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Short communication

Effect of Acetamizuril on enolase in second-generation merozoites of *Eimeria tenella*

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ABSTRACT

As an obligate intracellular apicomplexan parasite, *Eimeria tenella* (*E. tenella*) can rapidly invade chicken cecum epithelial cells and cause avian coccidiosis. Enolase, an essential enzyme that catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate, plays a very important role in glycolysis. In this study, each chicken was inoculated with 8×10^4 sporulated *E. tenella* oocysts suspended in 1 ml of distilled water to determine the effects of acetamizuril, a new triazine anticoccidial drug, on enolase in the second-generation merozoites of *E. tenella*. The chickens were divided into two groups: the untreatment group (challenged with *E. tenella* oocysts and provided with normal feed) and the treatment group (challenged with *E. tenella* oocysts and provided with 5 mg/kg of acetamizuril by oral gavage at 96 h after inoculation). The second-generation merozoites of *E. tenella* (mz-En) were obtained at 120 h after inoculation. Subsequently, quantitative real-time PCR and Western blotting were conducted to detect the enolase changes in mz-En at the transcriptional and translational levels. The results showed that enolase mRNA expression was downregulated, and the translational level was decreased in the treatment group. In addition, the subcellular localization of enolase demonstrated that enolase was distributed primarily at the top of the mz-En and that the fluorescence intensity was weak after treatment with acetamizuril. These findings indicated that enolase may be a promising target to prevent coccidiosis.

1. Introduction

Avian coccidiosis, an important parasitic disease caused by *Eimeria* spp., an apicomplexan protozoan parasite, is responsible for a substantial economic burden globally in the avian industry (Györke et al., 2013; Williams, 1999). As the most virulent *Eimeria* spp. species, *Eimeria tenella* has a complex lifestyle, including exogenous (sporogony) and endogenous developmental stages (schizogamy and gametogony, respectively). Although coccidian vaccines have been extensively studied as a novel strategy to provide protection against coccidiosis (Yin et al., 2014; Mansoori and Modirsanei, 2012; Song et al., 2013), these vaccines are not widely used in the poultry industry because of their poor stability. Thus, the control of coccidiosis still mainly depends on anti-coccidiosis drugs. Unfortunately, the large-scale and long-time use of anticoccidials has led to the emergence of anticoccidial resistance problems (Stephan et al., 1997; Kawazoe and Fabio, 1994; Arabkhazaeli et al., 2013). Therefore, studies have been conducted recently to search for novel therapeutic drugs and strategies against coccidiosis. Recently, a novel anti-coccidiosis triazine compound, acetamizuril, was synthesized by the Shanghai Veterinary Research Institute of the Chinese Academy of Agricultural Sciences; its structure is similar to those of diclazuril and toltrazuril. Previous studies have shown that this novel compound has a high anticoccidial activity with an anticoccidial index above 180 and an acceptable safety profile (unpublished data).

Enolase, a key glycolytic enzyme, is an essential cytoplasmic enzyme that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate in the final steps of the glycolytic pathway (Yang et al., 2014). Enolases have highly conserved amino acid sequences and possess a wide range of additional functions beyond their classical role in glycolysis (Raghunathan et al., 2014; Karina et al., 2012). In *E. tenella*, enolase also plays a vital role in the interaction of the parasites with host cells (Labbé et al., 2006).

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In this study, we aim to learn more about the role of enolase in mz-En to better understand whether acetamizuril effects the expression of enolase in mz-En.

2. Materials and methods

2.1. Reagents

Acetamizuril (>99.5%, No. 20141008) was provided by the Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences (CAAS).

2.2. Preparation of parasite

E. tenella was propagated by infecting 14-day-old coccidia-free chickens, and the unsporulated oocysts were isolated from the cecum of the chickens at day 8 after infection. Subsequently, the unsporulated oocysts were sporulated in a 2.5% potassium dichromate solution for 3 days.

The sporulated oocysts were washed with distilled water until the 2.5% potassium dichromate solution was removed completely and then counted using a cytometer before inoculation.

2.3. Second-generation merozoite collection

One-day-old Chinese yellow broiler male chickens purchased from the hatchery of Min You (Shanghai, China) were used in this experiment. The chickens were reared under coccidia-free conditions and provided with feed and water ad libitum. At two weeks of age, 120 chickens were randomly divided into two groups (60 chickens each), and each group was further subdivided into three blocks of 20 chickens each as biological replicates. All chickens were challenged by oral gavage with a single dose of 8×10^4 *E. tenella* sporulated oocysts and provided with normal feed. The first group, which did not receive any other treatments, was designated as the untreatment group. The chickens in the second group were given 5 mg/kg of acetamizuril by oral gavage at 96 h post-infection and designated as the treatment group. The mz-Ens were obtained by dissecting the chickens and collecting the ceca at 120 h after infection. All procedures were conducted according to the guidelines of the Animal Ethics Committee of the Faculty of Veterinary Medicine and Institutional Animal Care and Use Committee of China.

The mz-Ens were obtained from the ceca of the infected chickens following the procedures as described previously (Liu et al., 2006; Shen et al., 2012). Briefly, the ceca were harvested 120 h post-infection, and their contents were discarded. The ceca were then rinsed with PBS and cut into small pieces. The merozoites were released from the ceca by enzymatic digestion. Afterward, the merozoites were purified by filtration, centrifugation, erythrocyte disruption and Percoll density gradient centrifugation.

2.4. Quantitative real-time polymerase chain reaction analysis

Total RNA of the mz-Ens was extracted with TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer. To avoid DNA contamination, the extracted RNA was treated with RNase-free DNase I (40 U/mg RNA, Takara, China) and purified using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNAs were synthesized using the GoTaq® qPCR Master Mix Kit (Promega, China) following the instructions of the manufacturer after adjusting the samples to the same concentration for the untreatment and treatment groups. Quantitative real-time polymerase chain reaction analysis (qRT-PCR) was performed with the ABI 7500 real-time PCR System (Applied Biosystems, USA) and GoTaq® qPCR Master Mix Kit (Promega, China). The primers for enolase and the 18S housekeeping gene were as follows: enolase (sense 5′-GCGACAAGACCCGCTAC-3′, antisense 5′-TGCCGTCCAGCTCCTCCAC-3′); 18S (sense 5′-ATCGCAGTTGGTTCTTTTGG-3′, antisense 5′-CCTGCTGCCTTCCTTAGATG-3′). The qPCR reaction conditions consisted of an initial single cycle for 2 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C and extension for 15 s at 60 °C. The specificity of the amplification was confirmed using a melting curve and electrophoresis analysis. The data were analyzed with a normalized gene expression method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). Each reaction was performed in triplicate, and the entire experiment was carried out in triplicate.

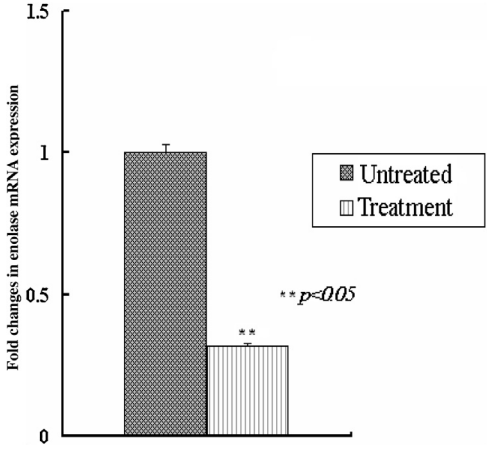

Fig. 1. qRT-PCR results of the mRNA level of enolase in mz-Ens for the untreated and treatment groups. The results are presented as the mean ± SD values of independent experiments performed in triplicate. $^{**}P < 0.05$ indicates statistically significant differences.

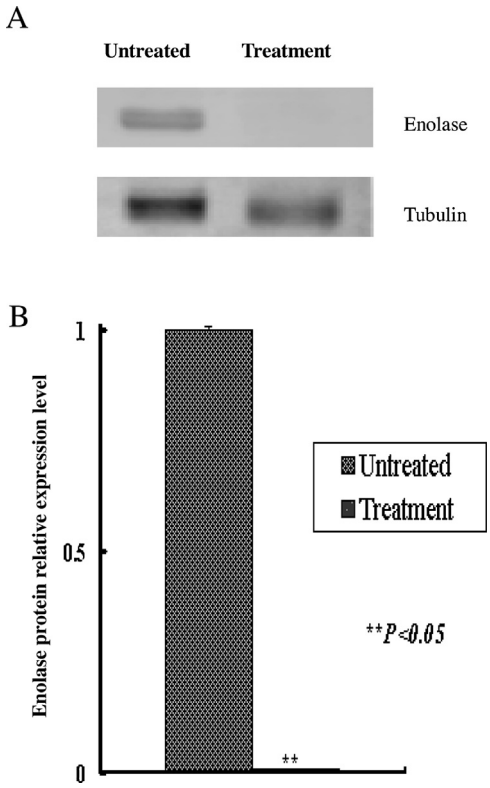

Fig. 2. The decrease in enolase protein levels. (A) Western blot analysis of the relative protein expression of enolase. Tubulin served as a loading control. (B) The relative quantification of mz enolase protein levels. $^{**}P < 0.05$ indicates statistically significant differences.