Research Article

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Gene expression, oxidative stress and apoptotic changes in rabbit ileum experimentally infected with *Eimeria intestinalis*

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Abstract: Coccidiosis is a parasitic disease caused by protists (apicomplexans) of the genus *Eimeria* Schneider, 1875 and is considered to be the most important disease faced by rabbit breeders due to its high morbidity. In the present study, the antioxidant status and changes in apoptosis and in the expression of some genes were quantified in rabbits' ilea following infection with *Eimeria intestinalis* Cheissin, 1948. Rabbits, orally infected with 1×10^5 sporulated oocysts of *E. intestinalis*, started to shed oocysts in their faeces on 8 days post infection (dpi) and reached maximum excretion on 10 dpi, with approximately 5 million oocysts. This was accompanied by a significant decrease in the live body weight of infected rabbits. Also, malondialdehyde and nitric oxide were significantly increased while catalase and glutathione were significantly decreased in the ileum tissues of the infected rabbits. In addition, a significant increase was observed in the percentages of apoptotic cells in the ilea of the infected rabbits. Furthermore, interleukin-1 β and interleukin-2 mRNA levels were significantly down-regulated and mRNA levels of interleukin-6, interferon gamma and inducible nitric oxide synthase were significantly up-regulated, while those of C-reactive protein remained unchanged. We conclude that infection with *E. intestinalis* induces oxidative stress, a significant increase in the percentage of apoptotic cells and a diverse and robust Th1 and Th1-related cytokine response in the ileum tissues.

Keywords: Coccidia, malondialdehyde, catalase, intestine, Real time PCR, apoptosis

Coccidiosis is a disease caused by obligate protistan intracellular parasites belonging to the genus *Eimeria* Schneider, 1875 and is considered to be the most important disease faced by rabbit breeders, because of its high morbidity (Oncel et al. 2011). So far, 15 species of *Eimeria* have been identified as infecting rabbits; 14 of these are parasites of the intestine whereas one exclusively invades the liver and the bile duct (Li and Ooi 2009). In Egypt, rabbit meat is a valuable source of animal protein and thus a survey was carried to determine the natural prevalence of infection with *Eimeria* spp. among domestic rabbits in Egyptian domestic farms (Balicka-Ramisz et al. 2014). El-Shahawi et al. (2012) detected eight species of *Eimeria*, with the overall prevalence reaching 70% and the predominant species being *E. intestinalis* Cheissin, 1948 and *E. coecicola* Cheissin, 1947.

Out of the 15 *Eimeria* species infecting rabbits in different parts of the world (Li and Ooi 2009), *E. intestinalis* has been recognised as one of the most pathogenic and highly immunogenic species (Pakandl et al. 2008). Vadlejšek et al. (2010) confirmed that *E. intestinalis* is lethal for rabbits and causes a high mortality rate. Sporozoites of *E. intestinalis* first in-

vade the mucosa of the duodenum of the rabbits, with further development occurring in the jejunum and ileum (Pakandl et al. 2006). The structural changes associated with the endogenous development has been previously investigated (Pakandl 2009), but gene expression, antioxidant status and apoptotic changes in the host target sites in response to infection with *E. intestinalis* have not yet been investigated. Accordingly, the main aim of the present study was to profile the antioxidant status and changes in apoptosis and in the expression of some genes following infection with *E. intestinalis*.

MATERIALS AND METHODS

Animals and infection

The animals used in the present study were sourced from the animal facilities of the Faculty of Science, Beni-Suef University, and were eight weeks old female rabbits of the New Zealand race (*Oryctolagus cuniculus* Linnaeus) and a mean weight of 1.5 kg $\pm$ 0.25 kg. Rabbits were housed individually in plastic cages and kept under constant conditions for one week before use. The faeces of these rabbits were examined daily during this week to affirm the absence of any parasitic infections.

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To calculate the day of maximum oocyst output during infection with *E. intestinalis*, eight rabbits were inoculated with 100 µl saline containing 1×10^5 sporulated purified oocysts of *E. intestinalis* isolated from a natural infection in Egypt using the single oocyst method (Kvičerová et al. 2008, Li and Ooi 2009). Fresh faeces were collected once every 24 h for 14 days from each rabbit individually. Then, oocyst output was determined using the modified McMaster technique (Schito et al. 1996) and the data were presented as the number of oocysts per gram of faeces.

Experimental Design

Rabbits were divided into two groups with eight animals per group. The first group was gavaged with saline and served as the control, uninfected, group. The second group was inoculated orally with approximately 1×10^5 sporulated purified oocysts of *E. intestinalis* in 100 µl saline for each animal. On 10 dpi, 16 rabbits were euthanised with ketamine (50 mg/kg) and dissected. Ileums of non-infected and infected groups were aseptically removed. Small pieces of ileum were rapidly washed in sterile physiological saline and immediately stored at -80 °C for the assay of oxidant/antioxidant markers and quantitative real-time PCR. Other parts of the ileums were used fresh to detect any apoptotic changes. For histology, small pieces of ileum were fixed in 10% buffered formalin and embedded in paraffin. Then, sections were cut and stained with hematoxylin and eosin.

Oxidative biomarkers

Oxidant/antioxidant markers, including malondialdehyde (MDA), nitric oxide (NO), catalase (CAT) and glutathione (GSH), were colorimetrically assayed using chemical kits (Biodiagnostic Co., Cairo, Egypt) and the absorbance of the reactions measured by Ultrospec 2000, UV/visible Spectrophotometer (Mettler-Toledo International Inc., Columbus, Ohio, USA), as follows: levels of MDA in ileum homogenate were estimated following the method of Ohkawa et al. (1979). In an acidic medium, thiobarbituric acid reacts with malondialdehyde for 30 min at a temperature of 95 °C to form thiobarbituric acid reactive species. The absorbance reading of the resultant pink product was performed at 534 nm.

Concentration of NO in ileum homogenate was assayed according to the method of Montgomery and Dymock (1961). In the acidic medium, the present nitrite forms nitrous acid diazotising sulphanilamide that can couple with N-(1-naphthyl) ethylenediamine to form azo dye which can be measured at 540 nm.

CAT in ileum homogenate was assayed according to Aebi (1984). Catalase is able to react with a known amount of H_2O_2 in one minute prior to stopping the reaction with a catalase inhibitor. The remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone in the presence of horseradish peroxidase to form a chromophore with a colour density inversely proportional to the quantity of catalase in the original specimen, which can be measured at 510 nm.

GSH in ileum homogenate was measured according to Beutler et al. (1963). The method is based on the principle that reduction of 5,5'-dithiobis (2-nitrobenzoic acid) with GSH will produce a yellow compound. The reduced chromogen is directly proportional to the amount of GSH and the absorbance can be measured at 405 nm.

Detection of apoptosis using propidium iodide and flow cytometry

The effect of infection with *E. intestinalis* on the induction of apoptosis in the intestinal epithelia was investigated using propidium iodide (PI) staining and flow cytometry according to Hishikawa et al. (1999) and Badr et al. (2012). In brief, the method is as follows: ileum cell suspensions were prepared according to Renaux et al. (2003). Small parts of the ilea were flushed with PBS. Fat and Peyer's patches were removed and samples were opened longitudinally and cut into small pieces. The mucosal layer was scraped and then dispatched by mechanical disruption for 15 min in RPMI-1640 using a magnetic stirring bar.

The separated cells were washed twice in RPMI-1640 by centrifugation and filtered to remove cell aggregates and tissue debris. Cells were counted using a trypan blue exclusion test. Cells (1×10^6 /tube) were washed in phosphate buffer saline (PBS), fixed and permeabilised by incubation in 100 µl of 70% ice-cold ethanol for 30 minutes on ice. After permeabilisation, 1 ml of PBS was added to each tube, gently centrifuged ($40 \times g$) for 5 min before the supernatants were aspirated. Cells were resuspended in 100 µl PBS and the DNA was stained by incubating the cells for 1 h at 4 °C in 1 ml of PBS containing equal volumes of PI and DNase free RNase. Cells were then washed twice in PBS and fixed in 0.5 ml PBS + 2% formaldehyde.

Stained cells were analysed by determining the FL2 red fluorescence on a linear scale. The percentage of apoptotic cells was estimated as the percentage of hypodiploid cells (sub G0/G1 peak). A four colour flow cytometry assay was carried out using standard FACSCalibur (BD Biosciences, San Jose, California, USA) and FACSDiva software (BD Biosciences). Data analysis was performed using FlowJo software, version 9 for Mac (Tree Star, Inc., Ashland, OR, USA).

Quantitative Real-Time PCR

Total RNA was isolated from frozen pieces of ileum using an animal tissue RNA purification kit (Norgen Biotek Corp., Thorold, Canada). A Nanodrop 8000 spectrophotometer (Thermo-Scientific, Wilmington, North Carolina, USA) was used to determine the concentration of the extracted RNA, while the RNA quality was detected on 1% agarose gel using a gel documentation unit (Universal Hood II, Bio Rad, Berkeley, USA).

RNA samples were converted into cDNA using a SCRIPT Reverse Transcriptase kit (Jena Bioscience GmbH, Jena, Germany) according to the manufacturer's protocol. The quantitative real-time PCR (RT-PCRq) was performed by QuantiTect Eva Green PCR kit (Qiagen, Hilden, Germany) using Light Cycler (Roche, Indianapolis, Indiana, USA). The present study evaluated the some genes encoding the mRNAs for the following proteins: interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and C-reactive protein (CRP). Primers were purchased from Qiagen.

PCRs were performed as follows: 10 min at 95 °C followed by 40 cycles including denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and elongation at 72 °C for 20 s. All the real-time PCR experiments were carried out in triplicate and data were presented as the mean of the results of these three independent experiments. The amplification data were quantitatively evaluated

using Light Cycle 480 software (Roche) and the 2- $\Delta\Delta$ CT method (Livak and Schmittgen 2001).

Statistical analysis

The student's *t*-test was performed to determine significant differences using Sigma Plot software version 11 (Erkrath, Rhine-Westphalia, Germany).

RESULTS

Light microscopic examination of hematoxylin and eosin-stained sections revealed that the epithelial cells of the ileum were infected with different developmental stages of *Eimeria intestinalis* including final oocysts enclosed in the parasitophorous vacuole (Fig. 1). All the infected rabbits shed oocysts in the course of patency. Oocyst shedding started on 8 dpi with a mean value of about 2×10^6 oocyst/g, and rose rapidly to reach a peak on 10 dpi, with mean value of about 5×10^6 oocyst/g. Oocyst excretion started to decline on 11 dpi and approximately 2.2×10^6 oocysts/g were excreted on 14 dpi (Fig. 2).

It was observed that all infected rabbits exhibited signs of diarrhoea, which was most evident on 9 dpi. The live body weight of the infected rabbits (1.30 ± 0.2 kg) had decreased significantly compared to the uninfected ones (1.75 ± 0.3 kg) on 10 dpi.

In the ileum homogenates, infection with *E. intestinalis* induced a highly significant increase in MDA and NO, and a highly significant decrease in CAT, as well as reduced GSH (Table 1).

The results showed that, on 10 dpi, the percentage of apoptotic cells in the ileum cell suspension from the infected group was significantly higher ($31.1\% \pm 2.2\%$) than in the uninfected group ($9.3\% \pm 1.8\%$) (Figs. 3).

The mRNA expression of the inflammatory cytokine IL-1 β and IL-2 were significantly down-regulated while that of IL-6 and interferon gamma (IFN- γ) were significantly up-regulated (Fig. 4). Also, iNOS-mRNA increased significantly upon infection, which was consistent with the significant increase in the content of nitric oxide in the ileum tissue (Table 1). However, the mRNA expression of CRP was not affected by the infection.

DISCUSSION

The pattern of oocyst shedding starting on 8 dpi, reaching a peak on 10 dpi before declining increasingly rapidly from 11 dpi confirms the work of Vadlejch et al. (2010), who obtained a similar pattern with *Eimeria intestinalis*. The presence of diarrhoea in the experimentally infected rabbits also accords with some previous studies (Vadlejch et al. 2010, Elfayoumi and Abdel-Haleem 2014). The fact that symptoms of diarrhoea were most evident on 9 dpi is in line both with Vadlejch et al.'s (2010) work and with the contention that gametogenesis begins to develop on 8–10 dpi (El-Shahawi et al. 2012).

By ten days post infection infected rabbits exhibited significant weight loss and severe growth retardation. Drouet-Viard et al. (1997) and Metwaly et al. (2013) described a similar weight loss rabbits. The significant body weight loss in the infected rabbits was probably partly due to the

Fig. 1. Histological section of ileum infected with different developmental stages (arrowheads) of *Eimeria intestinalis* Cheissin, 1948 enclosed in parasitophorous vacuole.

Fig. 2. Rate of shedding of oocysts of *Eimeria intestinalis* Cheissin, 1948 per day. All values are means $\pm$ SD.

Table 1. Levels of oxidative stress biomarkers in ileum homogenate of rabbits infected with *Eimeria intestinalis* Cheissin, 1948 on 10 dpi.

Parameter	Non-infect rabbit	Infected rabbit
Malondialdehyde (nmol/g)	32.3 $\pm$ 1.1	55.4 $\pm$ 1.7*
Nitric oxide (μ mol/g)	59.3 $\pm$ 4.3	142.7 $\pm$ 2.6*
Catalase (U/g)	5,273 $\pm$ 25	4,247 $\pm$ 50*
Glutathione (mg/g)	19.5 $\pm$ 0.5	14.9 $\pm$ 0.7*

Values are mean $\pm$ SD; * significant change at $P < 0.05$ with respect to the non-infected group.

deterioration in the food conversion rate and is doubtless also related to impaired endogenous enzymatic systemic defence (Chapman 1997, Georgieva et al. 2006). Metwaly et al. (2013) also attributed the reduction in rabbits' body weight during the course of intestinal coccidial infection to factors including depletion of growth hormones, distributed metabolic status and electrolyte homeostasis.

The results of the present study showed increased ileum tissue MDA and NO and decreased CAT and GSH con-

Fig. 3. One representative experiment of six rabbits for DNA fluorescence flow cytometric profile of PI-stained ileum cell suspension of non-infected and infected rabbits at day 10 post-infection. Abbreviations: FSC – forward-scattered light; G0–G2, M, S – representing the cell cycle phases; SSC – side-scattered light.

Fig. 4. Quantitative RT-PCR analysis of interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6), interferon gamma (IFN- γ), inducible nitric oxide synthase (iNOS) and C-reactive protein (CRP) in the ileum. Expression was analysed in non-infected and infected rabbits on day 10 post-infection, normalised to HPRT1 signals; relative expression is given as -fold increase compared to the non-infected control rabbit. Values are means $\pm$ SD. * Significant change at $p < 0.05$ with respect to non-infected rabbit.

centrations in the infected rabbits compared to controls. Similarly, Eraslan et al. (2004), Ahmed and Hassan (2007) and Dkhil et al. (2012) each noticed increased levels of MDA and NO as a result of the stressful conditions following infection with *E. tenella* Raillet and Lucet, 1891, *Eimeria* sp. and *E. coecicola*, respectively. The elevated MDA concentration in rabbits infected with *E. intestinalis* in our experiment could be due to increased production of

reactive oxygen species, resulting from lipid peroxidation (Georgieva et al. 2006). Dkhil et al. (2012) suggested that the increased levels of MDA and NO are a result of serious inflammatory response due to oxidative damage caused by coccidian infection.

Several studies, meanwhile, have postulated that the decrease in CAT and GSH activity during the course of eimeriosis might be due to the cellular defence systems being overwhelmed by excessive formation of reactive compounds, and that this is suggestive of oxidative stress (Eraslan et al. 2004, Ahmed and Hassan 2007, Çam et al. 2008). The increases in MDA and NO and decreases in CAT and GSH observed in this study tend to support this picture of impaired antioxidant status and the occurrence of oxidative stress in rabbits infected with *E. intestinalis*.

Our data also showed a significant increase in the percentages of apoptotic cells in the infected ileum compared to the control one. The intracellular stages of the coccidian parasites are known to modify their host cells quite extensively and to interfere with many host signalling pathways, including induction of host cell apoptosis in order to maximise their own chances of survival (Lüder et al. 2001, 2009).

Fisch et al. (2007) and Green (2000) postulated that the parasite invasion and replication might cause considerable stress to the host cell and that this stress, in turn, may trigger apoptosis. This contention was supported by Yan et al. (2015), who observed that infection with *E. tenella* promoted apoptosis during the different developmental stages, especially through gamogony and sporogony. This may be due to the fact that merogony of species of *Eimeria* requires a stable intracellular environment to obtain essential nutrients and evade host immune attack and therefore *Eimeria*

infection may directly activate the NF- κ B pathway in host cells to further inhibit host-cell apoptosis (Salminen et al. 2012).

Whereas gamogony and sporogony may prevent the expression of the NF- κ B response gene (Yan et al. 2015) then they further decrease the expression of the anti-apoptotic proteins Bcl-2 and BclXL, which accelerate host-cell apoptosis (Rasul et al. 2012). Liu et al. (2009) also realised that the merogony of *Cryptosporidium parvum* Tyzzer, 1912 upregulated the genes with antiapoptotic roles and downregulated the genes with apoptotic roles while gamogony and sporogony downregulated the anti-apoptotic genes and induced proapoptotic gene. Therefore, they suggested a biphasic regulation of apoptosis: antiapoptotic state in the early infection and apoptotic state in the mid and late infection.

Other factors that contribute to apoptosis during the course of eimeriosis are the elevation of reactive oxygen species and the presence of nitrogen intermediates and inflammatory cytokines within the infected cells (Major et al. 2011, Metwaly et al. 2014).

Our present data suggest remarkable inflammatory responses (Th1 cytokines), indicated by significantly down-regulated levels of IL-1 β and IL-2 mRNA (in line with Dkhil et al. 2011 and Hong et al. 2006), and significantly up-regulated levels of IL-6, IFN- γ and iNOS. Kaiser et al. (2000) have suggested that the down-regulation of IL-1 β and IL-2 might lead to a slower inflammatory response in the gut, thus allowing initial entry of the coccidia into epithelial cells.

In terms of the up-regulated levels of IL-6, Dkhil et al. (2012) reported significantly up-regulated mRNA expression of IL-6 in rabbit appendices infected with *E. coecicola*, and several authors have speculated that IL-6 plays a role in the initiation of anti-eimerian effector responses, particularly upon the generation of Th1 effector responses during primary infection (Lynagh et al. 2000, Dkhil et al. 2012). Swaggerty et al. (2004) and Hong et al. (2006) have also suggested that the increased expression of IL-6 may be to create a population of neutrophils more capable of responding to and eliminating pathogens.

IFN- γ , which is thought to activate the intracellular toxicity, is powerfully increased during infection, which suggests that it possesses a particular role in infection

with species of *Eimeria* (Dkhil et al. 2011). The notably increased levels of IFN- γ in the intestine during infection with *E. bovis* Zublin, 1928 and *E. alabamensis* Christensen, 1941, *E. maxima* Tyzzer, 1929 and *E. papillata* Ernst, Chobotar et Hammond, 1971 (see Alcalá-Canto and Ibarra-Velarde 2008, Cornelissen et al. 2009, Dkhil et al. 2011) provide a broad-based body of evidence in support of its role during eimeriosis. Dkhil et al. (2011) suggest that IFN- γ reduces the output of oocysts throughout primary infection with *Eimeria*. Additionally, IFN- γ is considered to be a major cytokine mediating resistance to many coccidian parasites including *Eimeria* (see Hong et al. 2006).

MacMicking et al. (1997) postulated that IFN- γ is a potent iNOS inducer; this would explain the concurrently high mRNA levels of IFN- γ and iNOS observed in the intestine of rabbits infected with *E. intestinalis* in the present study, as has also been reported in the case of infection with *E. maxima* (see Hong et al. 2006). Moreover, the detrimental effect of IFN- γ on the intracellular replication of *Eimeria* spp. is well documented, with NO being proposed as the effector molecule (Taubert et al. 2009). This suggests that iNOS up-regulation is coherent with the increase of nitrite/nitrate within tissue contents reported during the present study.

Many studies also proved that the increased IL-6 gene expression enhanced NO production through increasing the induced iNOS expression (Kang et al. 2007, Maalouf et al. 2010). CRP has been suggested as a preferable biomarker for chronic and acute inflammation (Dortay et al. 2011). CRP is primarily synthesised within the liver in response to IL-6, with the synthesis being enhanced synergistically by IL-1 β (Marnell et al. 2005). This synergistic relationship may imply that the low levels of IL-1 β mRNA and high levels of IL-6 mRNA found in this study may be working together to result in the unchanged CRP expression that was observed. Overall our data suggests that the infection with *E. intestinalis* induces oxidative stress, a significant increase in the percentage of apoptotic cells and a diverse and robust Th1 and Th1-related cytokine response in the ileum tissue.

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