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▣ BRIEF COMMUNICATION

Anticoccidial Activity of Berberine against *Eimeria*-Infected Chickens

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Abstract: Avian coccidiosis has a major economic impact on the poultry industry, it is caused by 7 species of *Eimeria*, and has been primarily controlled using chemotherapeutic agents. Due to the emergence of drug-resistant strains, alternative control strategies are needed. We assessed anticoccidial effects of berberine-based diets in broiler chickens following oral infection with 5 *Eimeria* species (*E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, and *E. praecox*). When 0.2% berberine, a concentration that does not affect weight gain, was added to the diet, the 4 groups infected with *E. acervulina*, *E. tenella*, *E. mitis*, or *E. praecox* showed significant reductions in fecal oocyst shedding ($P<0.05$) compared to their respective infected and untreated controls. In chickens treated 0.5% berberine instead of 0.2% and infected with *E. maxima*, fecal oocyst production was significantly reduced, but body weight deceased, indicating that berberine treatment was not useful for *E. maxima* infection. Taken together, these results illustrate the applicability of berberine for prophylactic use to control most *Eimeria* infections except *E. maxima*. Further studies on the mechanisms underlying the differences in anticoccidial susceptibility to berberine, particularly *E. maxima*, are remained.

Key words: *Eimeria* species, berberine, chicken, anticoccidial effect, different susceptibility

Coccidiosis is an enteric disease caused by infection with one or multiple species of *Eimeria* and is the most costly and prevalent disease in the poultry industry worldwide [1-3]. Several studies have shown that the worldwide prevalence of *Eimeria* infection varies from 10% to 90% in chicken farms [1,4]. The etiologic agents of avian coccidiosis are intracellular protozoan parasites of the genus *Eimeria* that infect different locations of chicken intestinal tracts. The prevalent poultry *Eimeria* species are *E. acervulina*, *E. tenella*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. mitis*, and *E. praecox* [3,5,6]. The infectious parasites invade intestinal epithelial cells, causing a variety of clinical signs, such as necrotic gut lesions, inefficient feed conversion rates, impaired growth rates, and, in severe cases, mortality [2,5,7]. To date, the poultry industry relies mainly on prophylactic in-feed anticoccidial agents to suppress the infection cycle and prevent coccidiosis outbreaks [8]. Although prophylactic drugs have been relatively successful in controlling outbreaks of avian coccidiosis, due to the development of drug-resistant parasites and increasing public health concerns about anticoccidial medication use, new approaches to fight this disease are needed [8-10].

Several natural products have been examined for potential therapeutic and prophylactic effects against *Eimeria*, one of which is berberine [9,11-14]. Berberine is a yellow isoquinoline alkaloid extracted from the stems and roots of various plants, such as *Berberis*, *Hydrastis canadensis*, and *Coptidis rhizoma*, and is used in Chinese medicine to treat gastrointestinal diseases [15,16]. Additionally, berberine has several different bioactivities including antiviral, antibacterial, anticancer, antidiabetic, analgesic, anti-hyperlipidemic, cardio protective, and anti-inflammatory effects [14,15,17-19].

Recently, berberine has received more attention due to its potential antiparasitic effects [20-23]. A limited number of studies have examined the effect of berberine in mice infected with *E. papillata* [12] and chickens infected with only 1 species *E. tenella* [14,24]. In chickens, coccidiosis can be caused by 7 species of *Eimeria* and each *Eimeria* strain invades the intestinal epithelium in a region-specific manner [2,3]. A commercial herbal formula containing a propylene glycol extract of *Allium sativum* and *Thymus serpyllum* was effective at reducing duodenal lesions caused by *E. acervulina* but is not effective in reducing cecal lesions caused by *E. tenella* [25]. Therefore, natural

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products may have different effects on the clinical symptoms caused by different species of *Eimeria*. Therefore, this study was conducted to further investigate anticoccidial activity of berberine-based diets in broiler chickens following oral infection with 5 *Eimeria* species, including *E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, and *E. praecox*.

All animal maintenance and experimental procedures were performed according to Gyeongsang National University Guidelines for the Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University (GNU-191111-C0058). Humane endpoint criteria were set for all animals such that severe moribund animals exhibiting severe weight loss and tremors or unresponsive and unaware of stimuli were euthanized immediately by atlanto-occipital dislocation. All remaining animals were euthanized at specific timepoints post-inoculation.

ROSS308 broiler chicks (Samhwa, Hongseong-gun, Korea) were raised in wire cages in a temperature-controlled environment with unlimited access to anticoccidial/antibiotic-free feed and water. Constant light was provided for the duration of the experiments, and infected and non-infected birds were housed separately in different rooms. The wild-type strains of *E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, and *E. praecox* developed and maintained at the Gyeongsang National University (Jinju, Korea) were used in this study. Feces were collected from *Eimeria*-infected chickens and diluted with phosphate buffered saline (PBS). Fecal samples were then passed through gauze to remove debris and washed 3 times with PBS by centrifugation. Precipitates were suspended in 2.5% potassium dichromate (Daejung Chemicals and Metals Co. Ltd, Siheung, Korea) and incubated at 28°C for 2 days for sporulation. Sporulated oocysts for experimental infections were enumerated using a McMaster counting chamber.

Berberine hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) at the highest available purity (≥97%). Male chickens were inoculated orally with 1×10^4 sporulated *Eimeria* oocysts and fed a standard diet supplemented with powdered berberine beginning 2 days prior to infection and throughout the experimental period. Oocysts for infection were cleaned by flotation on 5.25% sodium hypochlorite and washed 3 times with PBS.

Fecal samples were collected from 6 to 9 days post-infection and homogenized in a blade grinder. Two 30-ml samples were collected from each suspension. Samples were diluted in saturated NaCl, and oocysts were counted microscopically in a McMaster counting chamber. Total oocyst numbers were calculated as oocyst count × dilution factor × (fecal sample volume/counting chamber volume).

Data were analyzed using Student's *t*-test or one-way ANOVA, and Dunnett's multiple comparison tests using InStat statistical software (GraphPad, San Diego, California, USA). Differences were considered significant at $P < 0.05$. Data are expressed as the mean ± standard error (SE).

Chickens were treated with 4 different concentrations of berberine (0.05, 0.1, 0.2, or 0.3%) and infected with *E. tenella*. Fecal oocysts were collected from 6 to 9 days post-infection and oocysts were counted. The 0.2% and 0.3% treated groups showed a significant reduction in oocyst production compared to the untreated/infected group. However, the 0.05% and 0.1% groups had no suppressive effects on oocyst production (Fig. 1A). To determine if berberine treatment affects sporulation rates, the collected fecal oocysts were sporulated at 28°C for 2 days. Sporulation rates of all treatment groups were similar to that of the untreated/infected group ($P > 0.05$) (Fig. 1B).

Furthermore, fecal oocysts were collected from *E. maxima*- or *E. tenella*-infected chickens and then treated with 3 different concentrations of berberine. *E. tenella* samples were treated with berberine at concentrations of 0.1%, 0.2%, and 0.3% (Supplementary Fig. S1A). *E. maxima* samples were treated with berberine at concentrations of 0.1%, 0.2%, and 0.5% (Supplementary Fig. S1B). Sporulation rates of all treatment groups were similar to the untreated groups ($P > 0.05$) (Supplementary Fig. S1).

Because berberine treatment has the potential to cause reductions in weight gain [16,26], we monitored weight gain in chickens after feeding 3 different concentrations of berberine (0.1, 0.2, and 0.5%) for 2 or 6 days (Table 1). Body weight gain was not significantly affected by 0.1% and 0.2% berberine supplementation for 6 days. However, the 0.5% berberine-treated groups showed significant reductions ($P < 0.001$) in weight gain compared to the untreated chickens. The groups fed 0.5% berberine for 2 or 6 days showed weight loss of approximately 31.5% or 45.5%, respectively (Table 1). It is speculated that the weight loss of berberine may be partly mediated by the reduced rate of glucose absorption through delayed carbohydrate digestion and extended digestion time and by alleviating proliferation and differentiation of adipose tissue [16,26]. Therefore, 0.2% berberine treatment was used for subsequent experiments.

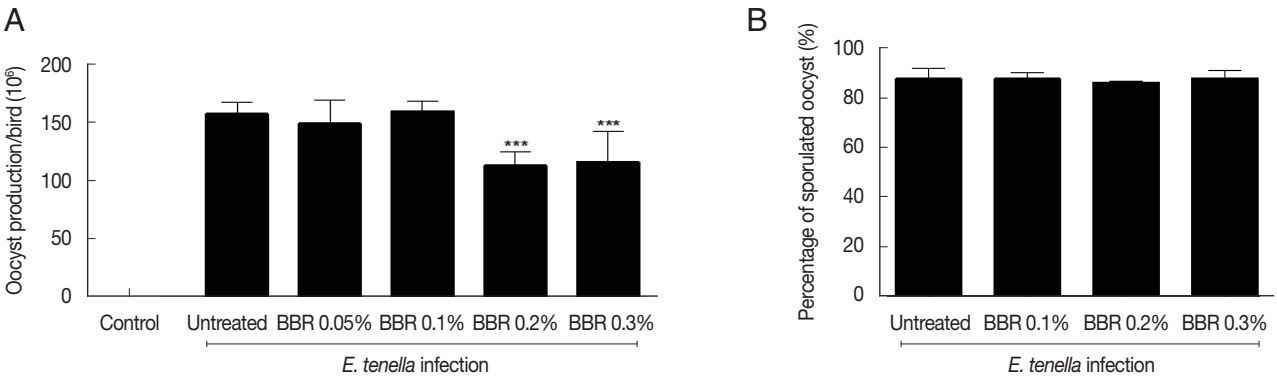

Fig. 1. Effect of berberine on oocyst shedding and sporulation. Twelve-day-old male chickens were inoculated orally with 1×10^4 sporulated oocysts of *E. tenella* and fed a standard diet supplemented with powdered berberine (0.05, 0.1, 0.2, or 0.3%) beginning 2 days prior to infection. (A) Fecal oocyst shedding to berberine treatment (n=48/group). Fecal materials were collected on days 6 to 9 post-infection and oocyst numbers were assessed. Data represent the mean±SE from 4 replicates, with 12 chickens in each replicate and one representative of 2 independent experiments. Control indicates uninfected, untreated healthy controls. ***$P<0.001$ compared to untreated and infected group. BBR, berberine. (B) Fecal materials were incubated at 28°C for 2 days for sporulation. Sporulated oocysts were enumerated using a McMaster counting chamber. Data represent the mean±SE from 3 replicates and one representative of 2 independent experiments.

Table 1. Effect of berberine treatment on body weight gain compared to untreated chickens

Period of berberine treatment	Concentrations of berberine			
	Control	0.1%	0.2%	0.5%
2 days	100±9.7	ND	108.4±12.1**	68.5±10.4***
6 days	100±22.6	103±21.8	98.3±22.2	55±12.2***

The body weight of ROSS308 male chickens (n=30/group) was measured on days 2 and 6 after initiation of feed supplemented with berberine. Body weight gains are expressed as the percentage of weight of the control chickens. Control chickens were fed the standard diet without berberine treatment. ND, not determined. ** $P<0.01$, *** $P<0.001$ compared to control group.

Seven species of *Eimeria* infect the intestinal epithelium in a region-specific manner. Thus, to evaluate the effect of berberine in chickens infected with different species of *Eimeria*, chickens were treated with 0.2% berberine and infected with 5 *Eimeria* species (*E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, or *E. praecox*). Fecal oocyst shedding was significantly reduced ($P<0.001$) in berberine-treated chickens infected with *E. acervulina* (Fig. 2A), *E. tenella* (Fig. 2B), *E. mitis* (Fig. 2C), or *E. praecox* (Fig. 2D) compared to the respective untreated/infected controls. However, in berberine-treated chickens infected with *E. maxima*, fecal oocyst shedding was similar to that of the untreated and infected control; however, treatment with 0.5% berberine significantly reduced fecal oocyst shedding (Fig. 2E).

The etiologic agents of chicken coccidiosis consist of more than 7 species of the genus *Eimeria*, an intracellular protozoan parasite. The various *Eimeria* species infect different areas of the chicken intestinal tract [5,6], thus *Eimeria* species may exhibit differences in susceptibility to alternative products, such as herbal products, essential oils, organic minerals, and probiotics [25]. One of the alternative products is berberine that has potential anticoccidial properties as demonstrated within a limited range, namely in *E. tenella* infected-chickens [14,24]. Therefore, this study investigated whether berberine differentially affects chickens infected with various species of *Eimeria*, *E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, and *E. praecox*. Furthermore, we monitored whether berberine affects sporulation with berberine-treated chicken stool samples or berberine-supplemented stool samples.

Berberine intercalates into viral DNA and inhibits DNA synthesis, protein biosynthesis, and enzyme activity, resulting in reduced virus replication [10,18]. Berberine also exhibits direct antimicrobial activity on Gram-negative bacteria, Gram-positive bacteria, filamentous fungi, and yeast [27], and dose-dependent inhibition of promastigote cell growth in the parasite *Leishmania* [28]. Furthermore, the sporulation rate of fecal oo-

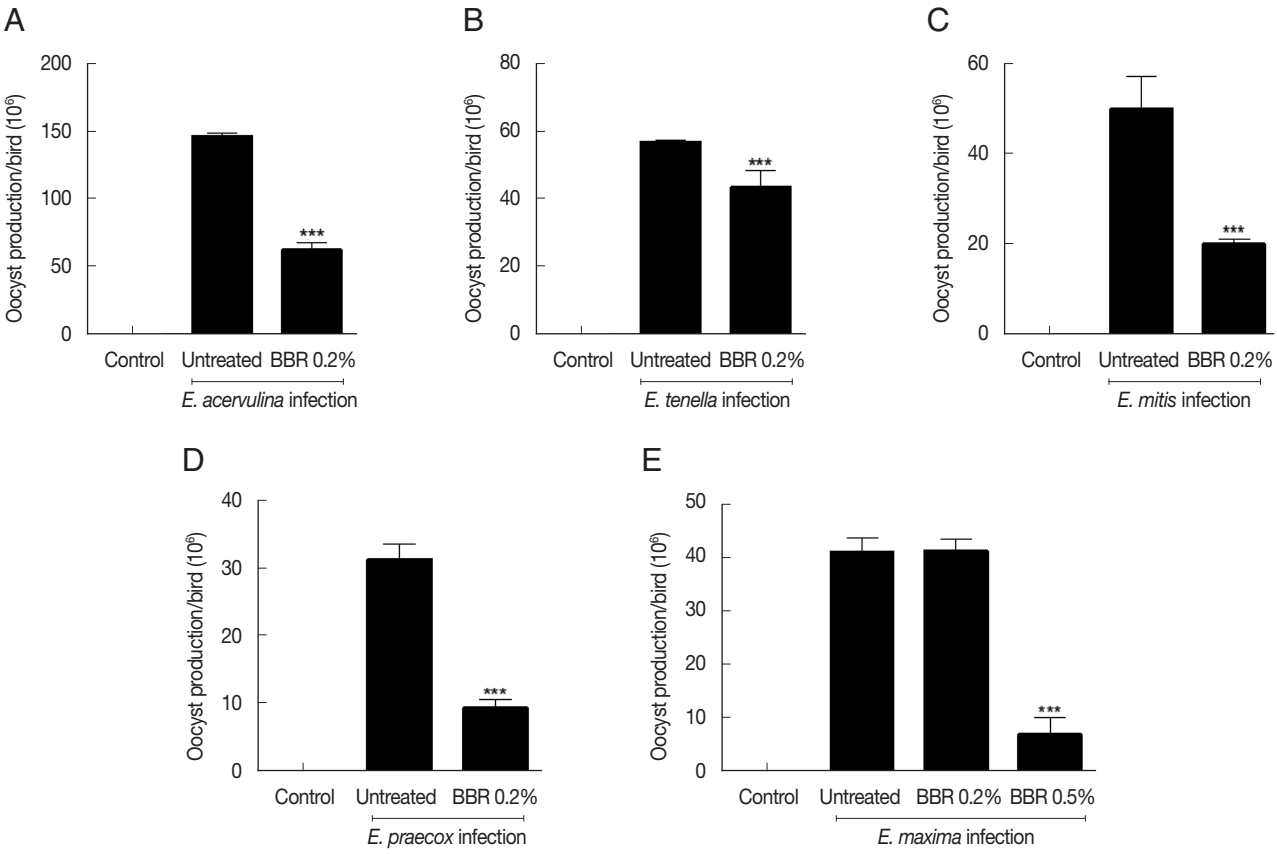

Fig. 2. Fecal oocyst shedding following berberine-based diets in chickens infected with *Eimeria* species. One week-old male chickens (n=28/group) were inoculated orally with 1×10^4 sporulated *E. acervulina*, *E. tenella*, *E. mitis*, *E. praecox*, or *E. maxima* oocysts and fed a standard diet supplemented with powdered berberine (0.2 or 0.5%) beginning 2 days prior to infection. Fecal oocysts were collected from 6 to 9 days post-infection and oocyst numbers were assessed. Data represent the mean±SE from 2 replicates, with 14 chickens in each replicate and one representative of 2 independent experiments. ***$P<0.001$ compared to untreated and infected group. BBR, berberine.

cysts is a critical factor affecting the epidemiology of *Eimeria* infection in a chicken flock because chickens can only be infected via ingestion of sporulated oocysts [6].

In our study, berberine did not inhibit oocyst sporulation rates in experiments with fecal samples obtained after berberine treatment and *Eimeria* infection (Fig. 1B) or in fecal samples supplemented with berberine (Supplementary Fig. S1). The oocyst sporulation rate was not inhibited in *E. maxima* samples treated with 0.5% berberine (Supplementary Fig. S1B). Therefore, oocysts excreted by berberine-treated birds did not have impaired sporulation and berberine at the concentrations used in the in vitro study did not affect the sporulation process of unsporulated oocysts. However, several natural extracts inhibit the formation of oocyst sporulation under in vitro conditions [29,30]. Artemisinin from *Artemisia annua* extracts inhibit the sporulation rate of mixed oocysts of *E. acervulina*, *E. necatrix*, and *E. tenella*; many of the sporulated oocysts were wrinkled or included abnormal sporocysts [29]. Similarly, *Aloe debrana* and *Aloe pulcherrima* leaf gels inhibited the sporulation rate of mixed oocysts of *E. acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella* [30].

Although berberine did not affect sporulation rates under in vitro conditions, berberine treatment significantly reduced fecal oocyst shedding in chickens infected with different *Eimeria* species (Fig. 2), indicating that berberine had anticoccidial activity only in vivo. Interestingly, previous studies of *E. tenella* showed that berberine-treated, *E. tenella*-infected chickens significantly reduced fecal oocyst shedding [14,24]. It is speculated that anticoccidial activity of berberine may be partly mediated by generation of a redox imbalance and depolarization of mitochondrial membrane [28], by impairment of intracellular development and multiplication of *Eimeria* [12] or by inhibi-

tion of telomerase activity [21]. Further analysis of the mechanisms or metabolic pathways would be necessary to know the precise anticoccidial effects of berberine.

Chickens can be infected with 7 species of *Eimeria* parasites that invade the intestinal epithelium in a region-specific manner [2,3]. In our study, berberine had differing anticoccidial activities depending on the *Eimeria* specie. Fecal oocyst shedding was significantly reduced in chickens treated with 0.2% berberine that were infected with *E. acervulina*, *E. tenella*, *E. mitis*, or *E. praecox*; whereas *E. maxima* oocyst shedding was only significantly reduced in chickens treated with 0.5% berberine. Interestingly, a commercially available herbal formula was effective in reducing duodenal lesions caused by *E. acervulian*, but not cecal lesions caused by *E. tenella* [25]. Therefore, it is possible that natural products have different efficacies for the various *Eimeria* species.

In conclusion, Berberine-based diets significantly inhibited fecal oocyst shedding in chickens infected with various *Eimeria* species. *E. maxima* unlike the other 4 species was more resistant to berberine treatment, suggesting that natural products may have different anticoccidial activities on the various *Eimeria* species. Taken together, our data illustrate the applicability of berberine for prophylactic use to control most eimeriosis in conventional and organic chicken industries. Further research remains on the mechanisms leading to differences in anticoccidial susceptibility to berberine, especially *E. maxima*.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest related to this work.

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