Increasing of serum nitric oxide metabolites in chicken *Eimeria* infection

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Abstract

In order to determine the involvement of nitric oxide in the pathogenesis of coccidiosis induced by *Eimeria*, 30 chickens were challenged with mixed sporulated oocysts of four species of *Eimeria* (*E. acervulina, E. maxima, E. necatrix,* and *E. tenella*) at 26 days of age. There was an increasing of oocyst shedding in the infected birds at 6, 10 and 14 days post-infection. Histopathological examination revealed a loss of epithelial tissue, congestion of blood vessels, severe muscular edema and necrosis of submucosa. The sum total of nitrite and nitrate was increased in the serum at 6, 10 and 14 days post-infection progressively, but was only significant ($P < 0.05$) at 10 and 14 days post-infection compared to earlier days. The nitrate amounts were also significantly higher at days 10 and 14. It can be concluded that infection by four species of *Eimeria* stimulated NO production after infection. It is therefore possible that NO is involved in the pathogenesis of coccidiosis.

Introduction

Chicken coccidiosis is a disease caused by one of the several prevalent enteric protozoan parasites of the genus *Eimeria*. Seven species have been recognized to infect chickens: *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis,* and *E. praecox.* Each species has its own characteristics with respect to preferred site of infection, pathogenicity and immunogenicity (Kitandu and Juranova, 2006) . It is the major parasitic disease in poultry, with a substantial economic burden estimated to cost the industry more than US$ 3 billion in annual losses worldwide (Dalloul and Lillehoj, 2006) . These estimates include the costs of prophylactic in-feed medication for broilers and broiler breeders, alternative treatments if medication fails, and losses due to mortality, morbidity, impaired growth rate, temporary reduction of egg production in layers and poor feed conversion of chickens that survive outbreaks (Kitandu and Juranova, 2006) .

The life cycle of *Eimeria* comprises intracellular, extracellular, asexual and sexual stages.

Therefore it is not surprising that host immunity is also complex and involves many facets of nonspecific and specific immunity (cellular and humoral immune mechanism) (Lillehoj 1998; Lillehoj and Lillehoj, 2000). *Eimeria* pathogenicity varies in birds of different genetic backgrounds. *Eimeria* species exhibit different tissue and organ specificity in the infected host. Thus, understanding the interplay between the host and the parasites in the intestine is crucial for the design of novel control approaches against coccidiosis (Dalloul and Lillehoj, 2005) .

Nitric oxide (NO) is a highly reactive free radical gas, which has been shown to possess an extraordinary variety of biological functions (Moncada *et al.,* 1997). NO is synthesized from L-arginine by a family of isoenzymes known as the nitric oxide synthases (NOS). There are three types of NOS. Two of these (NOS-1 and NOS-2) are constitutively expressed, while NOS-3 is expressed only in activated cells (Garcia and Stein, 2006; Hassanpour *et al.,* 2008) . NO is an important modulator of cellular functions, is a potent vasodilator and neurotransmitter and has been implicated in numerous physiological, pharmacological and pathological processes (Moncada *et al.,* 1997).

In the presence of oxygen, NO undergoes oxidation, which follows second-order kinetics. Hence, when NO levels are high, it is oxidized within seconds. The oxidation end-product of NO is nitrite (NO_2^-) in aqueous solutions, nitrate (NO_3^-) in the presence of oxyhemoglobins (e.g., in blood) and peroxynitrite in the presence of $O2^-$. NO and NO^- derived species (e.g., NO_2 or $ONOO^-$) can oxidize cell lipids (O´Donnell and Freeman, 2001).

Besides the process of mucosal disruption, inflammatory reactions may also contribute to the pathology of *Eimeria* infections, but they have not been

extensively studied. In particular, the role of bioregulator molecules such as nitric oxide (NO), which are known to be produced during immune responses to other parasites (Ovington and Smith, 1992; Gazzinelli *et al*., 1993; Allen and Teasdale, 1994; Oswald *et al.,* 1994; Petray *et al.,* 1994) , is unknown. The objective of this study was to evaluate nitric oxide metabolites (nitrite and nitrate) in the serum of broiler chickens that had been experimentally infected by four species of *Eimeria (E. acervulin, E. necatrix, E. maxima, E. tenella).*

Materials and Methods

Animals, management and treatment

Thirty one-day-old fast-growing chickens from Ross 308 breed were randomly divided into three groups for replicates. Chicks were reared at standard conditions for 40 days and provided *ad libitum* access to water and a standard ration (Starter: 13 MJ metabolisable energy (ME)/kg of diet, 230 g/kg crude protein (CP), Grower: 13 MJ metabolisable energy (ME)/kg of diet, 200 g/kg CP, Finisher: 13 MJ metabolisable energy (ME)/kg of diet, 180 g/kg CP formulated to meet requirements for broilers (NRC, 1994)

Challenge infection of chickens

In this study, the birds were challenged with mixed sporulated oocysts of four pathogenic species of *Eimeria* (1×10^4 *E. acervulina*, 4×10^4 *E. maxima*, 3×10^4 *E. necatrix*, and 3×10^4 *E. tenella*, purchased from the Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran) at an age of 26 days (Chapman, 1989). The challenge infection was carried out in the chickens via oral administration of oocysts suspension.

Birds were monitored for signs of disease and mortality during infection. Oocyst shedding was assessed as described by Min *et al.* (2001). Briefly, feces from each group were collected at 0, 6, 10 14 days post-infection. Fecal material was ground and homogenized. Then samples were diluted and the oocysts were counted microscopically, using a McMaster counting slide. The total number of oocysts per gram of feces (OPG) was calculated using the formula: total oocyst (OPG) = oocyst count × dilution factor × (fecal sample volume / counting chamber volume).

Nitrite and nitrate assessment in serum

Serum samples were prepared from seven randomly selected chicks at 0, 6, 10, and 14 days post-infection and stored at -20°C. Measurements of nitrite and nitrate were based on the reduction of nitrate to nitrite by cadmium. The nitrite produced was determined by Griess reaction. At this method, the serum sample was deproteinized by adding $ZnSO_4$ (75 mmol/l) and NaOH (55 mmol/l) solutions. After centrifuging, the supernatant was recovered and diluted in glycine buffer (45 g/l, pH 9.7). Cadmium granules (2 - 2.5 g) were rinsed three times with deionized distilled water and swirled in a CuSO4 solution (5 mmol/l) in glycine-NaOH buffer (15 g/l, pH 9.7) for 5 min to become activated. Freshly activated cadmium granules were added to pretreated deproteinized serum. After continuous stirring for 10 min, the samples were transferred to appropriately labeled tubes for nitrite determination by Griess reaction. Griess reagent 1 (1% sulfanilamide in 5% phosphoric acid) was added to the sample tubes and then incubated for 10 minutes at room temperature, protected from light. Griess reagent 2 was added (0.1% N-napthylethylenediamine dihydrochloride in water) to all samples and absorbance was measured within 10 minutes in a spectrophotometer at a wavelength of 540 nm (Navarro-Gonzalvez *et al.,* 1998). At this stage of the experiment, the sum of the nitrite and nitrate was measured. At the second stage, the amount of the nitrite in the serum was measured. For this method, all procedures described above were carried out except reduction by cadmium granules.

Histopathological assessment

At 0, 6, 10 and 14 days post-infection, seven randomly selected chicks were euthanized by decapitation and gross pathology dissection was performed; tissues were visualized and palpated for evidence of gross pathology. Different portions of the intestine were immersed in 10% phosphate-buffered formaldehyde. These samples were dehydrated in increasing concentrations of ethanol and xylene, embedded in paraffin and 5 µm thick sections were stained with hematoxylin and eosin and cover slipped. The tissues sections were reviewed for histopathological changes.

Statistical analysis

All results are represented as mean ± SEM. Comparisons were made using one-way ANOVA with Tukey's *post hoc* test (SPSS-14.0 package). *P*-values less than 0.05 were considered to be statistically significant.

Results

Dysentery and diarrhea were observed in the infected chickens at 6, 10 and 14 days post-infection. There was also an increasing of oocyst shedding (OPG) in the infected birds at those days, reaching a significant ($P < 0.05$) maximum at day 14. The OPG values were $1 \times 10^3 \pm 480$, $14 \times 10^4 \pm 3100$, and $13 \times 10^5 \pm 3 \times 10^4$ at 6, 10 and 14 days post-infection, respectively. All of them were significant ($P < 0.05$) compared with pre-infection levels at day 0. The lesions were noticeable at different

parts of the intestine, such as the ceca, jejunum and colon in the gross pathology examination. In case of the intestinal form, an extremely ballooned intestine and petechial hemorrhages could be easily seen while looking grossly without opening the gut. However, discrete hemorrhagic spots were also observed on the mucous membrane of the intestine when opened. The intestine was often found to be edematous, thickened, showing necrosis and sloughing of the friable intestinal epithelium. In almost all cases of cecal coccidiosis, enlargement of the cecum with clotted blood, hemorrhagic or whitish spots on the cecal wall, inflammation, necrotic patches, dilation of cecum with consolidation of cecal contents were observed. However, in some cases, ceca were found solid consisting caste of necrosed blood cells, epithelial cells and other debris. On opening the ceca, a bloody mass, a characteristic of cecal coccidiosis, was found in some cases. The changes in color from red to mottled reddish or milky white due to formation of oocysts were observed in some cases.

Histopathological examination revealed loss of epithelial tissue, congestion of blood vessels that indicated disruption followed by leakage of blood, severe muscular edema, necrosis of submucosa, loss of villi. The developmental periods of the growing parasite in the intestine are shown in Figure 1 and Figure 2.

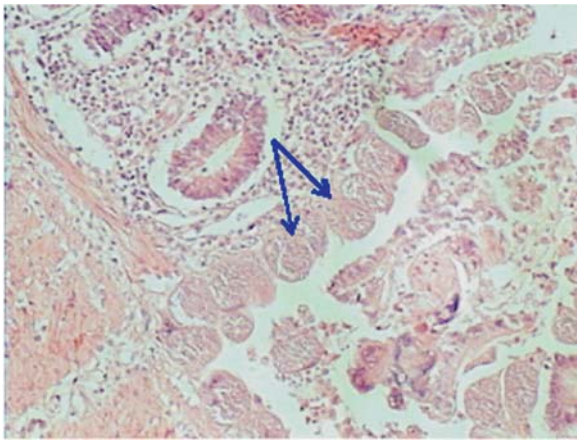

Figure 1: Intestine, Schizont of Eimeria spp in a histopathologic section. (H & E, 4×)

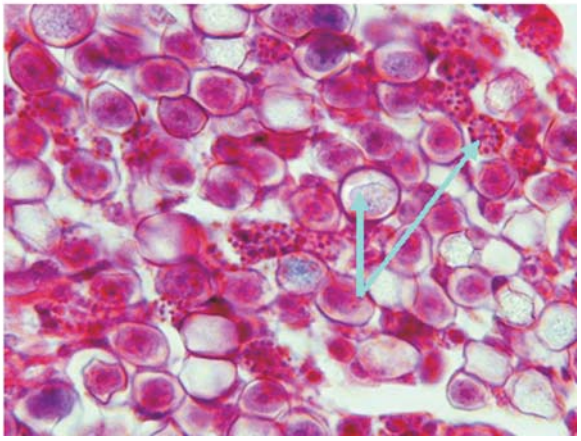

Figure 2: Intestine, Oocysts and gamonts of eimeria spp in a histopathologic section. (H & E, 10×)

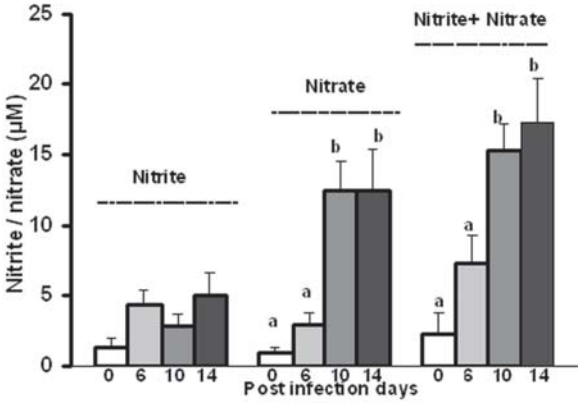

Figure3: Comparison of serum nitric oxide metabolites (nitrite+nitrate) at different days post infection in chickens with coccidiosis. Values are mean ± SEM. Different letters show significant difference (p<0.05)

Nitrite and nitrate as NO metabolites were measured in the serum samples. The sum of these metabolites increased progressively in the serum at 6, 10 and 14 days post-infection, and was significantly different at 10 and 14 days post infection. The nitrate amounts were also significantly ($P < 0.05$) higher at days 10 and 14. The nitrite amounts of serum showed no significant changes at different days after the infection (Figure 3).

Discussion

In this study, we induced coccidiosis in chickens at the age of 26 days. This induction was confirmed by clinical signs, oocyst shedding, gross pathology and histopathologic changes at 6, 10 and 14 days post-infection (Soomro *et al.,* 2001). The amounts of serum nitrite and nitrate as nitric oxide metabolites were also increased during this experimental coccidiosis. Allen and Teasdale (1994) found that infection with *E. acervulina* or *E. tenella* caused increases in plasma levels of nitrite and nitrate. Maximum values were found at a time of disorganization of the intestinal or cecal mucosa, and were associated with gamete formation, oocyst production, and shedding. Plasma levels of nitrite and nitrate also appeared to be dependent upon the dose of parasite oocysts given. Allen (1997b) reported that chickens infected with *E. maxima* exhibited levels of plasma and mucosal nitrite and nitrate that increased significantly at about 4 days post-infection and reached maximal levels at about 6 days post-infection, a time that coincides with maximum disorganization of the infected mucosa and shedding of oocysts. Allen (1997a) also determined that inoculation with 5×10^4 and 1×10^6 but not 1×10^3 oocysts of *E. tenella* caused significant increases in

concentrations of the plasma nitrite and nitrate when measured at 7 days post-inoculation. In chickens inoculated with 5×10^4 oocysts, significant increases in plasma nitrite and nitrate were seen at 5 and 7 days but not at 3 days post-inoculation. Allen and Fetterer (2000) found that plasma levels of nitrite and nitrate were positively correlated with infection dose and negatively correlated with weight gain. These studies are consistent with our reports, but we found that infection by a mixture of *Eimeria spp*. caused significant increases of serum level of nitrite and nitrate at 10 days post-infection. The mixture of four *Eimeria spp*. and different doses of them probably influenced on the production of nitric oxide and its metabolites (Allen and Fetterer, 2000) . There are different cellular sources of NO. A large number of cells (Moncada *et al.,* 1997), including stimulated macrophages, the enterocytes themselves, the vascular smooth muscle (Busse and Mulsch, 1990; Koide *et al.,* 1993) and endothelial cells (Oswaled *et al.,* 1994) have been reported to respond to inflammatory cytokines such as IFN and TNF with upregulation of iNOS. Large numbers of monocytic lymphocytes and macrophages were seen in mucosal smears from chickens infected with *E. maxima* at 6 days post-infection. Laurent *et al.* (2001) confirmed the strongest upregulation of iNOS expression in the intestine 7 days after *E. tenella* and *E. maxima* infections. Lillehoj and Li (2004) reported that macrophages produced high levels of NO in response to coccidia sporozoites. Mast cells can also be stimulated to synthesize NO (Salvemini *et al.,* 1990). A greatly increased number of cells interpreted as being intraepithelial mast cells (Lawn *et al.,* 1988) were observed in mucosal smears.

It can be concluded that infection by a mixture of four species of *Eimeria* stimulated NO production that was significant in 10 and 14 days post-infection. It is furthermore possible that NO is involved in the pathogenesis of coccidiosis.

Acknowledgments

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