EFFECT OF BERBERINE ON OXIDATIVE STRESS CAUSED BY *EIMERIA TENELLA* IN CHICKEN

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After coccidia infect chickens, the cecum of chickens produces oxidative stress, which is aggravated by the propagation of coccidia. The disruption of the balance of oxidant/antioxidant system causes chickens to suffer from diseases. Berberine has been shown to prevent cecum epithelial barrier damage from coccidia by suppressing the reproduction of coccidia and restore the normal functions of chicks' cecum. In the current study, effect of berberine (BBR) on the oxidative damage caused by coccidiosis was investigated. Forty-eight three-week-old chicks treated in different ways. All livers, serum and caeca were collected and the degree of oxidative stress and antioxidant enzyme activity were evaluated. The results showed that the increase in contents of free radicals in serum and cecum caused by *Eimeria tenella* were eliminated after daily treatment with berberine. Furthermore, level of lipid peroxidation was decreased, and superoxide dismutase activities were increased in the experimental groups treated by berberine compared to the infected birds. RT-PCR assay showed that mRNA level of superoxide dismutase was not affected by berberine. Taken together, berberine alleviates oxidative stress by increasing the activity of antioxidant enzymes such as superoxide dismutase. However, it is independent of mRNA expression of superoxide dismutase.

Key words: Antioxidant, Berberine, Chicken, Coccidiosis, Oxidative stress

Coccidiosis is a common parasitic disease caused by several species of *Eimeria* protozoa. Among the nine identified subspecies, *Eimeria tenella* is considered to

be one of the most pathogenic (Guo *et al.*, 2013). *E. tenella* is parasitic on the cecum, invading the intestinal cecum epithelium and destroying the integrity of intestinal mucosa

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(Vervelde *et al.*, 1993; Iacob and Duma, 2009). Infected chickens show typical clinical symptoms on day 4–5 post-infection, such as reduced weight gain, ruffled feathers and bloody diarrhea (Long *et al.*, 1976; Fang *et al.*, 2016). In severe cases, coccidiosis kills chicks.

Under normal physiological conditions, reactive oxygen species (ROS) produced by metabolism are maintained at low concentration (Masood *et al.*, 2013). Infecting coccidia destroy the antioxidant defense system composed of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and other enzymes, weakens the ability of removing ROS, and leads to ROS accumulation in the body. Excessive ROS acts on unsaturated fatty acids, generating toxic lipid peroxidation such as malondialdehyde, resulting in cell swelling and pathological damage to the body (Radi and Matkovics, 1988). These symptoms are attributed to oxidative damage caused by coccidia.

Most anticoccidial drugs, such as monensin and amprolium, inhibit the normal growth, metabolism and reproduction of coccidia. However, the widespread use of these drugs has led to the emergence of drug resistance and residues in poultry (Chapman, 1997; Tajick and Shohreh, 2006), and it's urgent to find new drugs with less drug residues and low drug resistance. Herbal plants and their byproducts have many advantages, such as few drug residues and side effects, less drug resistance and low prices. They are considered to have good anti-coccidial prospects (Abbas *et al.*, 2006). Berberine (BBR), a traditional

Chinese medicine, has also been proved to resist coccidiosis and protect the intestine of chicks (Fang *et al.*, 2016). In this study, effect of BBR on the oxidative damage induced by coccidia was investigated to clarify its protective mechanism against coccidia in chickens.

MATERIALS AND METHODS

Materials and animal treatments: Berberine chloride (reagent grade, >90% pure), amprolium and vitamin C (VC) were obtained from Bio Basic Inc (Shanghai, China). Forty-eight three-week-old chicks were purchased from Shanghai Pacific poultry breeders. They were divided randomly into six equal groups with four replicates per group (Table 1). Group 0 was used as a negative control indicating the uninfected without drug treatment. Group 1~2 were, used as positive controls, infected with low/high dosage of *E. tenella* oocysts (5×10^4 or 5×10^5 oocysts each chick) without drug treatment (Bahadoran *et al.*, 2014). Group 3~5 were infected with high dosage of *E. tenella* oocysts (5×10^5 oocysts each chick) and respectively treated with berberine (300mg/kg/day), amprolium (as a drug adding control, 120 mg/kg/day) and VC (500 mg/kg/day). Chicks were weighed every day and treated with berberine, amprolium or VC according to their weight. To avoid contamination, chickens of these groups were housed in six separate rooms. All of them had free access to water and feed and constant light was provided throughout experiment. All animal procedures in these experiments were approved by the Ethics Committee of the University of Donghua, Shanghai, China.

Table 1. Grouping and drug treatments

Group	Dosage of <i>E. tenella</i> oocysts	Treatments
0	0	No
1	5×10 ⁴ (low-dosage)	No
2	5×10 ⁵ (high-dosage)	No
3	5×10 ⁵ (high-dosage)	Berberine (300mg/kg/day)
4	5×10 ⁵ (high-dosage)	Amprolium (120 mg/kg/day)
5	5×10 ⁵ (high-dosage)	Vitamin C (500 mg/kg/day)

Weight gain and survival rate: Body weights were recorded before infection and on day 8 post-infection. The numbers of alive chickens were recorded on day 8 post-infection. Relative weight gain rate and mortality of each group were calculated as follows:

Rate of relative weight gain (%) = average weight gain of each group / average weight gain of group 0 × 100; Survival rate (%) = the number of alive chickens / total number of chickens × 100.

Oocysts production counts: Excreted oocysts were counted at day 5 post-infection according to previously described methods (Long *et al.*, 1976; Haug *et al.*, 2006) and referred to as oocytes per gram (OPG) in faeces.

Lesion score: At day 8 of post-infection, each chicken was euthanized by ether inhalation and cervical dislocation. Livers, serum and caeca were collected. Lesion scores were evaluated according to Johnson and Reid (Johnson and Reid, 1970). A score of 0 means no lesions, 1 means slight lesions, 2 means moderate lesions, 3 means severe lesions and 4 means very severe lesions or death owing to coccidiosis.

Anticoccidial index: Anticoccidial index (ACI), as an indicator of drug efficacy, was

calculated as follows (Fei *et al.*, 2013): ACI = (Survival rate + rate of relative weight gain) – (oocyst value + lesion score). Oocyst values were calculated as follows: (OPG output of each group/OPG output of group 2) × 100. An ACI <120 indicated inefficacy, from 120 to 159 indicated limited effect, from 160 to 179 indicated moderate effect and >180 indicated excellent effect (Mcmanus *et al.*, 1968).

Superoxide assay: The production of superoxide was detected according to the procedure previously described (Wang *et al.*, 2006; Wang *et al.*, 2010). Rubbed liver tissue or plasma samples were homogenized in 1 mL of 50 mM (pH 7.8) phosphate buffer followed by centrifugation at 10,000 g for 10 min. The supernatant (0.5 mL) was then collected and added 1 mL of 1 mM hydroxylamine hydrochloride. After 1 h reaction at 25°C, the mixture was added 1 mL of 1% α-naphthylamide and 1 mL of 17 mM p-aminophenylsulfonic acid. After reaction for 30 min, n-butanol (3 mL) was added into the above reaction mixture, and centrifuged at 6,000 g for 10 min. The specific absorption was measured at 530 nm. The content of superoxide was calculated with sodium nitrite as standard solution.

Lipid peroxidation evaluation: Since methane dicarboxylic aldehyde (MDA) is the end-product of lipid peroxidation, the production of MDA was measured to evaluate lipid peroxidation. Cells were centrifuged and collected. After washing, the wet weight of cell pellets was measured. Then, 2 mL of TBA–TCA reagent [0.25 M HCl, TBA 0.375% (w/v), trichloroacetic acid 15% (w/v)] was added to each pellet and the mixture was incubated in a boiling water bath for 30 min. After cooling, the suspension was centrifuged at 8,000 g for 10 min and the supernatant was separated. Then, the absorbance was measured at 450 nm, 532 nm and 600 nm. The concentration of MDA was calculated as follows (Dhindsa *et al.*, 1981):

$$\text{MDA } (\mu\text{mol/g}) = [6.452 \times (A_{532} - A_{600}) - 0.559 \times A_{450}] / \text{pellet weight.}$$

Superoxide dismutase (SOD) activity: SOD activity was assessed according to Babitha and Prakash (2002). The reaction mixture included 50 mM phosphate buffer (pH 7.8), 1.3 mM methionine, 63 mM nitroblue tetrazolium (NBT), 1.3 μM riboflavin and plasma samples or liver tissue protein extracts. After exposure to a cool white fluorescent light with an intensity of 4,000 lux for 15 min, the absorbance

of the mixture was measured at 560 nm. The reaction mixture without enzyme was taken as an internal control. From the resultant diagram, the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and regarded as an enzyme unit.

RT-PCR: The caeca samples (approximately 0.5 g) were cleaned and shredded. Then Total RNA was isolated using TRIzol reagent (TaKaRa, Tokyo, Japan). After extraction, the purified RNA was reverse-transcribed to cDNA using Reverse Transcription kit (TaKaRa, Tokyo, Japan). The cDNA and 2 $\times$ Hieff™ PCR Master Mix (YEASEN, Shanghai, China) were used for RT-PCR. RT-PCR was performed to evaluate changes of two key antioxidant genes (SOD1 and SOD2) expression. The primer sequences were shown in Table 2. Amplification and detection were carried out using equivalent amounts of the same cDNA samples used for PCR. Levels of individual transcripts were normalized by those of β -actin analyzed by PCR. Each RT-PCR experiment was conducted in triplicate.

Statistical analysis: The data were expressed as the mean $\pm$ standard deviation. Statistical

Table 2. Oligonucleotides used in this work

Primer	Sequence (5'-3')	Description
SOD1_F	TTGGAGACAACACAAATGGG	<i>SOD1</i> mRNA forward primer
SOD1_R	AGGTACAACGGTTAGCACTT	<i>SOD1</i> mRNA reverse primer
SOD2_F	ATCAGTTGGTGTTC AAGGAT	<i>SOD2</i> mRNA forward primer
SOD2_R	ATTCACCTGTATGCCACAGA	<i>SOD2</i> mRNA reverse primer
ACTB_F	CTCTTCCAGCCATCTTTCTT	<i>ACTB</i> mRNA forward primer
ACTB_R	GATTCATCGTACTCCTGCTT	<i>ACTB</i> mRNA reverse primer

analysis was performed using the Student's t-test. A P-value less than 0.05 was considered as statistically significant.

RESULTS

Anticoccidial effect of berberine: The chemical structure of berberine was presented in Fig. 1. The effect of berberine on bloody diarrhoea, survival rate, OPG and lesion score were tested and observed, and ACI was calculated (Table 3). From the data, the severity of bloody diarrhoea and cecum

lesions were positively correlated with dosage of *E. tenella* oocysts. The treatment with berberine was similar to the drug adding control and significantly alleviated these symptoms. The survival rate of group treated with berberine (group 3) was higher than that of positive controls (group 1-2). Berberine obviously reduced the amount of OPG to 4.21×10^6 oocytes per gram. Similar as amprolium, berberine exhibited excellent efficacy against *E. tenella*.

Table 3. The effect of berberine on chickens' performance*

Treatments	Bloody diarrhoea [#]	Survival rate (%)	OPG (*10 ⁶)	Lesion score	ACI
Uninfected + No treatment	-	100	0	0	200
Low dosage infected + No treatment	++	67±3.1	10.1±0.6	1.8±0.3	101.8
High dosage infected + No treatment	+++	50±5.7	14.2±1.7	3.1±0.3	68.0
High dosage infected + BBR	+	83±2.3	4.2±0.5	1.1±0.2	153.1
High dosage infected + Amprolium	-	100	0.1±0.03	0.6±0.1	195.6

*Data are presented as mean or mean±S.D.

[#]Bloody diarrhoea (- - +++ represents no, few, many and large amounts of blood)

Fig. 1. Chemical structure of berberine (BBR)

Berberine reduces coccidiosis-induced oxidative stress: Changes in superoxide contents were shown in Fig. 2. Superoxide was significantly increased in the serum, liver and cecum in the chickens exposed to high dosage of *E. tenella* oocysts (group 2), compared to the control group (group 0). Both berberine and amprolium effectively reduced the production of superoxide. Ascorbic acid (VC), is a physiologically multifunctional compound required for various processes in animals (Han *et al.*, 2018) and an antioxidant that protects tissues from ROS (Harrison *et al.*, 2012; Liu *et al.*, 2017). As seen in Fig. 2B and 2C, VC effectively reduced excess superoxide induced by coccidiosis in the liver and cecum. Compared with the positive control (group 2), there were no significant

changes of superoxide radical levels in serum of group 3-5. The above data indicated that the treatment of berberine helped eliminate ROS produced by coccidiosis in tissues, rather than in the blood.

Effect of berberine on lipid peroxidation induced by *E. tenella* in chickens: Excessive ROS can induce lipid peroxidation and cause membrane dysfunction and damage of membrane bound enzymes, further producing reactive oxygen species. MDA, one of the main products of lipid peroxidation, is a marker of radical-induced damage (Halliwell and Chirico, 1993). Contents of liver and cecum MDA were significantly increased in chickens infected by *E. tenella*, while berberine obviously inhibited their increase.

[Asterisks (*) indicate statistically significant differences between positive controls (group 1-2) and negative control (group 0) ($P < 0.05$). Hash marks (#) indicate statistically significant differences between infected/treated groups (group 3-5) and positive control (group 2) ($P < 0.05$). Error bars represent mean $\pm$ S.D.]

Fig. 2. Effect of berberine on superoxide radical levels in serum (A), liver (B) and cecum (C) of chickens

[Asterisks (*) indicate statistically significant differences between positive controls (group 1-2) and the negative control (group 0) ($P < 0.05$). Hash marks (#) indicate statistically significant differences between infected/treated groups (group 3-5) and positive control (group 2) ($P < 0.05$). Error bars represent mean $\pm$ S.D.]

Fig. 3. Effect of berberine on MDA contents in liver (A) and cecum (B) of chickens

Effect of berberine on SOD activity in chickens: In order to determine how berberine could inhibit the oxidative stress caused by *E.tenella* in chickens, the activity of SOD, a prominent antioxidant enzyme, was analyzed. As seen in Fig. 4, the SOD activities of positive control (group 2) were remarkably lower than those of negative control (group 0) in the serum and liver. The activities of SOD were significantly returned to normal level after treated by berberine. SOD activities also had a remarkable increase in the liver of chickens treated by ammoniapurin or VC.

Effect of berberine on mRNA levels of SOD: In animals, SOD has two isozymes, Cu/Zn SOD (SOD1) in cytoplasm and Mn SOD (SOD2) in mitochondria respectively. Their function is to remove O_2^- produced by the metabolism to prevent oxidation. Effect of berberine on expression of *SOD1* and *SOD2* was verified by RT-PCR (Fig. 5). Both mRNA levels of *SOD1* and *SOD2* were increased in high-dosage infected chickens compared to uninfected chickens. However, mRNA levels of SOD showed no significant increase in those infected chickens after treated by berberine,

[Asterisks (*) indicate statistically significant differences between positive controls (group 1-2) and the negative control (group 1) ($P < 0.05$). Hash marks (#) indicate statistically significant differences between infected/treated groups (group 3-5) and positive control (group 2) ($P < 0.05$). Error bars represent mean $\pm$ S.D.]

Fig. 4. Effect of berberine on SOD activities in serum (A) and liver (B) of chickens

[Gene expression changes of SOD related genes, *SOD1* and *SOD2* were measured. (A) Reverse transcription–polymerase chain reaction analyses of *SOD1* and *SOD2* expression. (B) Data (mean values of triplicate determinations with upper 95% confidence intervals) are representative of at least two independent experiments. Asterisks (*) indicate statistically significant differences between infected/treated groups (group 1-5) and the negative control (group 0) ($P<0.05$)]

Fig. 5. Effect of berberine on SOD related gene expression in chickens

ammoniaporin or VC. It is suggested that berberine did not increase SOD activity by increasing its mRNA expression.

DISCUSSION

Berberine is a traditional Chinese medicine and is effective on intestinal diseases. It has been shown to protect the intestinal barrier (Gu *et al.*, 2011; Li *et al.*, 2015) and is widely used in the treatment of bacterial dysentery and gastroenteritis (Sriwilaijareon *et al.*, 2002). Previous study has proved that berberine significantly relieves cecum lesions, reduces oocyst production in *E. tenella* infected chickens and alleviates the inflammation reaction (Dkhil *et al.*, 2015; Fang *et al.*, 2016), which is confirmed by our present result. *E. tenella* lives in the cecum of chicks and destroys

the intestinal epithelium tissue (Iacob and Duma, 2009). The blood seeps into the intestine, leading to bloody diarrhea (Inagaki-Ohara *et al.*, 2006). Berberine blocks the adherence of various pathogenic microorganisms to epithelial cells and inhibits their growth (Sun *et al.*, 1988; Pei *et al.*, 2019). This may be the reason why berberine can relieve cecum lesions and bloody diarrhoea and reduce oocyst production (Table 3). Therefore, berberine plays a protective role against coccidiosis infection in chickens.

Berberine was proved to be resistant to increased ROS and lipid oxidation (De Oliveira *et al.*, 2019). From our data, the increased level of superoxide radical and MDA in infected chickens indicates that oxidative stress is an

important aspect of the pathogenicity of coccidiosis (Fig. 2 and 3). Berberine reduced the excess superoxide radical by two-thirds and one-half respectively in the liver and cecum and decreased the excess of MDA to a fifth in the cecum. The effect of berberine was not weaker than VC, which has been shown to protect cells from oxidative stress and reduce inflammation (Han *et al.*, 2018). These data collectively suggested that berberine could provide antioxidative protection against oxidative stress induced by *E. tenella*. It is obvious that the antioxidation and antilipid peroxidation properties of berberine well explain its therapeutic effect on coccidiosis.

SOD, CAT, GPx and other enzymes form an antioxidant system against oxidation by scavenging ROS. In our current study, it showed that the antioxidation of berberine was closely related to the recovery of inhibited SOD activity (Fig. 4). Pirmoradi *et al.* (2019) observed the similar results that berberine alleviated the lowered activities of SOD in ischemic rats. However, berberine did not significantly enhance the mRNA expression of SOD (Fig. 5), which indicates that the increase of SOD activity is not due to the change of SOD content.

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- Cu/Zn SOD and Mn SOD are metal enzymes and their activities could be increased by increasing the concentration of metal ions, which alleviates oxidative stress (Bun *et al.*, 2011). The increase in SOD activity by berberine may be related to the metal levels. It was reported that berberine increases the mRNA level by prolonging the half-life of mRNA (Yuan *et al.*, 2017). Berberine may regulate metal concentrations by promoting the expression of metal-associated genes. The mechanism needs further exploration in the future.
- Collectively, these data indicate that berberine treatment of chicks infected with *E.tenella* could reduce oxidative stress through increasing activities of antioxidant enzymes such as SOD to prevent and control chicken coccidiosis.
- ## ACKNOWLEDGEMENTS
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