

Sanguinarine induces apoptosis in *Eimeria tenella* sporozoites via the generation of reactive oxygen species

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ABSTRACT *Eimeria tenella* (*E. tenella*) is the most pathogenic genus in *Eimeria* and can lead to a huge number of deaths of chickens, causing significant economic losses in the poultry industry worldwide. As a natural alkaloid, sanguinarine has many medicinal effects; to a certain extent, it can replace antibiotics and has good application prospects in veterinary medicine. To evaluate the effect of sanguinarine on sporozoites of *E. tenella*, we used flow cytometry and immunofluorescence staining to detect reactive oxygen species (ROS), mitochondrial membrane potential (MMP), calcium ion (Ca²⁺), and caspase-3 activation in *E. tenella* sporozoites treated with different concentrations of sanguinarine. The results of

flow cytometry showed that sanguinarine could inhibit the invasion of sporozoites of *E. tenella* in vitro ($P < 0.05$) and increase the reactive oxygen species and calcium ions in the sporozoites ($P < 0.05$). The results of immunofluorescence staining showed that sanguinarine could decrease the mitochondrial membrane potential of sporozoites. Our analysis suggests that sanguinarine can induce apoptosis of *E. tenella* sporozoites through reactive oxygen species-mediated reduction of the mitochondrial membrane potential and an increase in calcium ion concentration. It follows that sanguinarine is likely to be a novel type of anticoccidiosis drug with good research and clinical application prospects.

Key words: sanguinarine, *Eimeria tenella*, sporozoite, apoptosis

2022 Poultry Science 101:101771

<https://doi.org/10.1016/j.psj.2022.101771>

INTRODUCTION

Chicken coccidiosis is a common parasitic disease caused by obligate intracellular parasites of the genus *Eimeria* (Attree et al., 2021). *Eimeria tenella* (*E. tenella*) is the most pathogenic species of coccidiosis. It has a strict parasitic site, mainly in the caecum epithelial cells through schizonts, destroys the intestinal mucosa, and causes intestinal inflammation and epithelial cell disintegration (Augustine, 2001). The disease causes high morbidity due to acute bloody enteritis with high mortality (López-Osorio et al., 2020). Infection with *E. tenella* caused huge losses to the global poultry economy (Adams et al., 2021).

The life cycle of coccidia includes asexual reproduction and sexual reproduction. Sporozoite invasion of intestinal

mucosal epithelial cells is a very important period in the entire life cycle of *E. tenella* (Rose and Hesketh, 1991). Currently, the widely used methods for preventing and controlling chicken coccidiosis are mainly by using synthetic drugs and ionophorous compounds (Xie et al., 2020), including sulfonamide drugs, amide drugs, pyridine drugs (Noack et al., 2019), and polyether ionophore antibiotics, such as monensin, salinomycin, and madurasin (Mehlhorn et al., 1983). However, drug resistance has become a serious problem in the prevention and treatment of coccidiosis (Peek and Landman, 2011). In addition to drug resistance, environmental issues and food safety issues caused by anticoccidial drugs also need our attention. Therefore, people are constantly trying to find new anticoccidial methods (Fatoba and Adeleke, 2020; El-Shall et al., 2022).

Sanguinarine (C₂₀H₁₄NO₄) is a plant alkaloid. Alkaloids are nitrogen-containing compounds that may be found as “secondary metabolites” or “natural products” in plants (Mackraj et al., 2008). It is a phenanthridine isoquinoline alkaloid mainly found in the whole plant of *Macleaya cordata* (Willd.) R.Br. It has antibacterial, anti-inflammatory (Firatli et al., 1994), antitumor (Gaziano et al., 2016),

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Received November 24, 2021.

Accepted January 22, 2022.

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immunity enhancement (Zhang et al., 2013; Bussabong et al., 2021), and insecticidal effects (Zou et al., 2019). At the same time, sanguinarine also has the advantages of low toxicity (Singh and Sharma, 2018), low residue, and no environmental pollution as a plant-derived medicine (Lin et al., 2019). Sanguinarine showed antiparasitic efficacy against *Ichthyophthirius multifiliis* in vivo and in vitro (Yao et al., 2010). Sanguinarine has antischistosomal activities in vitro, and it is a promising alternative to chemotherapy for schistosomiasis (Zhang and Coultas, 2013). Sanguinarine also has anthelmintic activity against trichinella infection in infected mice by increasing the production of reactive oxygen species (ROS) in the host (Huang et al., 2020). A study showed that sanguinarine induced human cervical cancer cell apoptosis with caspase-3 activation (Ding et al., 2002).

To our knowledge, studies of sanguinarine against *E. tenella* have not been reported. Therefore, this study investigated the effect of sanguinarine on the invasion and apoptosis of *E. tenella* sporozoites in vitro.

MATERIALS AND METHODS

Purification of *E. tenella* Sporozoites

The strain of *E. tenella* was obtained from the laboratory of Jilin Provincial Engineering Research Center of Animal Probiotics (Liu et al., 2020) using 2.5% (w/v) potassium dichromate ($K_2Cr_2O_7$) for preservation before we studied it.

Sporulated oocysts were centrifuged at $2,900 \times g$ for 10 min at $4^\circ C$ and washed with distilled water to remove the $K_2Cr_2O_7$. The oocysts were treated with 10% (w/v) sodium hypochlorite for 15 min on ice and centrifuged at $2,900 \times g$ for 10 min. After washing with phosphate-buffered saline (PBS) 3 times, the precipitate was treated after centrifugation with 5% (w/v) sodium hypochlorite and centrifuged at $1,500 \times g$ for 20 min at $4^\circ C$, followed by washing with PBS 3 times. Then, the white flocs in the supernatant were collected and centrifuged at $2,500 \times g$ for 10 min at $4^\circ C$. After the last centrifugation, purified sporulated oocysts were obtained.

The purified sporulated oocysts were broken with a MagNA Lyser Instrument (MagNA Lyser, Roche, Basel, Switzerland). When the sporulated oocysts were more than 95% broken, 10 mL Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO) was added, and the oocysts were centrifuged 3 times at $1,500 \times g$ for 10 min at $4^\circ C$ to remove impurities. Digestion with 10% (v/v) chicken bile and 0.25% (w/v) trypsin was conducted with multiple microscopic examinations throughout the 3-h process. When we observed that released sporozoites reached more than 95%, we added HBSS and centrifuged at $1,500 \times g$ for 10 min at $4^\circ C$, repeated 3 times. The parasites were suspended in 10 mL HBSS and filtered through a G3 filter to obtain relatively pure sporozoites.

MDBK Cell Culture Growth Conditions

Madin-Darby bovine kidney (MDBK, ATCC CCL-22) cells were used. Cells were passaged using a 25-cm² cell

culture flask and then cultured overnight in a 48-well plate. An initial density of 5×10^4 per well was supplied with Dulbecco's modified Eagle's medium (HyClone, South Logan, UT) with newborn calf serum 10% (v/v) (Gibco, Thermo Fisher Scientific, Waltham, MA) to obtain semiconfluent monolayers at $37^\circ C$ and 5% CO₂.

Treatment of Sporozoites

Sporozoites and sanguinarine were incubated at $37^\circ C$ for 1 h in 1 mL DMEM containing 5% newborn calf serum, and the sporozoites were washed twice in PBS to remove the sanguinarine before we conducted invasion tests and fluorescent probe labeling tests at $2,900 \times g$ for 10 min at $4^\circ C$.

CFSE-Labeled Sporozoites Invade MDBK Cells

The purified sporozoites were suspended in 2 mL of sterile PBS containing 10 μM 5-(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma, St. Louis, MO) at $37^\circ C$ in a water bath for 10 min and centrifuged at $400 \times g$ for 5 min 3 times with PBS to stop the reaction.

The 10^5 sporozoites that were CFSE labeled and treated with sanguinarine (1, 5, 10 mg/L) for 1 h were used to infect MDBK cells at $37^\circ C$ and 5% CO₂ for 10 h. The cells were supplied with DMEM supplemented with 5% (v/v) newborn calf serum, and washed twice with PBS, used trypsin to digest the cells from the 48-well plate. Then they were fixed in 4% paraformaldehyde for 10 min, washed with PBS and centrifuged at $400 \times g$ for 5 min, then transferred to a 5-mL polystyrene test tube for flow cytometry analysis.

Detection of ROS of Sporozoites

The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) probe (KeyGEN BioTECH, China) was loaded in the sporozoites with reference to the manufacturer's instructions. The 10^5 sporozoites treated with sanguinarine (1, 2.5, 5 mg/L) were mixed with 1 mL DCFH-DA probes (10 μM) in 1.5 mL EP tubes at $37^\circ C$ for 20 min, centrifuged at $400 \times g$ for 5 min 3 times with serum-free cell culture medium, and transferred to a 5-mL polystyrene test tube for flow cytometry detection. Since the fluorescence spectrum of DCF is very similar to that of FITC, we measured the DCF with FITC parameters. The excitation wavelength was 488 nm, and the emission wavelength was 535 nm.

Detection of Mitochondrial Membrane Potential of Sporozoites

According to the manufacturer's instructions, a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1) (Beyotime, China) staining solution was prepared to label sporozoites treated with sanguinarine (1, 5,

10 mg/L). We mixed 0.5 mL JC-1 probe with 10^5 sporozoites in a 1.5 mL EP tube at 37°C for 20 min, centrifuged at $400 \times g$ for 5 min with JC-1 staining buffer ($1 \times$) and observed the sporozoites with a fluorescent microscope. JC-1 monomers and JC-1 aggregates were observed at the green emission channel and the red emission channel when excited at 488 nm.

Detection of Calcium ion (Ca^{2+}) Concentration in Sporozoites

The 2 mM Fluo-4 AM probe (Beyotime, China) was diluted to a $2.5 \mu\text{M}$ working solution with PBS. A total of 10^5 sporozoites were mixed with $50 \mu\text{L}$ of Fluo-4AM ($2.5 \mu\text{M}$) in 1.5 mL EP tubes. After incubation at 37°C for 30 min, the sporozoites were washed with PBS 3 times. Then, sanguinarine (1 and 2.5 mg/L) was added, incubated at 37°C for 30 min, and centrifuged at $400 \times g$ for 5 min with HBSS 3 times. After the last wash, each sample was resuspended in 200 mL PBS and transferred into a 5 mL polystyrene test tube for analysis by flow cytometry.

Detection of the Caspase-3 Activation of Sporozoites

We used GreenNuc Caspase-3 Substrate (Beyotime, China) to detect caspase-3 activation in the sporozoites. The GreenNuc Caspase-3 Substrate is a short peptide DEVD that is linked to a DNA binding fluorescence dye. Active caspase-3 could recognize the DEVD sequence and release green fluorescence. A total of 10^5 sporozoites treated with sanguinarine (1 mg/L, 2.5 mg/L, 5 mg/L) were mixed with $5 \mu\text{M}$ ($200 \mu\text{L}$) GreenNuc Caspase-3 Substrate in 1.5 mL EP tubes for 30 min and centrifuged

at $400 \times g$ for 5 min with HBSS three times. After the last wash, each sample was resuspended in $300 \mu\text{L}$ PBS and transferred into a 5 mL polystyrene test tube for analysis by flow cytometry.

Flow Cytometry

Flow cytometry was completed using standard settings of a BD LSRFortessa Cell Analyser at a laser excitation wavelength of 488 nm (for green fluorescence). The CFSE-, Fluo-4 AM-, and DCFH-DA-labeled samples were detected. Cell Quest Pro software was used to analyze the fluorescence signal of 20,000 events in each sample.

Statistical Analysis

Data analyses were performed using GraphPad Prism 5.0. One-way analysis of variance and Tukey's multiple comparison test were used to determine the significance of the differences between the test groups. When the probability (P) value was less than 0.05 ($P < 0.05$), the results were considered significant.

RESULTS

Sanguinarine Inhibited the Invasion of *E. tenella* Sporozoites

Flow cytometry was used to detect the invasion of MDBK cells by CFSE-labeled sporozoites treated with different concentrations of sanguinarine (1 mg/L, 5 mg/L, 10 mg/L). We used a single parameter histogram to indicate the intensity of the fluorescent signal, which can reflect the number of sporozoites invading cells (Figure 1A). The

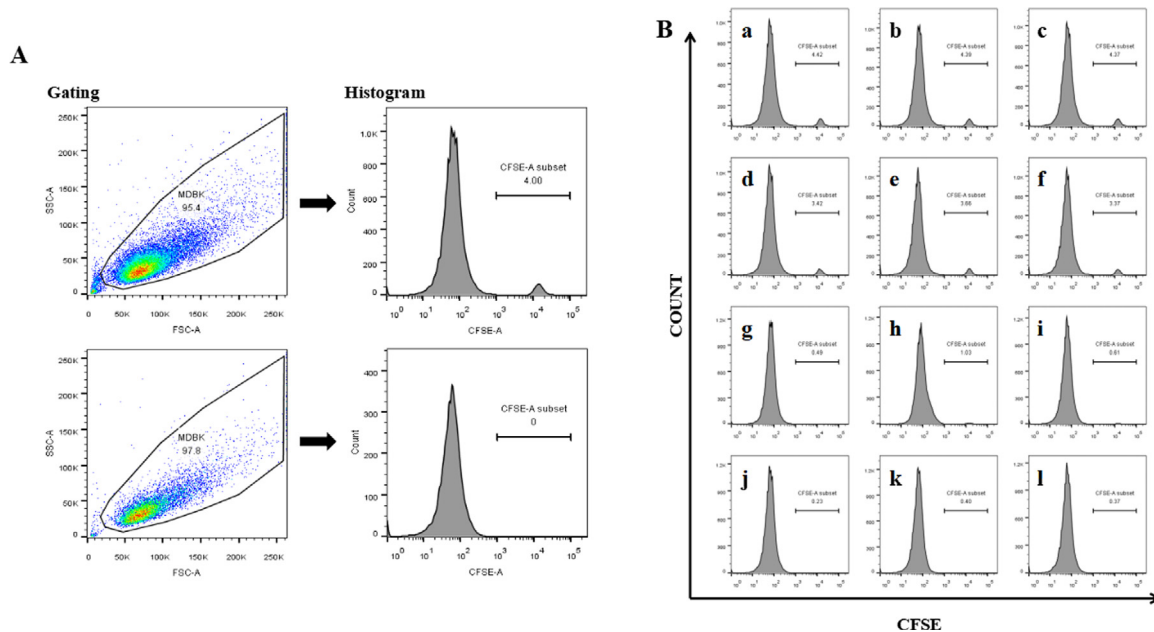


Figure 1. (A) Gating and single-parameter histogram of the flow cytometry. The gating of the sanguinarine-free group (only parasites) and the blank control (no parasites and sanguinarine-free). (B) Single-parameter flow cytometry histogram of sporozoite invasion of MDBK cells treated with different concentrations of sanguinarine. The histogram reflects the number of CFSE-labeled sporozoites invading the MDBK cells through the strength of the fluorescent signal. No treatment group (a, b, c); 1 mg/L sanguinarine treatment group (d, e, f); 5 mg/L sanguinarine treatment group (g, h, i); and 10 mg/L sanguinarine treatment group (j, k, l). (C) The difference in sporozoite invasion ability between the sanguinarine treatment group and the untreated control group was significant ($***P < 0.001$) (representative of 3 experiments, $n = 5$).

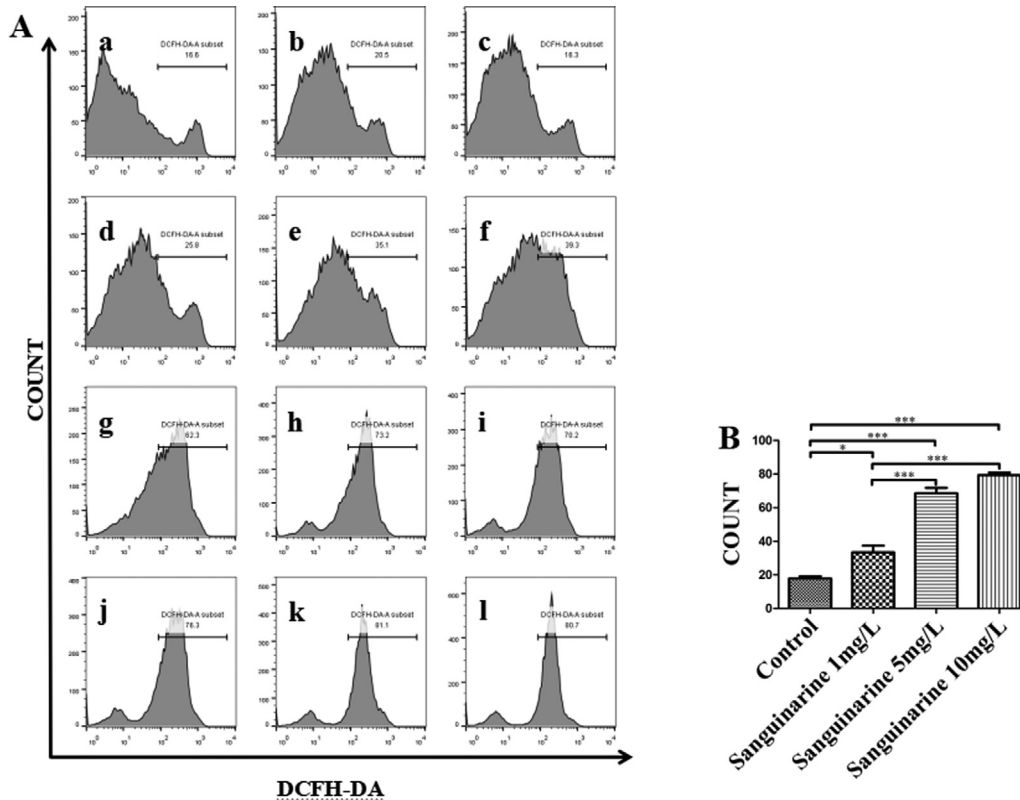


Figure 2. (A) Single-parameter flow cytometry histogram of reactive oxygen species in sporozoites after treatment with different concentrations of sanguinarine. No treatment group (a, b, c); 1 mg/L sanguinarine treatment group (d, e, f); 5 mg/L sanguinarine treatment group (g, h, i); and 10 mg/L sanguinarine treatment group (j, k, l). (B) The difference in sporozoite ROS levels between the sanguinarine treatment group and the untreated control group was significant (***) ($P < 0.001$) (representative of 3 experiments, $n = 4$).

results showed that the intensity of the fluorescent signal was dose-dependent with the concentration of sanguinarine (Figure 1B) and the difference was significant ($P < 0.05$; Figure 1C).

Sanguinarine Increased the ROS Levels of *E. tenella* Sporozoites

Flow cytometry was used to detect the ROS levels in sporozoites, and we used a single parameter histogram to represent the results. The results showed that sanguinarine significantly increased the ROS in sporozoites (Figure 2).

Sanguinarine Decreased the Mitochondrial Membrane Potential of *E. tenella* Sporozoites

JC-1-labeled sporozoites were observed using an inverted fluorescence microscope. Compared with the control group (without sanguinarine) and the CCCP group, we observed that the green fluorescence intensity of the experimental group became stronger with an increasing sanguinarine concentration, which proved that with increasing sanguinarine concentration, the MMP declined (Figure 3).

Sanguinarine Increased the Ca^{2+} Concentration in *E. tenella* Sporozoites

Fluo-4 AM-labeled sporozoite status was detected using flow cytometry with a single-parameter

histogram. The test results show that sanguinarine can increase the concentration of Ca^{2+} in sporozoites compared with the control group, showing a significant difference (Figure 4).

Sanguinarine Increased the Level of Caspase-3 Activation

Caspase-3 activation in sporozoites labeled with the GreenNuc Caspase-3 Substrate and treated with sanguinarine (1 mg/L, 2.5 mg/L, 5 mg/L) was analyzed by flow cytometry with a single-parameter histogram. Sanguinarine increased the level of caspase-3 activation in sporozoites compared with the control group, showing a significant difference (Figure 5).

DISCUSSION

E. tenella is an intracellular parasite whose sporozoites can invade host cells within 24 h after excystation from oocysts and then obtain essential nutrients from the host cell (Jiang et al., 2012). However, intrasporocystic sporozoites and extracellular sporozoites need to search for a new host cell to invade, resulting in a shortage of energy sources (Qi et al., 2019). Therefore, the invasion process is very important in *E. tenella* infection.

A well-established and widely used in vitro model for *E. tenella* infection is the Madin-Darby bovine kidney cell line (Burt et al., 2013; Hessenberger et al., 2016;

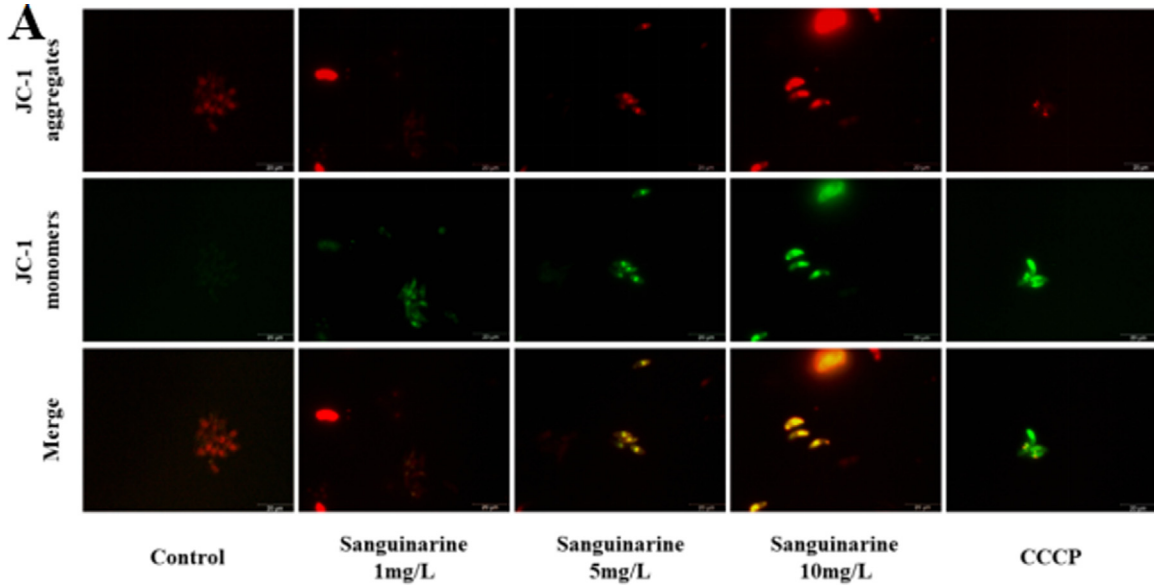


Figure 3. The fluorescence intensity of JC-1-labeled sporozoites under an inverted fluorescence microscope. Green fluorescence was produced when the MMP was lower, and red fluorescence was produced when the MMP was higher. CCCP is a mitochondrial electron transport chain inhibitor that can effectively induce a decrease in MMP. The control group was not treated with sanguinarine, and we observed that the fluorescence signal decreased with increasing sanguinarine concentration (representative of 3 experiments, $n = 3$). Abbreviation: MMP, mitochondrial membrane potential.

Ma et al., 2019; Taha et al., 2021). Flow cytometry data of sporozoites labeled with CFSE can reflect changes in invasion (Hermosilla et al., 2008; Khalafalla et al., 2011).

Many herbs have anticoccidial effects (El-Shall et al., 2022), and *Bidens pilosa* and *Artemisia indica* exert

their anticoccidial action in chickens via interference with the different stages of the coccidial life cycle (Yang et al., 2021). Sanguinarine has been shown to have a promoting effect on animal performance as a feed additive (Kosina et al., 2004; Kantas et al., 2015). It can induce partial protection of laying hens from the impact

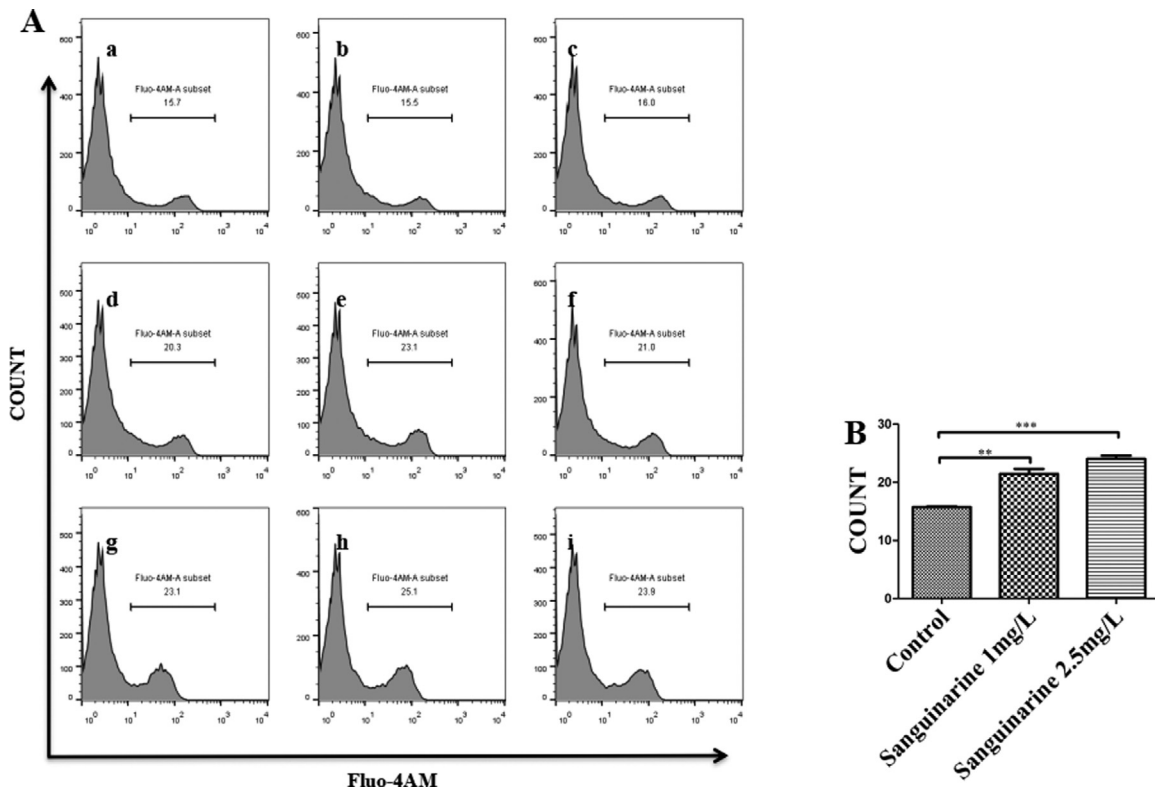


Figure 4. (A) Single-parameter flow cytometry histogram of the calcium ion concentration levels of Fluo-4-labeled sporozoites treated with different concentrations of sanguinarine. Untreated control group (a, b, c); 1 mg/L sanguinarine treatment group (d, e, f); and 2.5 mg/L sanguinarine treatment group (g, h, i). (B) The difference in sporozoite Ca^{2+} levels between the sanguinarine treatment group and the untreated control group was significant (** $P < 0.01$, *** $P < 0.001$) (representative of 3 experiments, $n = 5$).

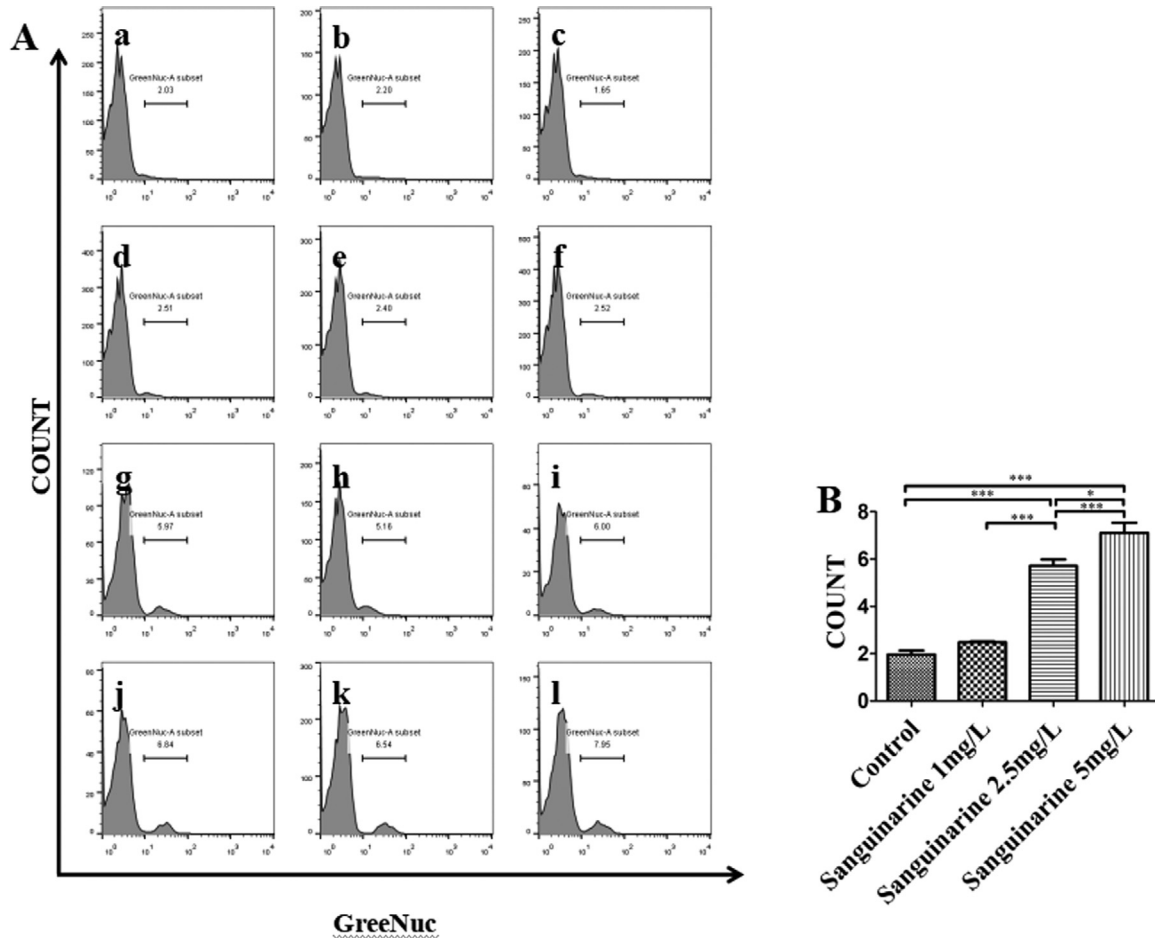


Figure 5. (A) Single-parameter flow cytometry histogram of the caspase-3 activation level of GreenNuc-labeled sporozoites treated with different concentrations of sanguinarine. Untreated control group (a, b, c); 1 mg/L sanguinarine treatment group (d, e, f); 2.5 mg/L sanguinarine treatment group (g, h, i); and 5 mg/L sanguinarine treatment group (j, k, l). (B) The difference in sporozoite caspase-3 activation levels between the sanguinarine treatment group and the untreated control group was significant (** $P < 0.01$, *** $P < 0.001$) (representative of 3 experiments, $n = 3$).

of *Campylobacter hepaticus* challenge (Quinteros et al., 2021) and improve nutrient digestibility and productive performance in laying hens fed low crude protein diets (Bavarsadi et al., 2021).

Although sanguinarine has many benefits, no studies have reported the anticoccidial effect of sanguinarine. We showed for the first time that sanguinarine concentrations of 1 mg/L, 5 mg/L, and 10 mg/L had a good effect on inhibiting invasion in vitro. These concentrations were obviously nontoxic to MDBK cells by flow cytometry scatter plots, which proved the potential of sanguinarine as a new anticoccidial drug.

Apoptosis plays an extremely important role in the parasite-host interaction (Lüder et al., 2001; Chain et al., 2020). As several important markers of apoptosis, ROS, MMP, Ca^{2+} , and caspase-3 have become essential for researchers to study apoptosis (Bock and Tait, 2020; Chen et al., 2020; Gong et al., 2020). ROS are involved in the process of parasite infection, and the activation of the macrophage respiratory burst in response to infection with *Trypanosoma cruzi* inflicts oxidative damage on the host's tissues. For decades, the role of ROS in the elimination of *Trypanosoma cruzi* was taken for granted (Paiva et al., 2018). The antiparasitic mechanism of artemisinin is to produce cytotoxic

reactive oxygen species that cause oxidative effects (Gold et al., 2017). The generation of ROS can destroy the mitochondrial membrane potential (MMP) and change the membrane permeability (Zorov et al., 2014). Sanguinarine-mediated acute lymphoblastic leukaemia apoptosis was found to be associated with the increased expression of proapoptotic Bax with a concomitant decrease in Bcl-2 expression leading to depolarization of the mitochondrial membrane, resulting in a loss of MMP (Kuttikrishnan et al., 2019). Our findings are in line with studies by others, and we found that sanguinarine concentrations of 1 mg/L, 5 mg/L, and 10 mg/L can increase the ROS level in *E. tenella* sporozoites.

Mitochondria are the main sites at which animal and plant cells produce ATP (Roger et al., 2017). They are the main organelles that promote cell energy conversion and participate in cell apoptosis (Sinha et al., 2013). The stability of the MMP is conducive to maintaining the normal physiological functions of cells (Zorov et al., 2014). When most cells undergo apoptosis in response to the action of different factors, the MMP decreases (Liu et al., 2015). ROS can act on the permeability transition pore between the inner and outer membranes of the mitochondria, causing the release of a large number of apoptotic factors (Danial and Korsmeyer, 2004). In

our study, we showed that sanguinarine concentrations of 1 mg/L, 5 mg/L, and 10 mg/L could reduce the MMP of *E. tenella* sporozoites.

The balance of intracellular Ca^{2+} concentration is crucial to maintaining the normal function of cells and the survival of cells (Zorov et al., 2014). ROS can affect the balance of intracellular Ca^{2+} concentration, causing mitochondrial Ca^{2+} overload and a decrease in MMP, which causes apoptosis through the mitochondrial pathway (Zhang et al., 2006). The increase in Ca^{2+} and the decrease in MMP can be caused by endoplasmic reticulum stress. Calcium in the endoplasmic reticulum is released into the cytoplasm, causing an overload of Ca^{2+} in the mitochondria and MMP reduction. Eventually, caspase-3 is activated and apoptosis is induced through the mitochondrial pathway (Takano et al., 2014). In our study, we found that sanguinarine concentrations of 1 mg/L and 2.5 mg/L increased sporozoite calcium ion concentrations. When the concentration of sanguinarine was 5 mg/L, its effect was the same as that of 2.5 mg/L (data not shown). No statistically significant differences were found between 2.5 mg/L and 5 mg/L. However, our inferences need further study.

Caspase-3 is a key downstream enzyme in the apoptotic cascade pathway. Caspase-3 activation is an important part of most apoptosis signal transduction pathways (Nagata, 2018). Activation of the proapoptotic protein caspase-3 leads to cell apoptosis (Park et al., 2012). Studies have shown that sanguinarine can induce apoptosis in U937 cells by activating caspase-3 (Han et al., 2008). Our study showed that sanguinarine at 10 mg/L increased the level of caspase-3; no significant differences were found between the 5 mg/L and 10 mg/L groups (data not shown). In other studies, sanguinarine had a strong lethal effect on *Trichinella spiralis* adults. A sanguinarine concentration from 5 to 15 mg/L significantly increased muscle larva mortality, and 15 mg/L killed all *Trichinella spiralis* adults. Compared with our experimental results, it was found that the sanguinarine inhibitory effect against *E. tenella* may be higher than that against *Trichinella spiralis*.

CONCLUSIONS

Although the results obtained in vitro appear promising, the efficacy in vivo remains to be determined. This study found that the invasion of *E. tenella* sporozoites is inhibited by sanguinarine in vitro. Our results show that sanguinarine can increase sporozoite ROS levels, reduce the MMP, increase intracellular calcium ion concentrations and caspase-3 activation, and trigger apoptosis. These results suggest that sanguinarine has certain potential as a new anticoccidial drug.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (32072888, 31941018, U21A20261), Science and Technology Development

Program of Jilin Province (20180201040NY, 20190301042NY, YDZJ202102CXJD029).

Availability of data and materials: The data supporting the conclusions of this article are included within the article.

Authors' contributions: G-L Y and C-F W designed this study. J-Y L, H-B H, NW, C-W S, T-X P, and BZ performed the experiments. J-Y L, G-L Y and C-F W drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate: This work was conducted on the basis of the National Guidelines for Experimental Animal Welfare. All animal experiments in this work were approved by the Laboratory Animal Welfare and Ethics Committee of Jilin Agricultural University. (No.2020 06 01 001).

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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