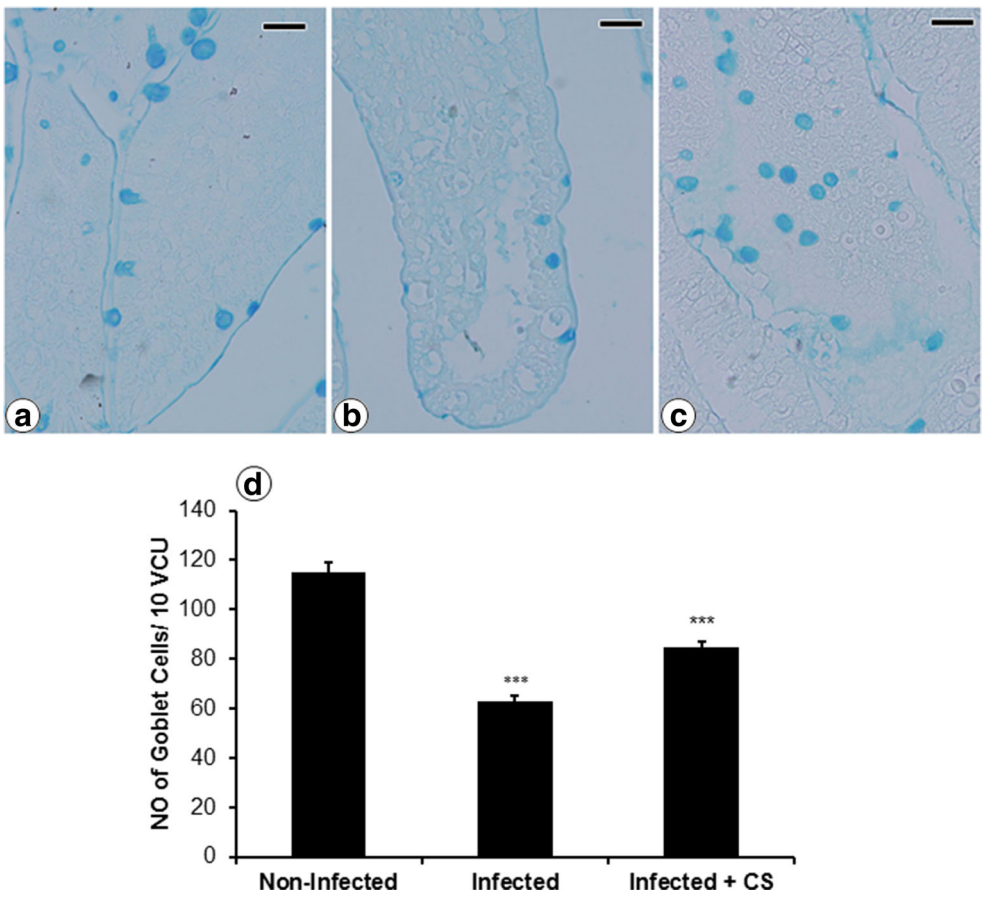

Fig. 3 Goblet cells stained with Alcian blue in non-infected, infected, and infected + CS groups at day 5 p.i. **a** Non-infected group shows high numbers of goblet cells. **b** Infected group shows decreased number of goblet cells compared to non-infected group; **c** CS treatment shows an increased occurrence of goblet cells compared to infected group; **d** statistical analysis shows a significant ($p < 0.001$) reduction in infected compared to non-infected but CS increased ($p < 0.001$) the number of goblet cells compared to infected. *Triple asterisks* indicates a statistical significance at $p < 0.001$. *Scale bar* = 10 μm

microscopy revealed that the epithelial cells of the jejunum were infected with different developmental stages of *E. papillata* parasites (meront, microgamont, macrogamont, and developing oocysts). The number of parasites per 10 villous-crypt units was significantly reduced ($p < 0.01$) by 47 % when the mice were treated with CS (Fig. 2). In addition, while there was a significant reduction ($p < 0.001$) in the number of goblet cells seen at the site of the *E. papillata* infection in the jejunum (Fig. 3), CS treatment significantly reversed this effect ($p < 0.001$).

E. papillata infections also induced a highly significant increase ($p < 0.001$) in jejunal NO and MDA (Fig. 4) but CS treatment significantly ($p < 0.001$) lowered the *E. papillata*-induced increase in both NO and MDA. MPO, which is a marker for neutrophil infiltration into the intestinal tissue was significantly ($p < 0.001$) increased in infection and decreased ($p < 0.01$) after CS treatment.

The immunohistochemically detected $CD3^+$ cells per 10 villous-crypt units were decreased significantly ($p < 0.05$) during infection (Fig. 5). Treatment with CS did not show a remarkable change from the infected group. The total IgA in sera and intestinal wash samples was significantly reduced ($p < 0.001$) after infection in comparison to the non-infected group (Fig. 6) but again CS treatment did not reverse this reduction in IgA levels.

Comparing the infected group to the non-infected one, the expression of TNF-α and TGF-β was increased ($p < 0.001$), while IL-10 and IL-4 was decreased ($p < 0.001$). The levels of expressed mRNA for TNF-α and TGF-β in the jejunum were significantly decreased ($p < 0.001$; Fig. 7), while the expression of both IL-10 and IL-4 increased ($p < 0.001$) after CS treatment compared to the infected group.

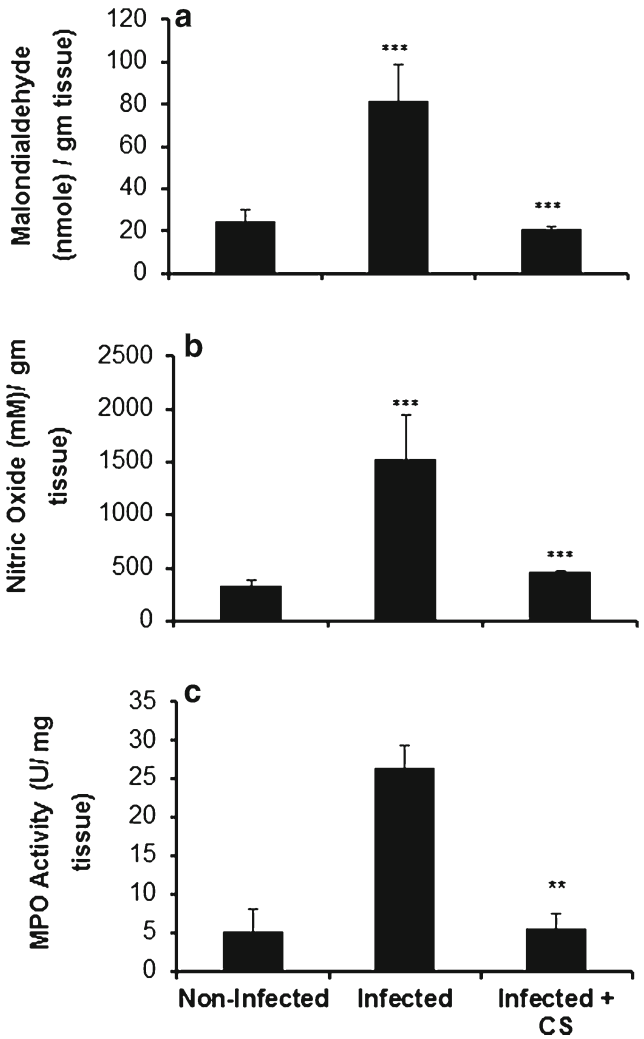

Fig. 4 Effect of CS on the levels of malondialdehyde (**a**), nitric oxide (**b**), and myeloperoxidase (**c**) in jejunum of infected mice with *E. papillata* at day 5 p.i. All had increased ($p < 0.001$) in infected compared to non-infected group. The levels were decreased ($p < 0.001$) in CS-treated when compared to infected group. *Triple asterisks* indicates a statistical significance at $p < 0.001$

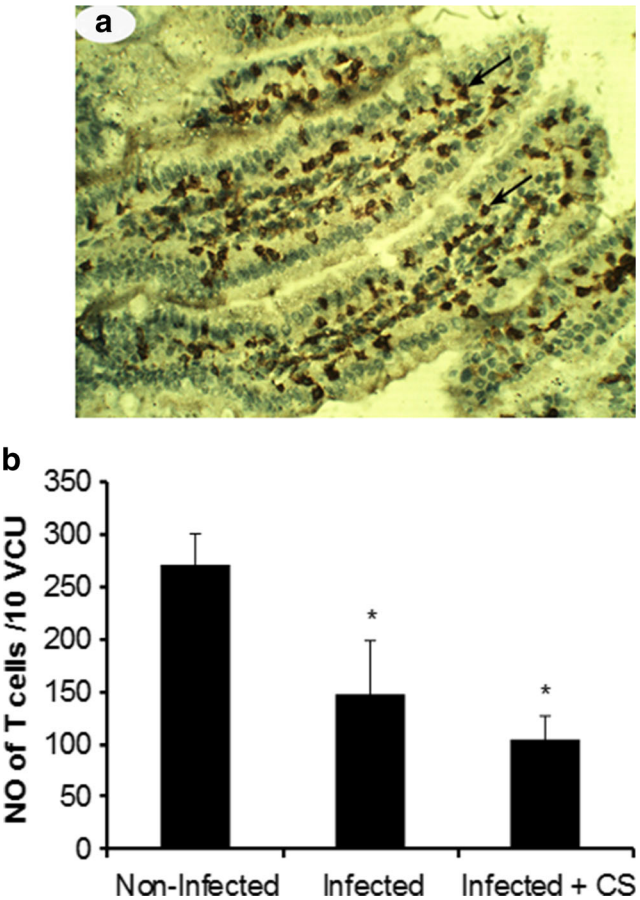

Fig. 5 Effect of CS treatment on the number of $CD3^+$ T cells in the mouse villi at day 5 p.i. **a** Immunohistochemical staining for T cells (*arrows* denotes to the stained cells). **b** The mean number of $CD3^+$ T cells ± SEM in 10 villi. The number was decreased in both of infected and infected + CS compared to the non-infected group. *Asterisk* indicates a statistical significance at $p < 0.05$

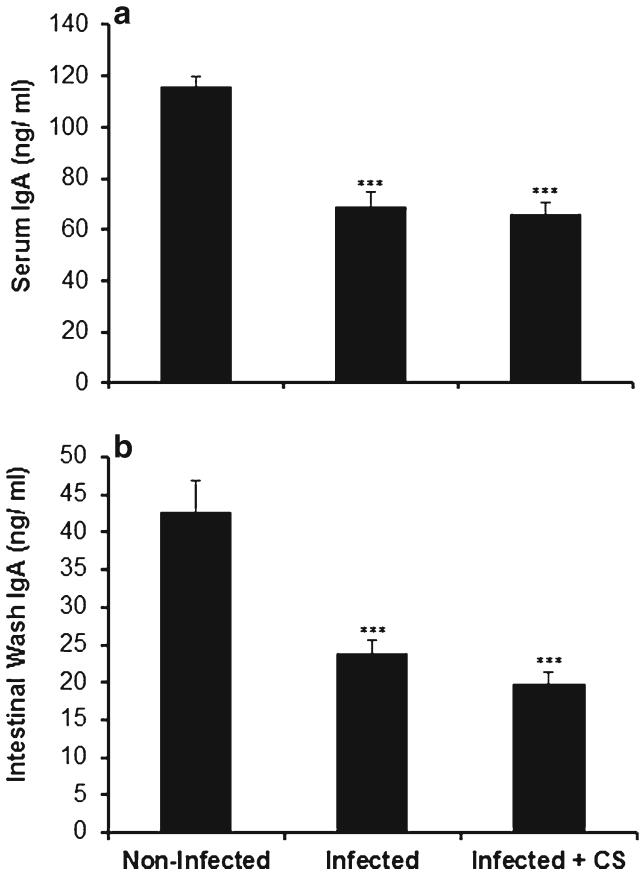

Fig. 6 Effect of CS on total IgA in serum (**a**) and intestinal wash samples (**b**) at day 5 p.i. In both samples, the infected and infected + CS groups showed a decrease ($p < 0.001$) in total IgA in comparison to non-infected group. *Triple asterisks* indicates a statistical significance at $p < 0.001$

Discussion

The current study indicates that CS exhibits anti-*Eimeria* activity in mice infected with *E. papillata*, perhaps due to its anti-inflammatory and anti-oxidant activity. The anti-*Eimeria* activity of CS is indicated by the reduction in the number of fecal oocyst shedding. This denotes that CS treatment impairs intracellular development and replication of *E. papillata* in the jejunal epithelium of mice. In addition, our results show that CS decreases oxidative stress in the infected jejunum, proven here as an impaired *E. papillata*-induced increase in both NO and MDA. These results are in line with Dkhil et al. (2015) and Khalil et al. (2015) that described similar effects for berberine and garlic treatments of *Eimeria* infections in mice. In the same time, CS also attenuates the inflammatory response, since it suppresses the release of the infection-induced MPO and decreases mRNA expression of TNF-α and TGF-β in the mouse jejunum infected with *E. papillata* (Kim et al. 2004).

The decrease in MDA, NO, and MPO in intestinal tissue confirms the anti-inflammatory effect of CS (Hierholzer et al. 2004). NO was found to play a role in intestinal injury while MPO was measured as an indicator for neutrophil infiltration (Ohtsuka et al. 2001; Barocelli et al. 2006). The increased NO and MPO in the infected group in this study may be related to the increased expression of TNF-α and TGF-β as suggested by Prabhu and Guruvayoorappan (2014).

A single dose of CS at 500 mg/kg was found to have anti-inflammatory properties against carrageenan-induced paw edema (Fernandes et al. 2010). In this study, a dose of 250 mg/kg over 5 days was found to be enough to induce an effect against *Eimeria* infection.

The ability of CS to restore the number of goblet cells in the infected intestine is important because of the role of goblet cells in protection against intestinal infection with *Eimeria* spp. (Yunus et al. 2005; Linh et al. 2009; Dkhil et al. 2015). The increased number of goblet cells after CS treatment has also been associated with increased expression of occludin which has been in turn linked to the increased expression of IL-4 and IL-10 which are markers of T helper cell type 2 (Th2) responses (Xiao et al. 2013). Furthermore, CS was found to be protective against *Toxoplasma*

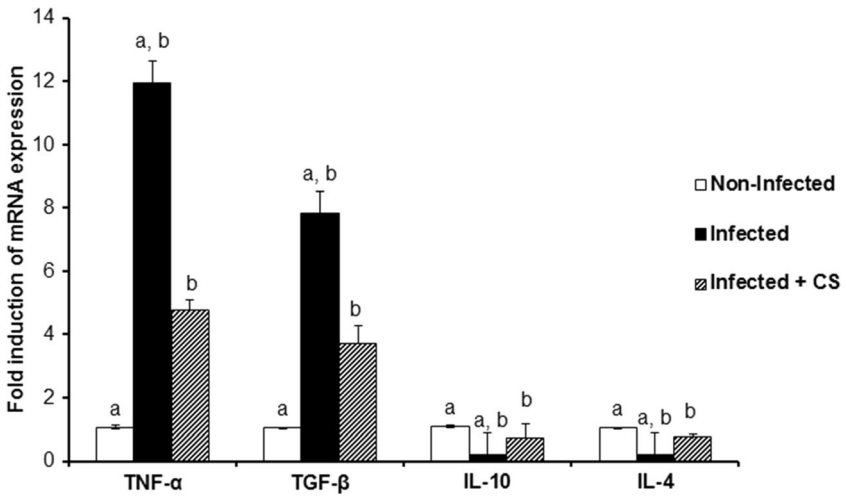

Fig. 7 RT-PCR analysis of TNF-α, TGF-β, IL-10, and IL-4 in the mouse jejunum at day 5 p.i. Fold induction of mRNA expression relative to non-infected group was given. All values are means ± SME. *a* indicates a significance ($p < 0.001$) for infected versus non-infected, while *b* indicates a statistical significance ($p < 0.001$) for infected + CS versus infected group

infections due to increased production of IFN-γ which is a Th1 cytokine (Gaafar et al. 2014). IL-4 was found to induce goblet cell hyperplasia in the intestine, which is related to increased secretion of mucin (Blanchard et al. 2004). The production of mucin maintains the integrity of mucosal surfaces and is considered to be one of the mechanisms of innate immunity against invading pathogens (Dharmani et al. 2009).

The number of T cells in intestinal tissue was decreased in infected groups compared to the non-infected, while CS treatment did not induce any changes, as was also the case in respect to decreased total IgA levels in both serum and intestinal wash in infected groups. This indicated that T and B cells did not play a role in protection after CS treatment because there was no stimulus or vaccine for their activation as effector arms in acquired immunity. It seems that T cells mediate their effects on the parasite in primary infections through secretion of cytokines while $CD8^+$ T cells play the greater role in secondary infections (Ovington et al. 1995). $CD8^+$ T cells, however, were found to be essential in garlic extract-induced protection in primary infection with *Eimeria vermiformis* (Khalil et al. 2015), while CS was found to be effective as an IgA inducer when coupled with the pathogen protein as an adjuvant or delivery system in immunization (Xu et al. 2015).

Although the secretion of TGF-β in mouse intestine has been found to be protective against *Eimeria* infection (Inagaki-Ohara et al. 2006), the current study has failed to demonstrate such a protective role. This refers to its significant decrease in treated-infected compared to the infected group and nearly normalized when compared to the non-infected group. Its expression coincided with the expression of TNF-α in mouse intestinal tissue. In fact, the production of TGF-β has been found to be combined with production of both IFN-γ and TNF-α following *Eimeria* infection (Lillehoj 1998). TGF-β4, in particular, has been found to increase in chickens after coccidian infection (Jakowlew et al. 1997).

In conclusion, CS appears to protect mice against *E. papillata* primary infection by reducing developmental stages and oocyst output. This protective effect was due to the anti-inflammatory action of CS and thus a reduction in epithelial cell damage which facilitates the parasite invasion into the host cell. The protective effect was also a result of increased production of IL-4 which serves to normalize goblet cell numbers. In addition, the development of Th2 response is protective against infection more than Th1 response. CS can be used as a food additive for poultry and other animals.

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Compliance with ethical standards

Conflict of interest statement We declare that we have no conflict of interest.

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