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Anti-parasitic activity of zinc oxide nanoparticles against *Eimeria tenella* in broilers experimentally infected

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Abstract

In the study, zinc oxide nanoparticles (ZNOPs) at concentrations of 20, 40, and 60 mg/kg were tested for their antimicrobial action against the oocysts of *Eimeria tenella*. The oocysts of *E. tenella* were isolated from the feces of broilers received at the veterinary hospital in Diwaniyah Province and initially diagnosed by compound optical microscopy. The oocysts were confirmed molecularly by polymerase chain reaction targeting the ITS1 gene with a molecular weight of 409 bp. The results in the first week showed that ZNOP concentrations of 20 and 40 mg/kg possess various activities against *E. tenella*, while 60 mg/kg was the most effective in reducing excreted oocysts compared to the positive control and amprolium group, along with the appearance of mild symptoms and a mortality rate of 0.8%. In the second week of infection, excreted oocysts and mortality rates generally decreased in all treated groups. A comparison of all groups showed that the 60 mg/kg ZNOP-treated group had a significantly lower number of excreted oocysts, and all birds in this group recovered during the second week of infection. These findings revealed the prospect of using ZNOPs against *E. tenella* in challenging situations of the appearance of resistance to anticoccidial agents.

Keywords: ZnO nanoparticles; broilers; *Eimeria tenella*; Iraq

Introduction

Nanomaterials play important roles in medicine, industry, and environmental sustainability [1,2]. Many experiments have been conducted in the field, including using nanoparticles as drug-carrying nanoparticles that deliver and transport medicinal drugs to treat cancer cells [3]. Nanomaterials have attracted considerable interest, especially in biological and medical applications, as several medications have poor efficacy. Various in vivo and in vitro treatment strategies with nanoparticles are useful because the scale of nanoparticles is close to biological molecules and forms [4]. On the other hand, nanoparticles may provide an alternative to antibiotics and inhibit pathogens [5]. Nanoparticle research has led to various applications that have improved productivity in poultry and animals [6,7]. The increasing resistance of microbes to many antibiotics has prompted researchers to use nanotechnology as a new solution. One of the most important nanoparticles to date is zinc oxide. Zinc oxide nanoparticles have low toxicity. In addition, they are

an antimicrobial, a growth enhancer, a nutritional supplement for humans and animals, an immunostimulant, and a catalyst for phytochemical reactions [8,9].

Eimeria tenella is an intracellular parasite that has a significant effect on poultry. The infections lead to malabsorption, diarrhea, and bleeding. Coccidiosis can be controlled through the preventive use of anticoccidial drugs. *Eimeria* vaccines are available, but their use is limited to a proportion of the international poultry industry because of production restrictions and high cost [10–12]. Coccidiosis is one of the important parasitic diseases and one of the difficult problems facing poultry farming in Iraq and the world because it causes significant economic losses despite the development of various means to limit its spread, such as good management, methods of improving breeding, and preventing chicks from eating mature oocysts through use of sterilizers. Nevertheless, most of them have not been proven effective [13,14]. Therefore, this study examined the anti-parasitic activity of zinc oxide nanoparticles (ZNOPs) against *E. tenella*-infected broilers.

Materials and Methods

Diagnosis of *E. tenella* oocysts

Oocysts were obtained from infected broiler specimens imported to a veterinary hospital in Al-Diwaniyah Province, where the stools were placed in a 2.5% potassium dichromate solution. Flotation was then performed by adding saturated saline (saturated sodium chloride) to float the oocysts.

A hemocytometer was used to calculate the number of oocysts used to infect the broiler with *E. tenella*, as described elsewhere [15,16]. Oocysts were counted in the boxes designated for counting white blood cells. The total number of oocysts was divided by eight to obtain the average per square, then multiplied by 10,000 to produce the number of oocysts in one milliliter.

$$\text{Account of oocysts in 1 mL} = \frac{\text{Account of oocysts in 8}}{8} \times 10,000$$

The diagnosis of the parasite was confirmed by a conventional polymerase chain reaction (PCR) using a pair of primers for the gene internal transcribed spacer-1 (ITS-1) (ITS1-F: AG-CAGGTAGTCGTCGGTGTT ITS1-R AGCAAAGTTCCAAG-CAGCAT), with a molecular weight of 409 bp based on Hamidinejat et al. [17]. The DNA was extracted using a Stool Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's protocol, and the concentration and purity of

the extracted DNA were measured using a Nanodrop spectrophotometer. The PCR mix was prepared using AccuPower Pre-mix Kit (Bioneer) according to the manufacturer's instructions. Electrophoresis was carried out using 1.5% agarose gel to read the PCR results.

Preparation of ZNOPs

This compound was purchased as ready-made oxide from Sky Spring Nanoparticles Inc. in the form of a white to light yellow powder with a purity of 99.8% and a size of 10 to 30 nm.

The nanocomposite (ZnO nanoparticles) was prepared in the form of a stock solution, where 5 g of ZNOPs were dispersed in 100ml of distilled water and sterilized in an autoclave. The colloid was mixed using an ultrasonic homogenizer for 15 minutes, and concentrations of 20, 40, and 60 mg/kg were prepared and kept in a refrigerator until used in the experiment.

Experiment animals

One hundred twenty-day-old broilers were obtained from the Diwaniyah hatchery (Turkish Rose strain), and all the chicks were reared on the floor with bedding from one day old until the end of the trial period. All groups of broilers were subjected to the same environmental conditions from feeding and lighting as well as a preventive health program.

Infection of the animals

Before infection, stool samples were collected daily for 21 days to check for the absence of *E. tenella* oocyst. Similarly, the clinical signs were observed daily. At 21 days, the experimental animals were divided into six groups, with 20 birds per group. G1 was left as a negative control group (without infection) and was given distilled water. G2 was dosed with 50,000 mature oocysts and left as a positive control (group infected). G3, G4, G5, and G6 were dosed with 50,000 oocysts. The feces were examined from the day after the infection until the oocysts appeared in their feces (4–6 days). Subsequently, G3 was dosed with amprolium drug (150 ppm), and G4, G5, and G6 were dosed with 20, 40, and 60 mg/kg of ZNOPs, respectively, for 14 days. An experimental infection with *E. tenella* was induced at 21 days old and dosed orally using gavage. Drugs were given daily orally for two weeks at the concentrations mentioned above.

Statistical analysis

A completely randomized design was adopted as the experimental laboratory design, in addition to comparing the means using the least significant difference test to determine the significant differences at the $p \leq 0.05$.

Ethical approval

All procedures conducted in animal studies were in accordance with the ethical standards of the institution at which the studies were conducted. The current study was approved by the Committee of the Department of Biology, Faculty of Education, University of AL-Qadisiyah (approval number: 345 in 3/6/2021).

Results

The diagnosis of the oocysts of *E. tenella* (Fig. 1), which was used in the experimental infection, DNA was extracted from stool samples taken from infected broilers imported to the veterinary hospital and subjected to PCR to confirm the diagnosis of the oocysts of *E. tenella* (Fig. 2). Of the 150 stool samples, 100 were positive for *E. tenella* ITS-1 gene (prevalence rate, 66.6%).

During the first week of infection, no pathological symptoms or fecal oocysts were observed in the negative control group (G1) (Table 1). On the other hand, birds in the *E. tenella*-infected group (G2) showed severe disease symptoms manifested by lethargy, general weakness, severe bloody diarrhea, and reduced feed consumption. Furthermore, G2 has a mortality rate and oocyst count of 10% and 6,080 oocysts, respectively. Compared to G2, amprolium-treated *E. tenella*-infected group (G3) showed mild symptoms, a reduced number of oocysts, and a mortality rate of 1.6%, whereas the ZNOP-treated-*E. tenella*-in-

fectured groups exhibited dose-dependent anticoccidial activity. G4 (20 mg/kg ZNOP group) showed similar symptoms to G2 and an oocyst count and mortality rate of 3,000 and 7.5%, respectively. In contrast, G5 (40 mg/kg ZNOP group) had moderate disease symptoms, a slight decrease in the number of oocysts, and a 3% mortality rate, while G6 (60 mg/kg ZNOP group) showed mild symptoms and a significantly reduced oocyst number ($p < 0.05$) and a 0.8% mortality rate.

Similarly, the second week of infection also indicated a decrease in the number of oocysts raised during the second week in the anticoccidial group and the groups treated with the three nano concentrations, with a decrease in the mortality rates of birds (Table 2). The concentration of 60 mg/kg recorded a clear superiority and a significant decrease ($p \leq 0.05$) in the number of oocysts excreted in the litter compared to all other groups, and all birds of this group recovered during the second week of infection. Weight changes (weakness) were observed visually only in the group infected with *E. tenella* (positive control), while groups treated with ZNOPs showed a normal weight and were close to the negative group (not infected).

Discussion

Recent research in the poultry industry indicates that adding zinc to the diet plays a vital role in the following: the growth of

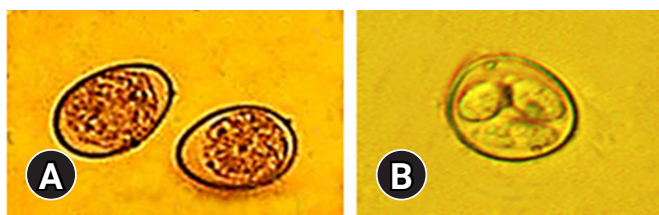


Fig. 1. *Eimeria tenella* oocyst isolated from infected broiler chickens. (A) Unsporulated oocysts. (B) Sporulated oocyst.

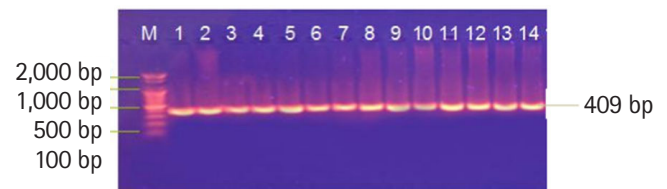


Fig. 2. Polymerase chain reaction product of the ITS-1 gene of *Eimeria tenella* electrophoresis. M, ladder; 1-14, representative samples.

Table 1. Comparison of the effects of ZNOPs on *Eimeria tenella*-infected birds at one week post-infection

Groups	No. of birds	Concentration (mg/kg)	Rate of oocyst (/mL)	Mortality rate (n, %)
G1	20	Water distilled	0	0
G2	20	5×10^4 oocysts	6,080 ^{a)}	12 (10)
G3	20	Amprolium	1,000	2 (1.6)
G4	20	ZNOPs, 20	3,000	9 (7.5)
G5	20	ZNOPs, 40	1,800	4 (3)
G6	20	ZNOPs, 60	500	1 (0.8)
Total	120			

ZNOP, zinc oxide nanoparticle; G1, negative control; G2, untreated and infected group; G3, amprolium-treated-*E. tenella*-infected; G4, 20 mg/kg ZNOP-treated *E. tenella*-infected; G5, 40 mg/kg ZNOP-treated *E. tenella*-infected; G6, 60 mg/kg ZNOP-treated *E. tenella*-infected.

^{a)} $p < 0.05$ indicates a significant difference compared to the untreated and infected group (G2).

Table 2. Comparison of the effects of ZNOPs on *Eimeria tenella*-infected birds at two weeks post-infection

Groups	No. of birds	Concentration (mg/kg)	rate of oocyst (/mL)	Mortality rate (n, %)
G1	20	Water distilled	0	0
G2	20	5 × 10 ⁴ oocysts	9,060 ^{a)}	2 (1.6)
G3	20	Amprolium	250	0
G4	20	ZNOPs, 20	1,600	1 (0.8)
G5	20	ZNOPs, 40	350	0
G6	20	ZNOPs, 60	20	0
Total	120			

ZNOP, zinc oxide nanoparticle; G1, negative control; G2, untreated and infected group; G3, amprolium-treated-*E. tenella*-infected; G4, 20 mg/kg ZNOP-treated *E. tenella*-infected; G5, 40 mg/kg ZNOP-treated *E. tenella*-infected; G6, 60 mg/kg ZNOP-treated *E. tenella*-infected.

^{a)} $p < 0.05$ indicates a significant difference compared to the untreated and infected group (G2).

bones, muscles, feathers, and skin; increasing the bioavailability of zinc; weight gain and improving the efficiency of food conversion. In addition, zinc is necessary for the activity of approximately 250 to 300 enzymes and participates in many enzymatic functions and metabolism in the body [18–20]. Furthermore, it increases IgY production and the total number of lymphocytes and improves immune responses in general [21,22]. It is preferable not to use high concentrations of ZNOPs because Zn is toxic when it exceeds the required level [23].

Dosing of the positive control group with oocysts to *E. tenella* led to the emergence of clinical symptoms represented by the general weakness of the bird, decline in growth, and the abstinence of some infected birds from eating feed and water, as well as severe bloody diarrhea. Bloody diarrhea results from a damaged capillary vessel in the ceca due to the penetration of spores and the development of the parasite during the schizogony phase in the epithelial tissue of the cecum [24–27].

The current study showed that the broilers infected with the oocysts of *E. tenella*, which were subsequently dosed at 20 mg/kg of ZNOPs solution during the first week, did not achieve a positive result in reducing the number of oocysts excreted in the stools. Most of the infected birds suffered from symptoms similar to those of the positive control group, in addition to the occurrence of death in birds of this group. When comparing this group with the anticoccidial group, anti-coccidiosis reduced the number of oocysts excreted by acting as an antibiotic that killed the developmental stages in the life cycle of the parasite.

At 40 mg/kg ZNOPs, there was a slight reduction in the number of oocysts excreted in the stool. Birds of this group showed satisfactory symptoms, but the symptoms were less severe than the positive control group. By contrast, 60 mg/kg revealed a significant decrease in the number of oocysts thrown into the litter and a mortality rate of 0.8%.

The results also showed a decrease in the number of oocysts excreted during the second week in the anticoccidial group and the treated groups with the three ZNOP concentrations, with a decrease in mortality. The 60 mg/kg concentration showed clear superiority and a significant decrease ($p \leq 0.05$) in the number of oocysts excreted in the litter compared to the other groups. All birds in this group recovered during the second week of infection.

The reason for the decrease in the oocysts in the broilers at 60 mg/kg of ZNOPs during the second week can be explained by the ZNOPs weakening the development of this parasite before the inactive oocysts were formed. This finding agrees with Dkhil et al. [28], 2015, regarding the effects of ZNOPs in significantly reducing oocyst excretion in the stool of *Eimeria papillata*-infected mice. These results may be explained by the ability of ZNOPs to weaken the development of the parasite before the inactive oocysts were formed or possibly the ability of ZNOP to cross the gastrointestinal tract and be distributed further in the blood and in the target organs, which in turn leads to increased immune responses and resistance to infection [29].

In addition, research has indicated that ZNOPs act as liver protective agents in rabbits infected with coccidiosis [30]. Recently, ZNOPs and L-carnitine were reported to have anti-neurological bilharziasis effects in infected mice [31]. Furthermore, Bafundo et al. [32] showed that zinc utilization use is diminished by *Eimeria acervulina* infection. General weakness was observed visually in the group infected with *E. tenella* (positive control) while groups treated with the ZNOPs showed a normal weight, similar to the negative group. Hence, the findings obtained from this study present a promising scenario for using ZNOPs as an effective alternative for treating coccidiosis. Zinc oxide nanoparticles play a role in reducing the incidence of coccidiosis, especially at 60 mg/kg.

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