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Apoptosis, mitochondrion membrane potential, Qing Chang oral liquid, second generation merozoite, ultra-microstructure.



Influence of Qingchang Oral Liquid on Second Generation Merozoite of the Chicken *Eimeria tenella*

ABSTRACT

To determine the effect of Qingchang Oral Liquid (QOL) on second generation merozoite of chicken *E. tenella*, healthy Roman pink chickens were randomly divided into model group and QOL group (drug group), and both groups of chicks were inoculated with 5×10^4 sporulated oocysts by oral gavage. Then, the drug group was given QOL at a dose of 2.4 ml/kg, and the model group was given the same volume of normal saline. After 120 hours of inoculation, both groups of experimental chickens were killed at the same time, their caecum tissues were collected, the second generation merozoite were separated, the ultra-microstructure of the second generation merozoite were observed with transmission electron microscope and the mitochondrial membrane potential and apoptosis proportion of the second generation merozoite were analyzed with flow cytometer. The current results suggested that QOL could cause swelling and vacuoles of mitochondria, swelling of endoplasmic reticulum and damage of outer membrane in the second generation merozoite of *E. tenella*. Compared with the model group, the drug group could increase the total apoptosis rate of the second generation merozoite ($p < 0.01$), and reduce the depolarization rate of mitochondrial membrane potential ($p < 0.01$). Conclusion: QOL can directly affect the outer membrane and mitochondria of the second generation merozoite of *E. tenella*, reduce the depolarization rate of mitochondrial membrane potential of the second generation merozoite and increase the apoptosis rate of the second generation merozoite.

INTRODUCTION

Chicken coccidiosis is a protozoan disease which is parasitized by one or several Eimeriida in the epithelial cells of chicken intestine, which is characterized by high mortality and hemorrhagic enteritis in chicks (Bachaya *et al.*, 2012; Kipper *et al.*, 2013). At the present moment, there are seven species of coccidia in chicken around the world, among which *E. tenella* is the most harmful (Shirley *et al.*, 2004; Rathinam *et al.*, 2014; Ritzi *et al.*, 2014; Yin *et al.*, 2015). Chicken coccidiosis is widely distributed and prevalent, which mainly occurs in the high temperature and high humidity seasons. The disease occurs in different degrees all over China. Chickens with different age and different breeds are susceptible, among which chickens aged 10-50 days are most susceptible, with the 20%-80% incidence rate, the 20%-30% mortality rate and even up to 80% in severe cases. After infected with coccidia, the chickens have some clinical manifestations, such as bad mental outlook, bloody stool, visible mucosal pallor, disheveling feathers, clustering and so on. Simultaneously, it can reduce the growth rate of chickens, reduce feed reward, and even cause death in serious cases (Blake & Tomley, 2014; Chaudhari *et al.*, 2020). It is reported that



the annual economic loss caused by coccidiosis in the world is as high as 10.36 billion pounds (Blake *et al.*, 2020). The annual economic loss in China is as high as more than 5 billion, and the drug expenditure alone is as high as 1.56-1.95 billion pounds, causing huge economic losses to the poultry industry.

Qing Chang oral liquid (QOL) is a Chinese veterinary drug compound with good therapeutic effect on chicken coccidiosis based on the theory of Chinese veterinary medicine and the principle of clinical syndrome differentiation. Chicken coccidiosis belongs to the category of damp-heat pouring downward and worm accumulation in the clinical practice of Chinese veterinary medicine, which is suitable to eliminate insect accumulation, clear away heat and stanch bleeding. The compound is composed of *Artemisiae Annuae Herba* and *Dichroae Radix* for clearing heat and interrupting malaria, *Agrimonia pilosa* for converging hemostasis and truncating dysentery and *Sanguisorbae Radix* for cooling blood and hemostasis, which has the efficacy of heat-clarifying and blood-cooling, removing insects and stopping dysentery (Cacho *et al.*, 2010; Zhang *et al.*, 2012; Jain *et al.*, 2015; Jin *et al.*, 2014). It has the characteristics of obvious curative effect, little toxic side effects, and lower drug resistance. In the early stage, our team infected healthy chickens with different doses of *E. tenella* sporulated oocysts and completed the replication and evaluation of chicken coccidia model (Cacho *et al.*, 2010). At the same time, in this study, the clinical dose of QOL (2.4ml / kg•BW) was chosen based on the dose of chicken coccidia powder in the People's Republic of China Veterinary Pharmacopoeia (2015 edition), a similar product which has used in the poultry industry, and the dose screening test was presented in our previous papers (Yan *et al.*, 2019; Yan *et al.*, 2021). In addition, clinical researches show that QOL can significantly reduce the measure of bloody stool in infected chickens, and bloody stool is mainly caused by invasion and damage of intestinal epithelial cells and intestinal mucosal tissue by merozoite (Li *et al.*, 2016). To further determine the mechanism of QOL against coccidiosis, our team artificially reproduced the chicken model of chicken coccidiosis, treated infected chickens with the compound, separated the second generation merozoite as the research objects, observed their ultra-microstructure with transmission electron microscope and detected their mitochondrial membrane potential and apoptosis proportion with flow cytometer.

MATERIALS AND METHODS

Trial drugs and preparation

After all the Chinese medicinal materials crushing, they were mixed at a ratio of *Artemisiae Annuae Herba*, *Dichroae Radix*, *Agrimonia pilosa* and *Sanguisorbae Radix*=5:2:2:1, and the mixtures were performed with water extraction according to the solid-liquid ratio of 1:10. The water extracts were implemented three times for 1 h each, combined each extract and concentrated to 1 ml, in which it contains 1 g of the crude drug in whole, and preparing for the next study.

Trial animals and eggs

Eighty 1-day-old healthy Roman pink chicks fed in a strictly disinfected environment until 16-day of age, were provided feed and water *ad libitum* during the entire experimental period. The *E. tenella* eggs, was provided by *ChongQing Academy of Animal Science*, separated from the cecum contents of natural infection chicks.

Effect of QOL on the ultra-microstructure of second generation merozoite of *E. tenella*

Packet treatment

Forty 16-day-old healthy Roman pink chicks were randomly divided into model group and drug group with 20 chicks in each group. Both groups were inoculated with 5×10^4 sporulated oocysts by oral gavage. The drug group was given QOL at a dose of 2.4mL/kg by gavage 108h after inoculation, and the model group was given the same volume of normal saline by gavage. And both groups of experimental chickens were killed at the same time and their caecum tissues were collected after 120 hours of inoculation.

Fabrication and observation of ultrathin section

1 mm³ caecum tissue was cut and fixed with 2.5% glutaraldehyde at 4 °C overnight. The tissue was rinsed 3 times with 0.1 M PBS buffer, fixed with 1% osmic acid for 2 h, rinsed 3 times with 0.1 M PBS buffer, treated with 30%, 50%, 70%, 80% and 90% acetone respectively once, and treated with 100% acetone for 3 times. The samples were soaked, embedded, cut into ultrathin sections of 60nm after trimming, stained with lead citrate and uranium dioxide acetate, and then observed and recorded by transmission electron microscope (JEM-1400, JEOL).



Effect of QOL on apoptosis and mitochondrial membrane potential of the second generation merozoite of *E. tenella*

Experiment grouping and treatment

Forty 16-day-old healthy Roman pink chicks were randomly divided into model group and drug group with 20 chicks in each group. Both groups of chicks were inoculated with 5×10^4 sporulated oocysts by oral gavage. The drug group was given QOL at a dose of 2.4ml/kg by gavage and the model group was given the same volume of normal saline by gavage, once a day. Both groups of experimental chickens were killed at the same time.

Release and separation of the second generation merozoite of coccidian

The cecum was taken according to the literature method (Zhou *et al.*, 2020) and the cecum contents were washed with PBS solution containing 2000U penicillin streptomycin double antibody. The small pieces of cecum were placed in a 1L beaker, and 10 times of the volume of hyaluronidase digestion solution was added. The cecum was placed in a 37 °C water bath shaker for shaking digestion lasting 1h, filtered, centrifuged, the supernatant was discarded, added FACS lysing solution according to the ratio of 1:3, fixed at 4 °C, centrifuged and the supernatant was discarded. And the precipitate was added with appropriate volume of PBS to make suspension and then 100% Percollstoste was added to make 30% Percollmerozoite suspension. 30% Percollmerozoite suspension was slowly added to 50% Percoll liquid surface, centrifuged, washed with sterilized PBS, centrifuged and repeat twice to collect the precipitate. The precipitate was the second generation merozoite and adjusted to the concentration to 106 pieces/ml with PBS solution.

Determination of mitochondrial membrane potential in second generation merozoite

In flow cytometric tube, 100ul cell suspension and 500ul JC-1 fluorescent probe (BTMMitoScreen (JC-1)(BD)) was added, whirled, incubated at 37 °C for 15 min in the dark, 1 ml PBS solution was added, centrifuged at 350g for 5 min, the supernatant was discarded and repeated once; Then 400ul PBS solution was added and whirled, detected by CytoFLEX flow cytometer and analyzed by Kaluza 2.1 software.

Determination of apoptosis in the second generation merozoite

100ul 10^6 pieces per ml merozoite suspension was centrifuged and the supernatant was discarded. 200ul freshly prepared buffer was added to resuspend the cells and 5ul Annexin V-FITC fluorochrome (Invitrogen) was added to dye for 10 min in the dark at room temperature. 10ul PI was added to dye for 5 min at the same condition. 400ul binding buffer was added to resuspend the cells and the cells were immediately detected on the computer. The data was detected by CytoFLEX flow cytometer and analyzed by Kaluza 2.1 software.

Data analysis

SPSS20.0 was used for statistical analysis of the trial data and analyzed with the independent sample test method, Graphpad prism mapping. Value ($p < 0.01$) was considered extremely significant difference and value ($p < 0.05$) was considered significant difference.

RESULTS

Effect of QOL on the ultra-microstructure of second generation merozoite of *E. tenella*

As showed in Figure 1 A and B, the membrane structure of the second generation mitozoite was normal, and the subcellular structures such as mitochondria, endoplasmic reticulum, rod-like bodies and nuclei were normal without abnormality in the model group. In the drug group, the second generation mitozoite showed swelling and vacuoles of mitochondria, swelling of endoplasmic reticulum (Figure 1 C) and damage and incompleteness of outer membrane (Figure 1 D).

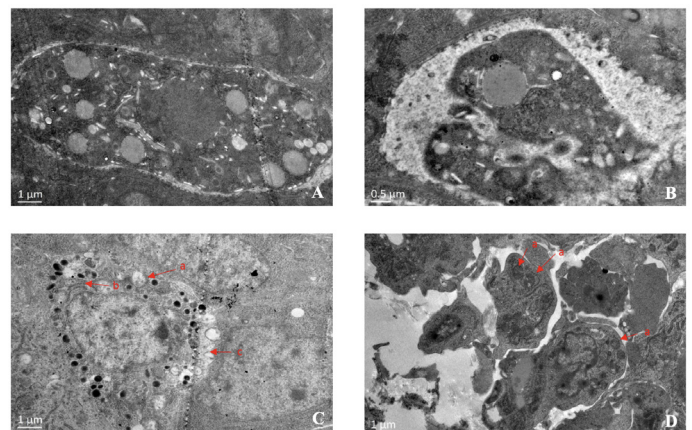


Figure 1 – Effect of Qing Chang oral liquid on the ultra-microstructure of second generation merozoite of *E. tenella*.

A. Model group. B. Model group. C. Drug group: a: swelling and vacuoles of mitochondria; b: swelling of endoplasmic reticulum; c: damage and incompleteness of outer membrane. D. Drug group: a: significant swelling of mitochondria.



Effect of QOL on mitochondrial membrane potential of the second generation merozoite of *E. tenella*

The depolarization rate of mitochondrial membrane potential in the second generation merozoite was (21.73 ± 1.53) % in the drug group, which was significantly lower than that in the model group (28.49 ± 2.80) % ($P < 0.01$) (Figure 2).

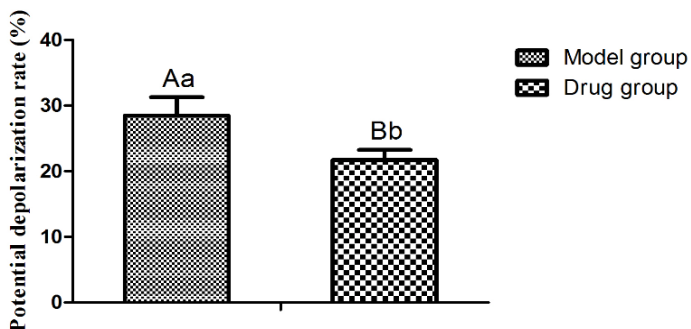


Figure 2 – Effect of QOL on mitochondrial membrane potential of the second generation merozoite of *E. tenella*.

Note: different lower case letters on the column mark indicate significant difference ($p < 0.05$), and different capital letters on the superscript indicate extremely significant difference ($p < 0.01$), the same below.

Effect of QOL on apoptosis of the second generation merozoite of *E. tenella*

As seen from Figure 3, the early apoptosis rate of the second generation merozoite in the drug group was much higher than that in the model group ($p < 0.01$), while there was no significant difference in the late apoptosis rate between the two groups ($p > 0.05$). The total apoptosis rate of the second generation merozoites in the drug group was significantly higher than that in the model group ($p < 0.01$).

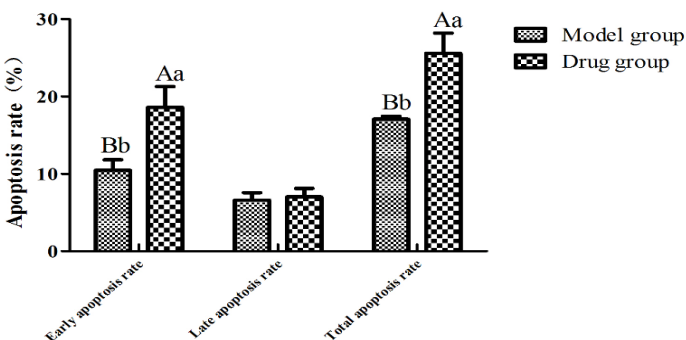


Figure 3 – Effect of Qing Chang oral liquid on apoptosis of the second generation merozoite of *E. tenella*.

DISCUSSION

The life cycle of *E. tenella* mainly includes sexual reproduction and asexual reproduction, the former also mains gametic reproduction and the latter includes spore formation and fission. Fission and gametic

reproduction are endogenous reproduction stages which are completed in chicken intestinal epithelial cells, while spore formation is carried out in an appropriate external environment (Lal *et al.*, 2010; Zhou *et al.*, 2013). According to the life history of *E. tenella*, the damage of *E. tenella* mainly occurs in the fission stage where the schizont expand repeatedly and the mature schizont releases a large number of merozoites which invade and damage the intestinal epithelial cells. It resulted in the excretion of bloody flux, destruction of intestinal mucosa tissue, digestive disorder, anemia, emaciation and other clinical symptoms (Matsubayashi *et al.*, 2019; Zhou *et al.*, 2020). The fission stage is the most serious hazard developmental stage, which mainly occurs 3-5 days after infection.

At the present moment, the ultra-microstructure of *E. tenella* at each stage has been well studied (Cacho *et al.*, 2010; Wang *et al.*, 2013; Liu *et al.*, 2016). By studying the changes of the ultra-microstructure of coccidia at each stage after drug action, we can reveal the possible drug action stage and target from the perspective of morphology. The study showed that QOL could damage the outer membrane of the second generation of merozoite, and simultaneously result in mitochondrial swelling, vacuoles and endoplasmic reticulum swelling. It was inferred that the anti-coccidiosis effect of QOL might be realized by directly acting on the outer membrane and mitochondria of the second generation of merozoite.

The mitochondrion is the main place for cells to carry out aerobic respiration and provide energy, which is involved in cell differentiation, cell information transmission and cell apoptosis, and has ability to regulate cell growth as well as cell cycle. Gu *et al* found that apoptosis in cells infected with *E. tenella* is positively correlated with changes in mitochondrial structure (Gu *et al.*, 2010). Furthermore, blocking the mitochondrial apoptotic pathway may also inhibit the *E. tenella*-induced apoptosis (Yang *et al.*, 2015). It has been reported that an increase in mitochondrial membrane permeability is one of the key events in apoptotic and necrotic death (Kinnally *et al.*, 2010; Xu *et al.*, 2016). But the mitochondrial membrane permeability directly affects mitochondrial membrane potential. Mitochondrial membrane potential generally refers to the potential difference generated from two solutions which are spaced by mitochondrial membrane, and it plays an important role in nerve cell communication. It is an important parameter, reflecting the mitochondrial functions in cells (Nair *et al.*, 2014), and the decreasing of mitochondrial membrane potential is an early sign of apoptosis



initiator (Cai *et al.*, 2001). The study showed that the mitochondrial membrane potential depolarization rate of the second generation merozoite in the drug group decreased, which has extremely significant difference between both groups. It is indicated that QOL can change the mitochondrial membrane potential of the second generation merozoite of *E. tenella*, promote mitochondrial apoptosis, and then realize anti-coccidial effect.

Apoptosis refers to the orderly and autonomous death of cells controlled by genes so as to maintain the stability of the internal environment, which is an active process, involved in a series of activation, expression and regulation of genes. It is not a kind of self-injury in pathology condition but an active death process to pursue a better accommodation to living environment (Deng *et al.*, 2016). Apoptosis can occur in both physiological and pathological states and it plays an important role in maintaining the homeostasis of the internal environment and impeding cell damage, aging and tumors caused by diseases or poisoning (Singh *et al.*, 2021). When early apoptosis occurs, the phosphatidylserine (PS) on the inner surface of the cell membrane is turned over and exposed to the outer surface, which is one of the biochemical characteristics of cell apoptosis (Yang *et al.*, 2015). The study results found that the early and total apoptotic rates of the second generation merozoite in the drug group were significantly increased and the difference was extremely significant between the two groups. It showed that QOL can promote the apoptosis of the second generation merozoite of *E. tenella*. It is supposed that QOL can directly act on the mitochondria of the second generation merozoite, affecting mitochondrial function and promoting mitochondria apoptosis, ultimately promoting the apoptosis of the second generation merozoite of *E. tenella*.

CONCLUSION

QOL can act on the outer membrane and mitochondria of the second generation merozoite of *E. tenella*, reduce the depolarization rate of mitochondrial membrane potential of the second generation merozoite and increase the apoptosis rate of the second generation merozoite.

CONFLICT OF INTEREST

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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