The role of Ca²⁺ in the injury of host cells during the schizogenic stage of *E. tenella*

Xiao-Ling Lv,[*] Yong-Yan Wang,[*] Ming-Xue Zheng,[*,1] Rui Bai,[*] Li Zhang,[*] Bu-Ting Duan,[*] Xuan Lei,[*] Xue-Song Zhang,[*] Yong-Juan Zhao,[†] Kai-Ling Cui,[*] and Tong Xu[*]

[*]College of Veterinary Medicine, Shanxi Agricultural University, Taigu, Jinzhong, 030801, China; and [†]School of Food and Environment, Jinzhong College of Information, Taigu, Jinzhong, 030801, China

ABSTRACT Cecal epithelial cell damage is a key factor in host injure during the development of *E. tenella*. The intracellular free Ca²⁺ of the host cell is closely related to the invasion, development and proliferation of intracellular parasites, and cell damage. To determine the relationship between Ca²⁺ and host cell damage in the schizogenic stage of *E. tenella*, we established a chick embryo cecal epithelial cells model of *E. tenella* infection. Fluorescence staining, flow cytometry, transmission electron microscopy, inhibition and blocking experiments were used to detect the damage effect and mechanism of host cells during the schizogenic stage of *E. tenella*. The results showed that the host cells cytoskeletal remodeling, cell and organelle structure was destroyed, and apoptosis and necrosis were increased during the schizont stage of *E. tenella*. Furthermore, the above-mentioned effects of the schizogenic stage of *E. tenella* on cells can be alleviated by reducing the intracellular Ca²⁺ concentration in the host cells. These observations indicate that the effect of host cell injury was closely related to Ca²⁺ during schizont stage of *E. tenella*.

Key words: *Eimeria tenella*, Ca²⁺, cell injury, schizogenic stage

INTRODUCTION

Chicken eimeriosis is a serious global protozoal disease. *Eimeria tenella* is the most toxic and harmful of the seven species of coccidia found in chickens which mainly affects the caecum (Sandholt et al., 2021). *E. tenella* sporozoites rapidly develop into schizonts after entering the cecal mucosal epithelial cells. After the schizont matures, a large number of merozoites are released from the cells to damage the cecal epithelial cells, causing the mucosal epithelial cells to undergo rapid degeneration, necrosis, disintegration and shedding (Zhang et al., 2019). In addition, the apoptotic rate of cecal mucosal epithelial cells in infected chickens increased (Xu et al., 2017). Studies have confirmed that the increase of Ca²⁺ in the cecal mucosa and glandular epithelial cells of chickens can promote the apoptosis of cecal cells after infection with *E. tenella* (Cui et al., 2016).

Ca²⁺ plays an important role in maintaining the normal function and structure of cells. It is the most common and important signal transduction component in cells that acts as a second messenger together with cyclic adenosine monophosphate (**cAMP**) (Lock and Parker, 2020). Studies have shown that Ca²⁺ plays an important role in causing fatal damage to cells (Sharma et al., 2021). Ca²⁺ overload is the "last common pathway" for cell damage caused by many reasons. Ca²⁺ overload can induce degradation of cytoskeleton proteins to destroy the integrity of the connection between the cytoskeleton and the cell membrane; damage to membrane phospholipids makes the bilayer structure disorder and membrane fluidity changes; damage DNA; cause lipid peroxidation of cell membrane; promote the production of oxygen free radicals to aggravate membrane damage (Modesti et al., 2021). Furthermore, Ca²⁺ plays a crucial role in parasite differentiation, cell scaffold dynamics, migration and colonization and is also a relevant factor in the interaction between the parasite and the host cell (Docampo and Moreno, 2021). The intracellular free Ca²⁺ of the host cell increases significantly during the process of host cell development of various intracellular parasites, such as *Trypanosoma cruzi, Plasmodium falciparum, Leishmania*, and *Toxoplasma gondii* (Docampo and Huang, 2021; Fu et al.,

2021; Orrego et al., 2021; Scarpelli et al., 2021). Therefore, the process of intracellular parasites damaging host cells may be closely related to Ca^{2+} signal transduction.

Chicken cecal epithelial cells are natural host cells of *E. tenella* (Zhang et al., 2020), but the damage effect of *E.tenella* on host cells during the schizogenic stage has not been reported. Furthermore, the relationship between Ca^{2+} and *E. tenella*-induced cell damage was also unclear. The purpose of this study was to explore the damage effect of *E. tenella* on host cells during the schizogenic stage and its relationship with Ca^{2+}. Based on the *E. tenella*-infection, a chick embryo cecal epithelial cells model, fluorescence staining, flow cytometry, transmission electron microscopy, inhibition and blocking experiments, etc. were used to explore the damage and mechanism of *E. tenella* to host cells during the schizogenic stage. This study reveals the relationship between Ca^{2+} and host cell damage during the schizogenic stage of *E. tenella*, and also provide a theoretical basis for the search for anticoccidial drugs that intervene and control the occurrence and development of *E. tenella* in chickens.

MATERIALS AND METHODS

Ethics Statement

All experiments involving animals were carried out in accordance with institutional animal care and use committee (**IACUC**) follow the guidelines. And it was approved by the Animal Protection and Utilization Committee of Shanxi Agricultural University, China.

Experimental Animals and Parasite

15-day-old SPF chicken embryos were obtained from Beijing Meri Avigon Laboratory Animal Technology Co., Ltd. (Beijing, China). The *E. tenella* Shanxi virulent strain (EtSX01) was the strain used in this experiment, which was provided by the Laboratory of Veterinary Pathology in the College of Veterinary Medicine, Shanxi Agricultural University.

Reagents

Thermolysin, Hematoxylin, Eosin, and Trypan Blue were purchased from Sigma (St. Louis, MO); Recombinant murine EGF was purchased from PeproTech (East Windsor, NJ); MTT, Heparin sodium, Paraformaldehyde, 4%, TritonX-100, EGTA were purchased from Solarbio (Beijing, China); Trifluoperazine was purchased from Wuhan Xinhuayuan Technology Development company (Wuhan, China); BAPTA/AM were purchased from ENZO (New York City, NY); FITC-phalloidin was purchased from Cytoskeleton (Denver, CO); ApoDETECT Annexin V-FITC Kit was purchased from Invitrogen (Carlsbad, CA); Mouse monoclonal to beta Actin, Anti-alpha Tubulin antibody, Mouse monoclonal to GAPDH were purchased from abcam(Cambridge, UK); Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L) secondary antibodies was purchased from Proteintech (Chicago, IL); bicinchoninic acid (**BCA**) protein assay kit was purchased from Thermo Scientific (Waltham, MA).

Primary Culture of Chicken Embryo Caecal Epithelial Cells

The chicken embryo caecal epithelial cells were isolated and cultured according to the reported method (Li et al., 2017). Briefly, the cecum was removed from 15-day-old SPF embryos and striped the mesentery carefully; the cecum was fully washed by the PBS buffer, then cut into 1 mm^3 size tissue and washed; the tissue was resuspended and mixed by thermolysin (50 mg/L), and digested at 41°C for 2 h; PBS was used to stop the digestion, then centrifuge at 1,200 r/min for 5 min and discard supernatant; the cell pellet was resuspended in 10% FBS low-glycemic DMEM cell culture medium, seeded in a cell culture flask and adhered to the culture for 70 min; the liquid in the flask was collected, centrifuged at 1,200 r/min for 5 min, and then resuspended and counted in DMEM/F12 culture medium containing 2.5% fetal bovine serum (**FBS**); the cells were seeded in a cell culture plate and could be used for subsequent experiments when the cell attachment rate was reached about 85%.

E. tenella Sporozoite Preparation

The preparation of *E. tenella* sporozoite was based on the method of Xu et al. (2017). *E. tenella* sporulated oocysts were centrifuged at 2,000 r/min for 5 min, the supernatant was discarded, and PBS was added to wash thoroughly. The oocysts were suspended in 2 mL PBS and ground with a homogenizer until the excystation rate reached 80%. The above solution was centrifuged at 1,800 r/min for 5 min. Then, precipitate were added suitable quantum spore digestion solution and digest with shaking at 41°C (150 r/min) until 80% of the sporozoites are released. After filtration, the digestion solution was removed by centrifugation at 3,000 r/min for 10 min, and the precipitate was suspended in DMEM solution.

Cytotoxicity Assay

The chicken embryo caecal epithelial cells were plated in 96-well plates at a density of 2×10^4 cells/well in primary cell culture medium containing 2.5% FBS. Then, cells were treated with different inhibitors (8 μmol/L trifluoperazine, 10 μmol/L BAPTA/AM) at the indicated concentrations for 24 h when the cell attachment rate was reached about 85%. After 2 washes with PBS, 15 μL of the MTT solution was added to 100 μL of primary cell culture medium in each well of a 96-well plate and incubated at 41°C for 4 h, then add 150 μL DMSO to each well. The absorbance was measured at 492 nm with a microplate reader.

Host Cell Morphology Determination

The cells were divided into blank control group (C), *E. tenella* group (T1), trifluoperazine group (T2), BAPTA/AM group (T3) and BAPTA/AM+trifluoperazine group (T4). T2, T3, T4 were incubated respectively with 8 μmol/L trifluoperazine , 10 μmol/L BAPTA/AM or 8 μmol/L trifluoperazine and 10 μmol/L BAPTA/AMfor 4 h. The sporozoites were inoculated into the cells (48-well plate, 1×10^5 sporozoites/well), except for blank control group. Cell slides were taken out at different time points. H&E staining was used to detect the morphological and structural changes of cells. The morphology of 100 cells in each slide was randomly observed and images were captured using a 14.0 MP digital microscope camera.

Cells were grouped and processed as described above. The sporozoites were inoculated into the cells (25 cm^2 cell culture flask, 1 million sporozoites/flask), except for blank control group. Cells were taken out at different time points. The cell suspension was centrifuged at 1,500 r/min for 5 min, the supernatant was discarded, and the cell pellet was collected; fixed with 2.5% glutaraldehyde for 2 h; fixed with 1% osmic acid for 3 h; gradient dehydration with 30%, 50%, 70%, 85%, 95%, 100% I, 100% II alcohol; immersion in isoamyl acetate for 20 min; embedding, polymerization, sectioning, and staining. Transmission electron microscopy observes cellular ultrastructure and captures images.

Apoptosis Detection

At indicated times of infection, cells were washed 3 times with PBS, Trypsin-EDTA combined digestion and DMEM containing 10% fetal bovine serum was used to terminate the digestion, and the cells were collected; cells were washed with Binding Buffer; resuspend cells in
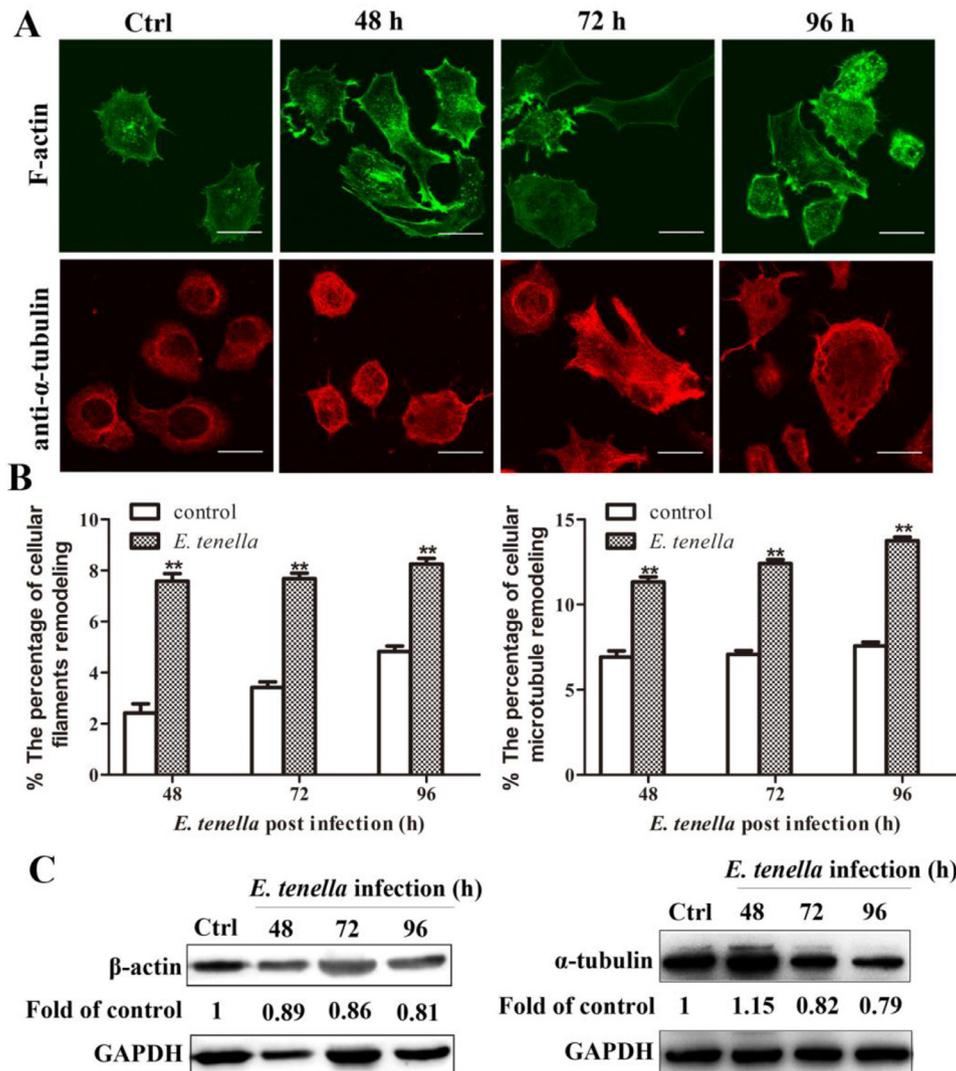
Figure 1. The schizogenic stage of *E. tenella* induces host cells cytoskeletal remodeling. (A) *E. tenella* infection induces cytoskeletal remodeling. chicken embryo cecal epithelial cells were incubated with *E. tenella* and fixed at the indicated time points. The untreated cells were used as a control. F-actin was stained with FITC-phalloidin (green) and α-tubulin was stained with anti-α-tubulin (red) and observed by confocal microscopy. Scale bar = 10 μm. (B) Quantitative detection of cytoskeleton changes at different time points after infection. each value represents the average value from 60 to 80 cells from at least 5 regions of 3 representative experiments. (C) β-actin and α-tubulin protein levels in chicken embryo cecal epithelial cells at different infection time points. All of the data are representative of at least three independent experiments. *P < 0.05, **P < 0.01 vs. normal controls.

500 μL Binding Buffer; 5 μL Annexin V-FITC and 1μL PI were added to samples for 30 min at room temperature; Finally, put it into the flow cytometer for detection. Data analysis with CellQuest software.

Immunofluorescence Staining and Confocal Microscopy

The chicken embryo caecal epithelial cells were plated in 24-well plates with coverslips at a density of 8×10^4 cells/well in primary cell culture medium containing 2.5% FBS. The sporozoites were inoculated into the cells (2×10^5 sporozoites/well). At indicated times of infection, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and then permeabilized with 0.2% Triton X-100 for 5 min and blocked with 5% nonfat milk powder for 1 h at 37°C. To stain actin, FITC-phalloidin was added to samples for 30 min at 37°C, hoechst was used to stain the nuclei. To stain tubulin, the cells were incubated overnight at 4°C with anti-alpha Tubulin antibody (1:100). After washing with PBS three times, Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L) secondary antibody was incubated with PBS at 37°C for 1 h. Hoechst was used to stain the nuclei. After washing with PBS 3 times, the coverslips were mounted onto glass with antifade solution before visualization on a confocal microscope. All images were acquired randomly using a laser scanning confocal fluorescence microscope.

Western Blotting

At indicated times of infection, cells were washed three times with PBS and lysed for 30 min in RIPA buffer (1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride [**PMSF**] in PBS) on ice. The concentration of protein was determined using a bicinchoninic acid (**BCA**) protein assay kit. The protein samples (50 mg/lane) were separated using 10% polyacrylamide gels and were transferred to 0.22 μm polyvinylidene fluoride membranes using the Bio-Rad wet transfer system. After blocking for 1 h at 37°C with 5% nonfat dry milk in PBS and reacting with the indicated primary antibodies at 4°C overnight, membranes were exposed to species-specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence detection by autoradiography. GAPDH was used as a loading control.

Image and Statistical Analyses

Values are presented as the arithmetic mean ± standard error. Each experiment was repeated at least 3 times. The SPSS 17.0 statistical software package (Chicago) was used to perform ANOVA analysis of all data. Histograms were prepared with GraphPad Prism 5.0 software (San Diego, CA). Fluorescence intensity was analyzed by ImageJ software (National Institutes of Health). All results were considered statistically significant at a P value < 0.05.

RESULTS

The Schizogenic Stage of E. tenella Induces Chick Embryo Cecal Epithelial Cells Cytoskeletal Remodeling

The changes in cytoskeleton of chick embryo cecal epithelial cells caused by *E. tenella* infection were assessed by confocal microscopy. The remodeling rate of microfilament skeleton of host cells inoculated with *E. tenella* sporozoites was extremely significantly higher than that of the control group from 48 to 96 h, and the remodeling rate increased sequentially. In addition, the microtubule remodeling rate was consistent with the above results (Figure 1A, B). The average expression of actin protein in *E. tenella* sporozoite host cells was significantly lower than that in the control group from 48 to 96 h. Furthermore, the expression of tubulin significantly lower than that in the control group from 72 to 96 h (Figure 1C).
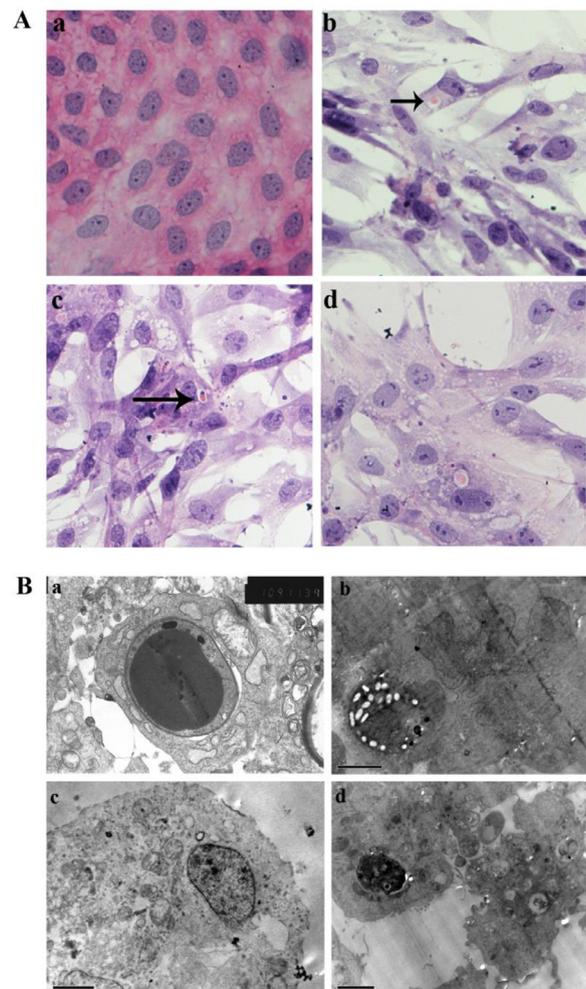
Figure 2. *E. tenella* damages host cell and organelle structure during schizogenic stage. (A) Host cell changes during the schizogenic stage of *E. tenella*. The sporozoites were added to chicken embryo caecal epithelial cells and observe under an optical microscope at at different time points. a: normal cells; b: cells infected with *E. tenella* at 48 h, trophozoites are indicated by black arrows; c: cells infected with *E. tenella* at 72 h, merozoites are indicated by black arrows; d: cells infected with *E. tenella* at 96 h (H&E staining, × 400). (B) Changes in host cell organelles during the schizogenic stage of *E. tenella*. a: normal cells; b: cells infected with *E. tenella* at 48, 72, 96 h.

The results indicated that the *E. tenella* schizogenic stage induced host cytoskeletal remodeling and affected the expression of cytoskeletal proteins.

E. tenella *Damages Host Cell and Organelle Structure During Schizogenic Stage*

H&E staining of chick embryo cecal epithelial cells showed pale red cytoplasm, clear nuclei, and clear intercellular boundaries (Figure 2Aa). The cells were inoculated with sporozoites for 48 h, and the sporozoites developed into oval trophozoites. The cells shrunken, the intercellular spaces expanded, some cells swelled, vacuoles appeared in the cytoplasm, the chromatin was reduced in basophilia, and the cell density decreased compared with the control group (Figure 2Ab). The cells were inoculated with sporozoites for 72 h, the cell boundary was not obvious, and merozoites of about 3 μm appeared in the cells, which were oval or round in shape (Figure 2Ac). The cells were inoculated with sporozoites for 96 h, the damage to the cell structure is more serious, and the nucleus even disappears (Figure 2Ad). The results indicated that *E. tenella* schizogenic stage could destroy the morphology of cecal epithelial cells in chick embryos.

Changes of various organelles in host cells observed by transmission electron microscopy during the schizogenic stage of *E. tenella*. 48 h after inoculation, the microvilli on the cell surface of the host cells become thinner, and then the microvilli shedding decreased, mitochondria swelled, ridges disappeared, vacuolar degeneration, and the rough endoplasmic reticulum degranulated and expanded, and in severe cases burst into Large vacuoles (Figure 2Bb). After 72 h of inoculation, cells detach from adjacent cells, lost microvilli, and develop irregular pseudopodia-like cell protrusions. At the same time, the host cell undergoes different degrees of apoptosis, the cell membrane shrinked and saged, the cytoplasm was divided and surrounded and protrudes outward to "germinate." The cell body fragments were surrounded by complete cell membranes and contain concentrated cytoplasm, complete organelles or nuclear fragments, etc., forming apoptotic bodies (Figure 2Bc). After 96 h of inoculation, the cell junction structure on the cell membrane adjacent to the cell disappeared, the desmosomes decreased or
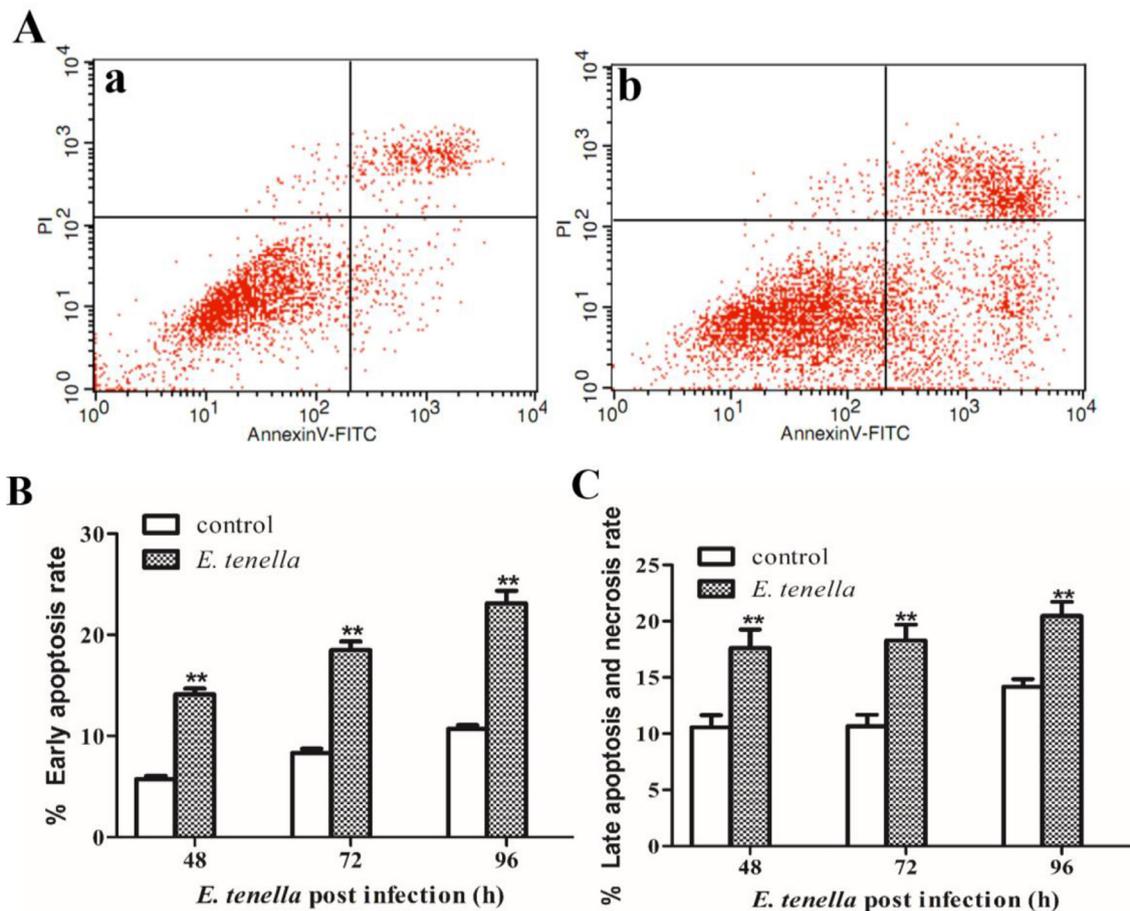
Figure 3. The apoptosis and necrosis of host cells during the schizogenic stage of *E. tenella*. (A) Apoptosis scatter plot. a: normal cells; b: cells infected with *E. tenella* at 72 h. (Populations of cells, cell that not undergoing apoptosis were on left bottom panels; cell that undergoing early apoptosis were on right bottom panels; on right top bottom, cell twere in end stage apoptosis or already dead). (B-C) Quantitative detection of early, late apoptosis at different time points after infection. All of the data are representative of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. normal controls.

disappeared, the intercellular connection was lost, and the gap was widened (Figure 2Bd). The results indicated that *E. tenella* in schizoreproductive stage could severely damage the host cell structure.

E. tenella *in Schizogenic Stage Promotes Apoptosis and Necrosis of Host Cells*

The effect of *E. tenella* schizogenic stage on apoptosis and necrosis was quantitatively determined by flow cytometry. The early, late apoptosis and necrosis rates of host cells inoculated with *E.tenella* sporozoites were higher than those in the control group from 48 to 96 h, and were most significant at 72 h (Figure 3A). the results are consistent with the above by using CellQuest software to analyze the data at 3 time points (Figure 3B, C). It was confirmed that the *E. tenella* schizogenic stage can promote the occurrence of host cell apoptosis and necrosis.

Ca^{2+} *is Closely Related to the Damage of Host Cells During the Schizogenic Stage of E. tenella*

To explore the mechanism of *E. tenella* schizogenic stage to host cell injury, chicken embryonic cecal epithelial cells were treated with an intracellular calcium chelator (BAPTA/AM), a calmodulin inhibitor (trifluoperazine) or BAPTA/AM+trifluoperazine to alter intracellular Ca^{2+} concentration. First, we tested the effect of chemicals used in this study on cell viability, cell structure, apoptosis rate. The results showed that none of the chemicals used in this experiment had a significant effect on the cell growth rate, cell structure, apoptosis rate (Figure 4).

Then, the effects of those drugs on host cell damage during the schizogenic stage of *E. tenella* were examined. The results showed that BAPTA/AM, trifluoperazine, BAPTA/AM+trifluoperazine all could reduce the cytoskeletal rearrangement, decrease of cytoskeletal proteins, changes in cell morphology and organelle
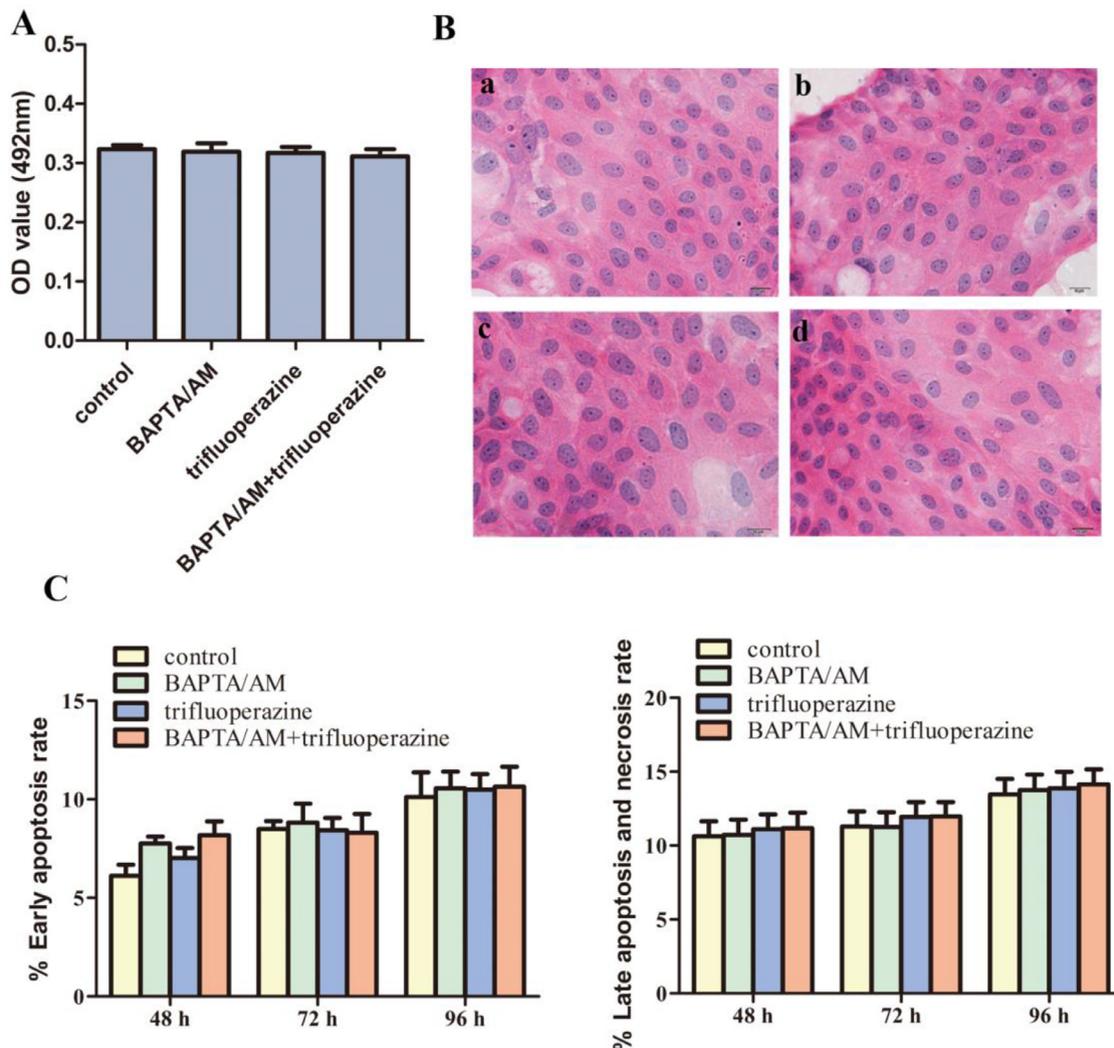
Figure 4. Ca^{2+} channel inhibitors do not affect cell viability. (A) Different inhibitors did not effect on cell growth rate of chicken embryo cecal epithelial cells. (B) Different inhibitors did not effect on cell structure. a: control group; b-d: BAPTA/AM, trifluoperazine, BAPTA/AM+trifluoperazine groups. (C) Quantitative detection of apoptosis in each group. All of the data are representative of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. normal controls.
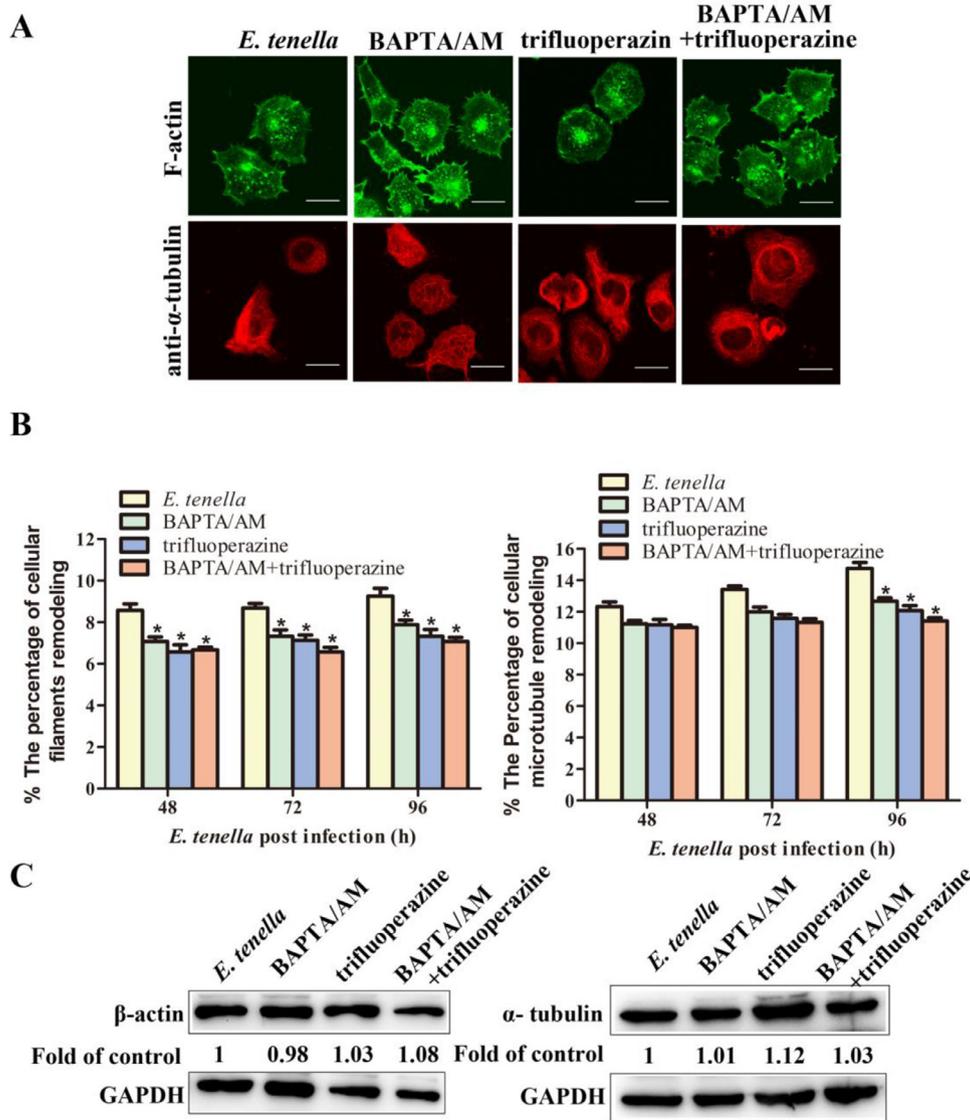
Figure 5. Ca^{2+} affect cytoskeletal remodeling during the schizogenic stage of *E. tenella*. (A) Inhibitors attenuate *E. tenella* infection-induced cytoskeletal remodeling. (B) Quantitative detection of cytoskeletal changes in each group. each value represents the average value from 60 to 80 cells from at least 5 regions of 3 representative experiments. *$P < 0.05$ vs. controls. (C) β-actin and α-tubulin protein levels in each group. Cells in each group were infected with *E. tenella* at 72 h. All of the data are representative of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. normal controls.

structure, apoptosis and necrosis of host cells caused by *E. tenella* schizogenic stage, while the effect of BAPTA/AM+trifluoperazine was stronger (Figures 5and 6). The above results indicated that Ca^{2+} is closely related to the damage of host cells during the schizogenic stage of *E. tenella*.

DISCUSSION

E. tenella is a typical intracellular parasite. The parasite invades the cecal mucosal epithelial cells and releases merozoites through schizont. *E. tenella* inhibits host cell apoptosis during pre-infection. But, the cecum will appear microvilli shedding, swelling of mucosal epithelial cells, necrosis and disintegration, etc during the reproduction of merozoites (Zhang et al., 2015). However, the damage effect of *E. tenella* to cells during the schizogenic stage is not clear. The cytoskeleton plays an important role in all stages of the pathogen's life cycle. Cytoskeleton remodeling often occurs during pathogen development, such as *Toxoplasma gondii, Cryptosporidium* (Stradal and Schelhaas, 2018). Cytoskeleton remodeling affects cellular protein secretion, signaling and other functions (Jones et al., 2019). In this study, immunofluorescence staining and western blotting were used to observe the changes of cytoskeleton during the schizogenic stage of *E. tenella*. The results showed that *E. tenella* caused cytoskeleton remodeling and decreased cytoskeletal protein expression during the schizogenic stage. However, whether this abnormal remodeling and reduced protein expression affect cell function still needs to be further studied.

Intact cells provide cells with a relatively stable internal environment. When cells are damaged, their permeability will change, and intracellular membranous organelles (mainly mitochondria and endoplasmic reticulum) will also be damaged (Wylot et al., 2021). Many
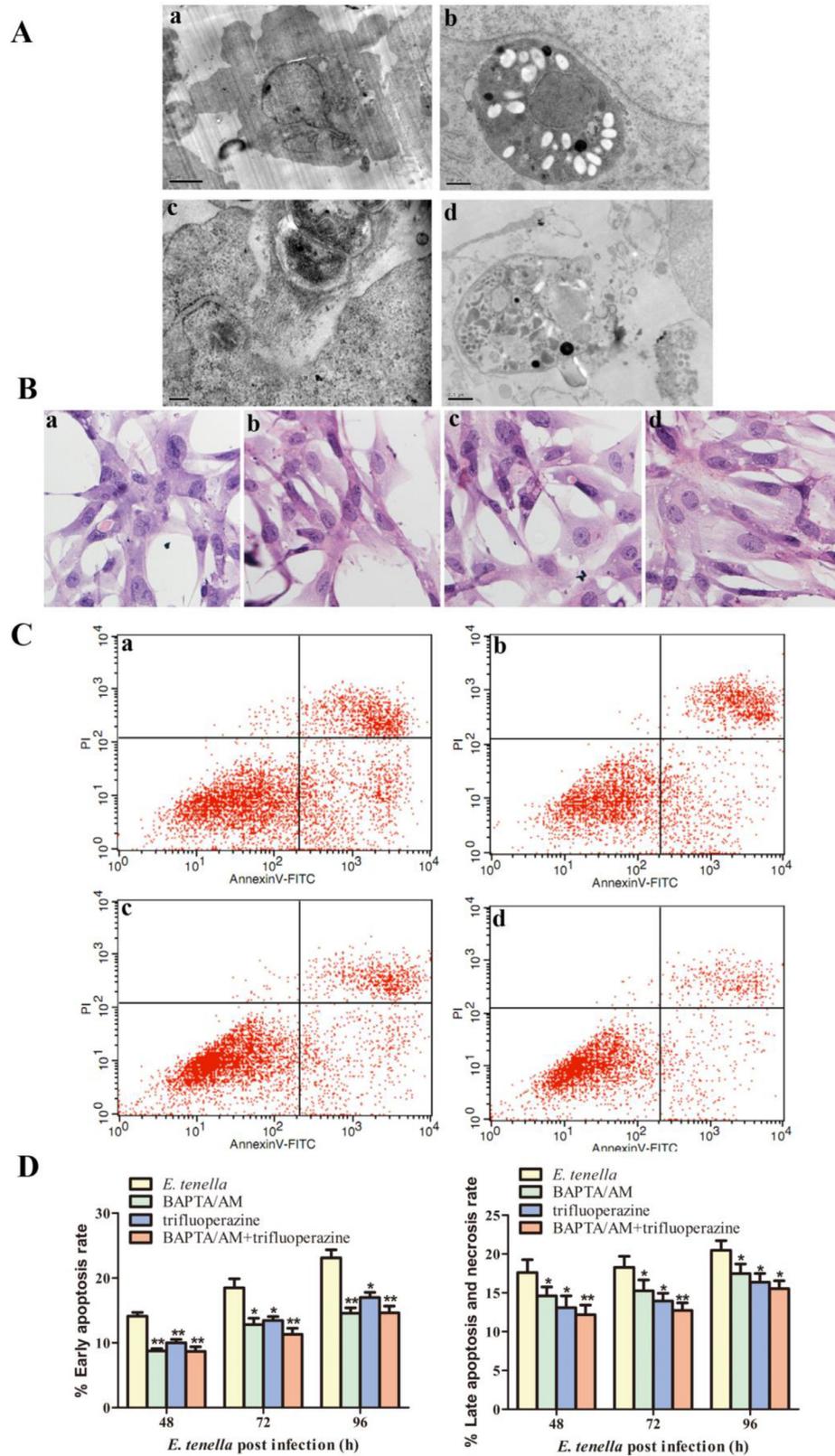
Figure 6. Ca^{2+} affect cell injury during the schizogenic stage of E. tenella. (A-C) Changes in host cell and organelle structure and apoptosis in each group. a: *E. tenella* groups; b-d: BAPTA/AM, trifluoperazine, BAPTA/AM+trifluoperazine groups, respectively. Cells in each group were infected with *E. tenella* at 72 h. (D) Quantitative detection of apoptosis in each group. All of the data are representative of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. normal controls.

extracellular pathogens, for example, *C. botulinum, C. tetani*; many intracellular pathogens, for example, *T. cruzi, L. pneumophila*, infection by these pathogens disrupts cell membrane integrity (Welch, 2015). In this

study, H&E staining and transmission electron microscopy were used to observe the changes in the morphology and organelles of host cells during the schizogenic stage of *E. tenella*. The results showed that the host cells

were swollen, the cell boundary was not obvious, and the nucleus was lysed during the schizogenic stage of *E. tenella*. Furthermore, host cell microvilli were shed, mitochondrial ridges disappeared, the rough endoplasmic reticulum was degranulated and enlarged, irregular pseudopodia-like cell protrusions were formed, intercellular junctions were lost, and gaps were widened. It was confirmed that the morphological structure of host cells could be severely damaged during the schizogenic stage of *E. tenella*.

To survive, parasites interact with host cells and regulate host cell apoptosis through multiple pathways, a process that has been shown to be one of the pathogenic mechanisms of parasites (Kapczuk et al., 2020). *Trypanosomes* can induce apoptosis in mature CD^{4+} T cells (Ana et al., 2021). *Schistosoma japonicum* can promote cell apoptosis by increasing the expression of Fas and FasL in CD^{4+} and CD^{8+} T cells (Lundy et al., 2001). When *Toxoplasma gondii* infected the embryo trophoblast, the expression of Bax and P27 proteins in host cells increased, and the apoptosis rate of host cells increased (Elsalam et al., 2021). After chickens were infected with *E. tenella*, the concentration of calcium ions and reactive oxygen species in the cecal mucosa and intestinal gland epithelial cells of chickens increased, thereby promoting the apoptosis of cecal cells (Zhang et al., 2015). However, it is not clear whether host cell apoptosis is induced during the schizogenic stage of *E. tenella*. This study confirmed that the schizogenic stage of *E. tenella* can cause apoptosis of host cells and increase the necrosis rate. Further confirmation that the schizogenic stage of *E. tenella* can cause host cell damage.

Mitochondria are the most important calcium reservoirs in cells and are crucial for the regulation of the balance of Ca^{2+} in the cytoplasm (Alevriadou et al., 2021). Ca^{2+} plays an important role in maintaining the normal physiological function of cells, which can regulate various types of cell apoptosis (Sukumaran et al., 2021). Under the action of some stimulatory factors, the endoplasmic reticulum releases its stored Ca^{2+}, which are then taken up by mitochondria. Mitochondrial calcium overload leads to its damage, followed by the release of cytochrome c, activation of caspases, and ultimately apoptosis (Marchi et al., 2018). In addition, the schizogenic stage of *E. tenella* can cause mitochondrial damage was confirmed. In this study, it was shown that changing the intracellular Ca^{2+} concentration can affect the damage of cells caused by the schizogenic stage of *E. tenella*. The results confirmed that the schizogenic stage of *E. tenella* damage host cells are closely related to Ca^{2+}. However, whether this damage is caused by mitochondrial dysfunction through elevated Ca^{2+} remains to be investigated.

Collectively, our results confirm that *E. tenella* can severely damage host cells during the schizogenic stage of *E. tenella*, and this effect is closely related to Ca^{2+}. The results can provide a theoretical basis for a better understanding of the invasion mechanism of *E. tenella* and the study of new anticoccidial drugs.

AUTHOR CONTRIBUTIONS

Xiao-ling Lv carried out most of the experiments, wrote the manuscript, and should be considered as primary author. Ming-xue Zheng critically revised the manuscript and the experiment design. Yong-yan Wang, Rui Bai, Li Zhang, Bu-ting Duan, Xuan Lei, Xue-song Zhang, Yong-juan Zhao, Kai-ling Cui, Tong Xu helped with the experiment. All the authors read and approved the final version of the manuscript.

DISCLOSURES

Xiao-ling Lv carried out most of the experiments, wrote the manuscript, and should be considered as primary author. Ming-xue Zheng critically revised the manuscript and the experiment design. Yong-yan Wang, Rui Bai, Li Zhang, Bu-ting Duan, Xuan Lei, Xue-song Zhang, Yong-juan Zhao, Kai-ling Cui, Tong Xu helped with the experiment. All the authors read and approved the final version of the manuscript.

REFERENCES

Alevriadou, B. R., A. Patel, M. Noble, S. Ghosh, V. M. Gohil, P. B. Stathopulos, and M. Madesh. 2021. Molecular nature and physiological role of the mitochondrial calcium uniporter channel. Am. J. Physiol Cell Physiol. 320:C465–C482.

Ana, Y., J. D. Rojas Marquez, L. Fozzatti, R. E. Baigorri, C. Marin, B. A. Maletto, F. M. Cerban, R. Radi, L. Piacenza, and C. C. Stempin. 2021. An exacerbated metabolism and mitochondrial reactive oxygen species contribute to mitochondrial alterations and apoptosis in CD4 T cells during the acute phase of Trypanosoma cruzi infection. Free Radic. Biol. Med. 163:268–280.

Cui, X. Z., M. X. Zheng, Y. Zhang, R. L. Liu, S. S. Yang, S. Li, Z. Y. Xu, R. Bai, Q. H. Lv, and W. L. Zhao. 2016. Calcium homeostasis in mitochondrion-mediated apoptosis of chick embryo cecal epithelial cells induced by Eimeria tenella infection. Res. Vet. Sci. 104:166–173.

Docampo, R., and G. Huang. 2021. The IP3 receptor and Ca(2+) signaling in trypanosomes. Biochim. Biophys. Acta. Mol. Cell Res. 1868:118947.

Docampo, R., and S. N. Moreno. 2021. Calcium signaling in intracellular protist parasites. Curr. Opin. Microbiol. 64:33–40.

Elsalam, S. A., A. E. Mansor, M. H. Sarhan, A. M. Shalaby, M. A. Gobran, and M. A. Alabiad. 2021. Evaluation of apoptosis, proliferation, and adhesion molecule expression in trophoblastic tissue of women with recurrent spontaneous abortion and infected with Toxoplasma gondii. Int. J. Gynecol. Pathol. 40:124–133.

Fu, Y., K. M. Brown, N. G. Jones, S. N. Moreno, and L. D. Sibley. 2021. Toxoplasma bradyzoites exhibit physiological

plasticity of calcium and energy stores controlling motility and egress. Elife 10:e73011.

Jones, M. C., J. Zha, and M. J. Humphries. 2019. Connections between the cell cycle, cell adhesion and the cytoskeleton. Philos. Trans. R. Soc. Lond. B Biol. Sci. 374:20180227.

Kapczuk, P., D. Kosik-Bogacka, P. Kupnicka, E. Metryka, D. Siminska, K. Rogulska, M. Skorka, I. Gutowska, D. Chlubek, and I. Baranowska-Bosiacka. 2020. The influence of selected gastrointestinal parasites on apoptosis in intestinal epithelial cells. Biomolecules 10:674.

Li, S., M. X. Zheng, H. C. Xu, X. Z. Cui, Y. Zhang, L. Zhang, S. S. Yang, Z. Y. Xu, R. Bai, and X. G. Sun. 2017. Mitochondrial pathways are involved in Eimeria tenella-induced apoptosis of chick embryo cecal epithelial cells. Parasitol. Res. 116:225–235.

Lock, J. T., and I. Parker. 2020. IP3 mediated global Ca(2+) signals arise through two temporally and spatially distinct modes of Ca(2+) release. Elife 9:e55008.

Lundy, S. K., S. P. Lerman, and D. L. Boros. 2001. Soluble egg antigen-stimulated T helper lymphocyte apoptosis and evidence for cell death mediated by FasL(+) T and B cells during murine Schistosoma mansoni infection. Infect. Immun. 69:271–280.

Marchi, S., S. Patergnani, S. Missiroli, G. Morciano, A. Rimessi, M. R. Wieckowski, C. Giorgi, and P. Pinton. 2018. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium. 69:62–72.

Modesti, L., A. Danese, V. Angela Maria Vitto, D. Ramaccini, G. Aguiari, R. Gafa, G. Lanza, C. Giorgi, and P. Pinton. 2021. Mitochondrial Ca(2+) signaling in health, disease and therapy. Cells 10:1317.

Orrego, P. R., M. Serrano-Rodriguez, M. Cortez, and J. E. Araya. 2021. In Silico characterization of calcineurin from pathogenic obligate intracellular trypanosomatids: potential new biological roles. Biomolecules 11:1322.

Sandholt, A. K. S., F. Xu, R. Soderlund, A. Lunden, K. Troell, S. G. Svard, and E. Wattrang. 2021. Dual RNA-Seq transcriptome analysis of chicken macrophage-like cells (HD11) infected in vitro with Eimeria tenella. Parasitology. 148:712–725.

Scarpelli, P. H., M. F. Pecenin, and C. R. S. Garcia. 2021. Intracellular Ca(2+) signaling in protozoan parasites: an overview with a focus on mitochondria. Int. J. Mol. Sci. 22:469.

Sharma, A., G. T. Ramena, and R. C. Elble. 2021. Advances in intracellular calcium signaling reveal untapped targets for cancer therapy. Biomedicines 9:1077.

Stradal, T. E. B., and M. Schelhaas. 2018. Actin dynamics in host-pathogen interaction. FEBS Lett. 592:3658–3669.

Sukumaran, P., V. Nascimento Da Conceicao, Y. Sun, N. Ahamad, L. R. Saraiva, S. Selvaraj, and B. B. Singh. 2021. Calcium signaling regulates autophagy and apoptosis. Cells 10:2125.

Welch, M. D. 2015. Why should cell biologists study microbial pathogens? Mol Biol Cell. 26:4295–4301.

Wylot, M., D. T. E. Whittaker, S. A. C. Wren, J. H. Bothwell, L. Hughes, and J. L. Griffin. 2021. Monitoring apoptosis in intact cells by high-resolution magic angle spinning (1) H NMR spectroscopy. NMR Biomed. 34:e4456.

Xu, Z. Y., M. X. Zheng, L. Zhang, X. Gong, R. Xi, X. Z. Cui, and R. Bai. 2017. Dynamic expression of death receptor adapter proteins tradd and fadd in Eimeria tenella-induced host cell apoptosis. Poult. Sci. 96:1438–1444.

Zhang, L., M. X. Zheng, R. Xi, Z. Y. Xu, X. S. Zhang, L. L. Zheng, R. Bai, C. L. Mi, F. F. Hao, and Y. P. Feng. 2019. Comparison of the host cells apoptosis induced by precocious strains and virulent strains of Eimeria tenella. Poult. Sci. 98:4384–4390.

Zhang, X., S. Li, M. Zheng, L. Zhang, R. Bai, R. Li, S. Hao, B. Bai, and H. Kang. 2020. Effects of the PI3K/Akt signaling pathway on the apoptosis of early host cells infected with Eimeria tenella. Parasitol. Res. 119:2549–2561.

Zhang, Y., M. X. Zheng, Z. Y. Xu, H. C. Xu, X. Z. Cui, S. S. Yang, W. L. Zhao, S. Li, Q. H. Lv, and R. Bai. 2015. Relationship between Eimeria tenella development and host cell apoptosis in chickens. Poult. Sci. 94:2970–2979.