

# Ultrastructural effects of acetamizuril on endogenous phases of *Eimeria tenella*

Lili Liu<sup>1</sup> · Huiya Chen<sup>1</sup> · Chenzhong Fei<sup>1</sup>  · Xiaoyang Wang<sup>1</sup> · Wenli Zheng<sup>1</sup> · Mi Wang<sup>1</sup> · Keyu Zhang<sup>1</sup> · Lifang Zhang<sup>1</sup> · Tao Li<sup>1</sup> · Feiqun Xue<sup>1</sup>

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**Abstract** To explore the primary stage or site of action of acetamizuril (AZL), a novel triazine anticoccidial compound, the ultrastructural development of *Eimeria tenella* at different endogenous stages was studied in experimentally infected chickens treated with a single oral dose of 15 mg/kg AZL. As a result of drug action, the differentiations of second-generation schizonts and microgamonts were largely inhibited and merozoites became irregular in shape. Meanwhile, the outer membrane blistering and perinuclear space enlargement were obvious in the second-generation schizonts and microgamonts, which were never observed in the classic triazine anticoccidiosis drug diclazuril-treated *E. tenella*. The chromatin aggregation, anachromasis, and marginalization were visible in merozoites and microgamonts. Furthermore, the abnormal evagination of microgametes finally resulted in the degeneration of microgamonts and the failure of subsequent fertilization. The most marked micromorphological alteration occurring in the macrogamonts was the WFB2. Even if the fertilization occurred, the formation of oocyst wall became malformed and the zygote proceeded to the obvious degeneration. In addition, mitochondria swelling and cytoplasm vacuolization were discerned in respective intracellular stages, while endoplasmic reticulum and Golgi body swelling was less seen. These alterations may be the causes leading to the final death

of *E. tenella* and also provide some information for further exploring the mechanism of action of AZL at the molecular level.

**Keywords** Acetamizuril · *E. tenella* · Endogenous phase · Ultrastructure

## Introduction

*Eimeria* species are protozoan parasites that could cause the severely intestinal disease known as coccidiosis, especially the *Eimeria tenella*, which is the most significant and pathogenic species (Alcala-Canto et al. 2014; Matsubayashi et al. 2012). *E. tenella* is an obligate intracellular parasite that infects caecum epithelial cells and results in the extensive destruction and the great economic loss to the poultry industry annually (Dalloul and Lillehoj 2006; Zaman et al. 2012). Until now, the control of coccidiosis heavily depends on the anticoccidials including polyether ionophore antibiotics and synthetic chemicals, but the frequent emergence of resistant strains brings the huge challenge for the control of coccidiosis (Abbas et al. 2012; Quiroz-Castaneda and Dantan-Gonzalez 2015; Tewari and Maharana 2011). These have prompted a constant search for products that could replace drugs against which organisms have acquired resistance.

Acetamizuril (AZL), a novel triazine anticoccidial compound, is developed by Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences (CAAS). Preclinical safety and effectivity studies showed that it has an acceptable safety and high efficacy on the coccidiosis with the anticoccidial index (ACI) above 180 and possesses a wide development prospect (unpublished data).

To learn about the efficacy and the mechanism of action of drugs from the perspective of morphology, many researchers

✉ Chenzhong Fei  
aries@shvri.ac.cn

✉ Feiqun Xue  
fqxue@shvri.ac.cn

<sup>1</sup> Key Laboratory of Veterinary Drug Safety Evaluation and Residues Research, Chinese Academy of Agricultural Sciences, Shanghai Veterinary Research Institute, CAAS, No.518 Ziyue Rd, Minhang Dist., Shanghai 200241, People's Republic of China

have investigated the effects of coccidiostats on the *Eimeria* spp. ultrastructure (Danforth et al. 1997; Leathem and Engle 1970), especially the ionophore drugs (Daszak et al. 1991; Mehlhorn et al. 1983). Although the ultrastructural effects of diclazuril on the endogenous phases of *E. tenella* have been evaluated previously (Verheyen et al. 1988), whether the novel triazine compound AZL elicits different alterations is unknown. In the present study, we aim to observe the ultrastructural effects of AZL on endogenous phases of *E. tenella* and expect to find the primary stage or site of action of AZL. Finally, we hope that this study would provide some information for the subsequent explorations.

## Materials and methods

### Experimental materials

**Parasites** *E. tenella* was maintained at the Key Laboratory of Animal Parasitology of the Ministry of Agriculture. Sporulated oocysts were stored at 4 °C in 2.5 % potassium dichromate. These cells were washed three times with phosphate-buffered saline (PBS; pH 7.2) before use and diluted to the required concentration in 1-mL solution.

**Chickens** One-day-old Pudong yellow broiler chickens were purchased from a local hatchery. The birds were reared in cages under hygienic conditions with ad libitum access to water and a standard diet without drug supplements.

**Experimental compound** AZL (>98 %) was synthesized by our laboratories (Shanghai Veterinary Research Institute, CAAS).

### Experimental methods

At 14 days of age, chickens were randomly divided into the control group and the AZL group: All chickens were challenged with  $8 \times 10^4$  *E. tenella* sporulated oocysts per chicken by oral gavage. For the AZL group, the chickens were given a single dose of 15 mg/kg b.w. AZL at either 8, 20, 32, 44, 56, 68, 80, 92, 104, 116, 128, 140, or 152 h post-infection (p.i.). Two chickens in each time point were killed at 16 h after the medication; meanwhile, two unmediated and inoculated chickens from the control group served as controls.

For electron microscopical observation, the caecum tissue samples (about 1 mm<sup>3</sup>) were rapidly collected from anatomized chickens and immersed in 2.5 % glutaraldehyde in phosphate buffer at pH 7.4 overnight at 4 °C for fixing. After washing with PBS, the samples were subsequently post-fixed with 1 % osmium tetroxide (OsO<sub>4</sub>) and then dehydrated in a graded alcohol series, saturated in acetone, and embedded in Epon. The ultrathin sections were obtained

and stained with uranyl acetate and lead citrate and, finally, the sections were examined with a Philips CM 120 microscope.

## Results

### The schizogamy stage

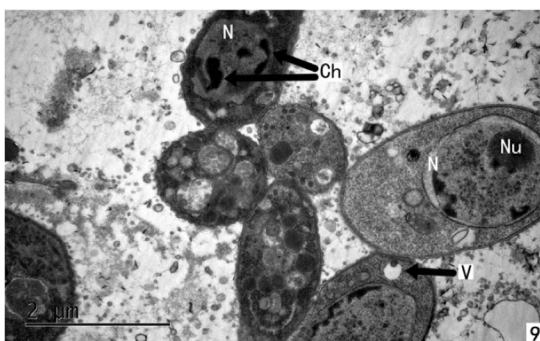
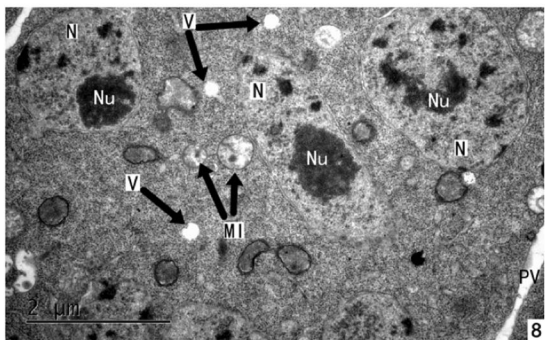
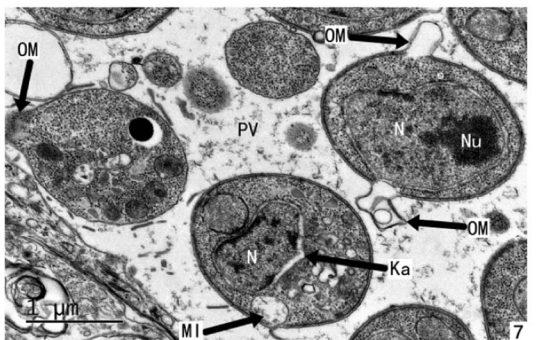
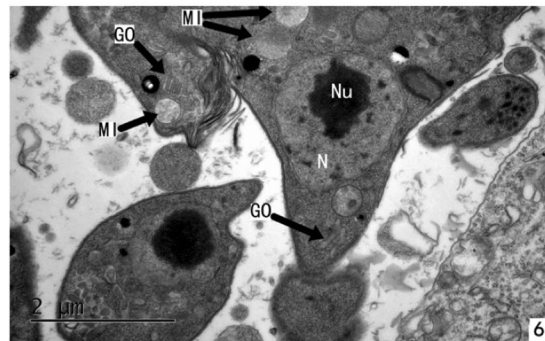
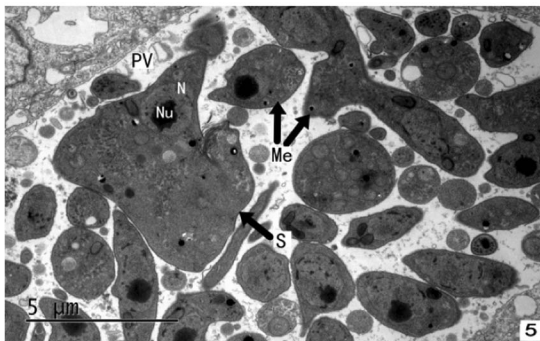
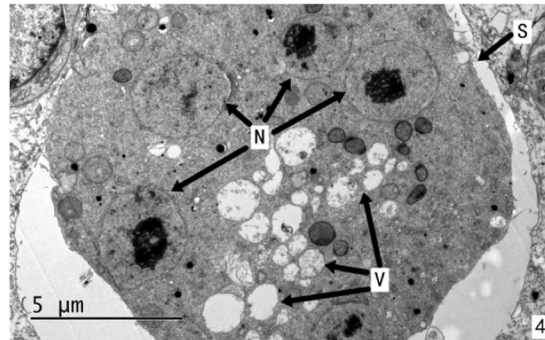
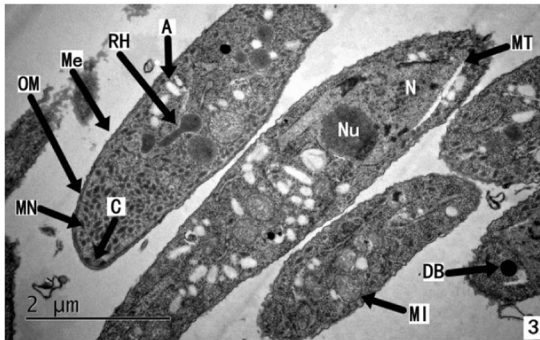
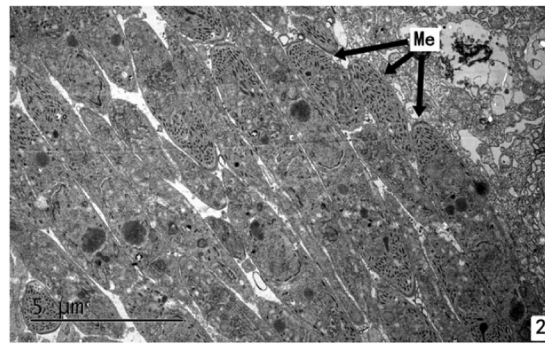
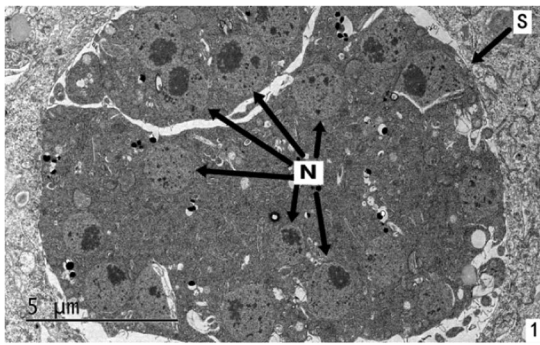
As we could see from the electron microscopical graphs, the second-generation schizont divisions and the micromorphology of merozoites were normal in the control group (Figs. 1, 2, and 3). At the apical end of merozoites, the subcellular structures such as microneme, rhoptry, and microtubules were clear and normal. In the cytoplasm of merozoites, we could also observe that the mitochondrias and nuclei have no ultrastructural changes (Figs. 2 and 3).

After the AZL treatment, the second-generation schizonts divided into merozoites were progressed abnormally and many large vacuoles appeared in the cytoplasm (Figs. 4 and 8). The micromorphology of merozoites was heavily affected and became irregular, and the internal structures of merozoites were vague. Microneme reduction, rhoptry disappearance, mitochondria swelling, and crista structure blur occurred (Figs. 5 and 6). Sometimes, the slight swelling Golgi body was also visible in the merozoites (Fig. 6). Another significant damage in the merozoites was the outer membrane blistering and perinuclear space enlargement (Figs. 7 and 9). The condensation of the chromatin material was found in the nuclei of merozoites (Fig. 9).

### The gametogony stage and the oocyst wall formation

For the unmedicated chickens, microgametogenesis and macrogametogenesis were proceeded normally to form the macrogametes and microgametes. In the process of microgametogenesis, the nuclear divisions of microgamonts

**Fig. 1–9** Transmission electron micrographs of the schizogamy stage of *E. tenella* (96–120 h p.i.). Untreated controls (Fig. 1–3). Dividing schizonts (Fig. 1, 96h p.i.). Merozoites (Fig. 2, 108h p.i.). Merozoites (Fig. 3, 120h p.i.). Sections from AZL mediated chickens (Fig. 4–9). Abnormal schizonts with many large vacuoles (Fig. 4, treated at 80 h p.i. and killed at 96 h p.i.). Divided abnormally schizonts and aberrant merozoites (Fig. 5–6, treated at 92 h p.i. and killed at 108 h p.i.). Merozoites with the injured outer membrane and karyolemma (Fig. 7, treated at 104 h p.i. and killed at 120 h p.i.). The formation of merozoites was inhibited and the nuclei were remained in the large residual cytoplasm (Fig. 8, treated at 104 h p.i. and killed at 120 h p.i.). Merozoites with the condensed and hyperchromatic chromatin (Fig. 9, treated at 104 h p.i. and killed at 120 h p.i.). Abbreviations of figures: *A*: amylopectin; *C*: Conoid; *Ch*: chromatin; *DB*: Dark body; *ER*: endoplasmic reticulum; *F*: flagellum; *GO*: Golgi body; *Ka*: karyolemma; *L*: fatty granule; *Ma*: macrogamont; *Me*: Merozoite; *Mi*: microgamont; *MN*: Microneme; *Mie*: microgamete; *MI*: mitochondria; *MT*: microtubules; *N*: nucleus; *Nu*: nucleolus; *OM*: the outer member; *OOW*: the outer layer of oocyst wall; *OW*: the oocyst wall; *PV*: the parasitophorous vacuole; *RH*: Rhoptry; *S*: Schizont; *V*: vacuole; *WFB1*: the wall formation body1; *WFB2*: the wall formation body1; *Z*: zygote



were not affected and the nuclei were normally migrated to the periphery of microgamonts (Fig. 10). As the differentiation of microgamonts, the flagella began to form surrounding the microgamonts (Fig. 11). Finally, the nucleus and mitochondria with associated flagella evaginated into the parasitophorous vacuole and formed the mature microgametes (Fig. 12). Meanwhile, the obvious characteristic was the appearance of wall-forming body 1 (WFB1) and wall-forming body 2 (WFB2) in the development process of macrogamonts. A large number of amylopectin granules and microtubules began to appear in the cytoplasm of macrogamonts (Figs. 11 and 13). There is no obvious boundary between macrogamont and macrogamete. The formation of oocyst wall also occurred normally after the macrogamete fertilization (Figs. 14 and 15).

In AZL-treated chickens, the outer membrane blebbing, especially the nuclear envelope enlargement, was marked in the microgamonts (Figs. 16, 17 and 19). In addition, cytoplasm vacuolization (Fig. 17), Golgi body, and endoplasmic reticulum cistern slight swelling (Figs 17 and 19) could also be observed in the microgamonts. The prominent abnormality in the microgamonts was chromatin aggregation, anachromasis, and marginalization, and the chromatin presented in crescent shape was attached around the nuclear membrane (Figs. 20 and 21), which showed the signs of apoptosis. The evagination of microgamete was further prevented and finally resulted in the degeneration of microgamonts (Figs. 20 and 21).

The ultrastructural alterations in the developmental process of macrogamonts were also seen in chickens with the AZL treatment. The micromorphology of macrogamonts was irregular and subcellular organelles were blurred (Figs. 22 and 23). The WFB2 formation was disturbed to a great degree. The number of WFB2 was apparently reduced and even partial WFB2 were fused (Fig. 23). The internal granules of WFB2 were disintegrated and the cytoplasm presented vacuolization, while WFB1 seemed to be less affected (Fig. 24). The aberrant WFB2 further hindered the formation of the oocyst wall and finally resulted in the zygote degeneration or necrosis (Fig. 25). Moreover, we also observed that the oocyst wall shrinking occurred in the AZL group (Fig. 26).

## Discussion

AZL, as a novel triazine anticoccidial compound, is likely to be a potential novel anticoccidial agent. In this article, we found that the ultrastructural alterations in the schizogamy and gametogony stages of *E. tenella* were obviously detected after the AZL treatment. These subcellular structure changes in parasite can provide direct clues or evidence for action site and the possible mechanism of action of drugs.

In the untreated controls, we could observe that the second-generation schizonts were normally divided into the

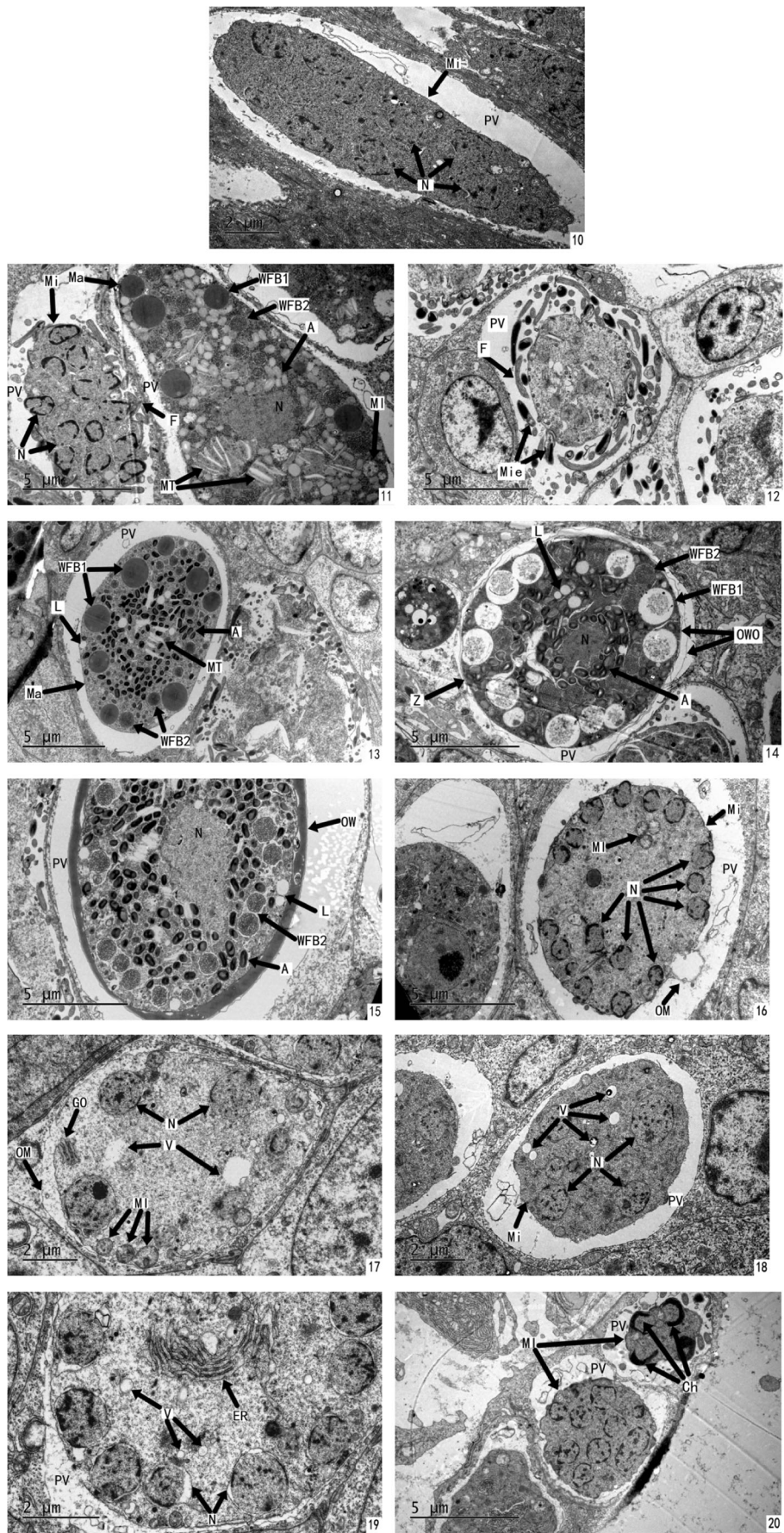
**Fig. 10–26** Transmission electron micrographs of the gametogony stage of *E. tenella* (132–168 h p.i.). Untreated controls (Fig. 10–15). Microgamonts with the dividing nucleus (Fig. 10, 132h p.i.). Developing microgamonts and macrogamont (Fig. 11, 144 h p.i.). Macrogamonts being differentiated into macrogametes (Fig. 12, 156 h p.i.). Developing macrogamont (macrogamete) (Fig. 13, 156 h p.i.). Zygote showing the oocyst wall formation has begun (Fig. 14, 156 h p.i.). Oocyst with the forming oocyst wall (Fig. 15, 168 h p.i.). Sections from AZL mediated chickens (Fig. 16–26). Developing microgamonts. The outer membrane and karyolemma of microgamonts were severely damaged and many vacuoles appeared in the cytoplasm wherein the slight swelling of Golgi apparatus and endoplasmic reticulum was seen (Fig. 16–19, treated at 128 h p.i. and killed at 144 h p.i.). Degenerating microgamonts with chromatin aggregation, anachromasis, marginalization (Fig. 20, treated at 140 h p.i. and killed at 156 h p.i.). For abbreviations, see Fig. 1

merozoites. The morphology of merozoites and the subcellular structures such as conoid, microneme, and rhoptry have been described by McLaren (1969) and Long (1971). With the effect of AZL, the second-generation schizont differentiation into merozoites was clearly affected, which was coherent with that previously described for *E. tenella* (Ball et al. 1985, 1997; Verheyen et al. 1988). A few merozoites matured from second-generation schizonts in AZL-treated chickens infected by *E. tenella* were presented in abnormal shape. This is possible that AZL affected the membranous skeleton structure of *E. tenella*. It is reported that actin depolymerizing factor (ADF) plays a critical role in remodeling the actin cytoskeleton and helping the apicomplexan parasites invade host cells (Xu et al. 2008). Furthermore, the previous study in our laboratory indicated that diclazuril could affect the expression of ADF in the second-generation merozoites of *E. tenella* (Zhou et al. 2010b). Therefore, we think that AZL may have the similar role with diclazuril in this respect.

Microneme and rhoptry were two secretory organelles, which localized at the apical end of apicomplexan protozoans. They play different roles in the process of parasite invasion by secreted proteins: Micronemes are responsible for host cell recognition, binding, and motility, and rhoptries involve in the parasitophorous vacuole formation (Dubremetz et al. 1998; Gubbels and Duraisingh 2012). These two subcellular organelles observed in the second-generation schizonts and merozoites were evidently reduced and even disappeared after AZL treatment. This will disturb the invasion of *E. tenella* to a certain extent.

Different from diclazuril, AZL caused the outer member blister of merozoites and microgamonts, which was the typical damage caused by ionophore antibiotics (Daszak et al. 1991; Smith et al. 1981) due to the imbalanced osmotic pressure. The enlargement of the perinuclear space was another common micromorphological change in the merozoites and microgamonts. This phenomenon was not also observed in *Eimeria* species with the diclazuril action (Verheyen et al. 1989) but was found after the application of arprinocid (Ball et al. 1985).

As the center of energy metabolism, the major function of mitochondria is ATP generation via oxidative phosphorylation

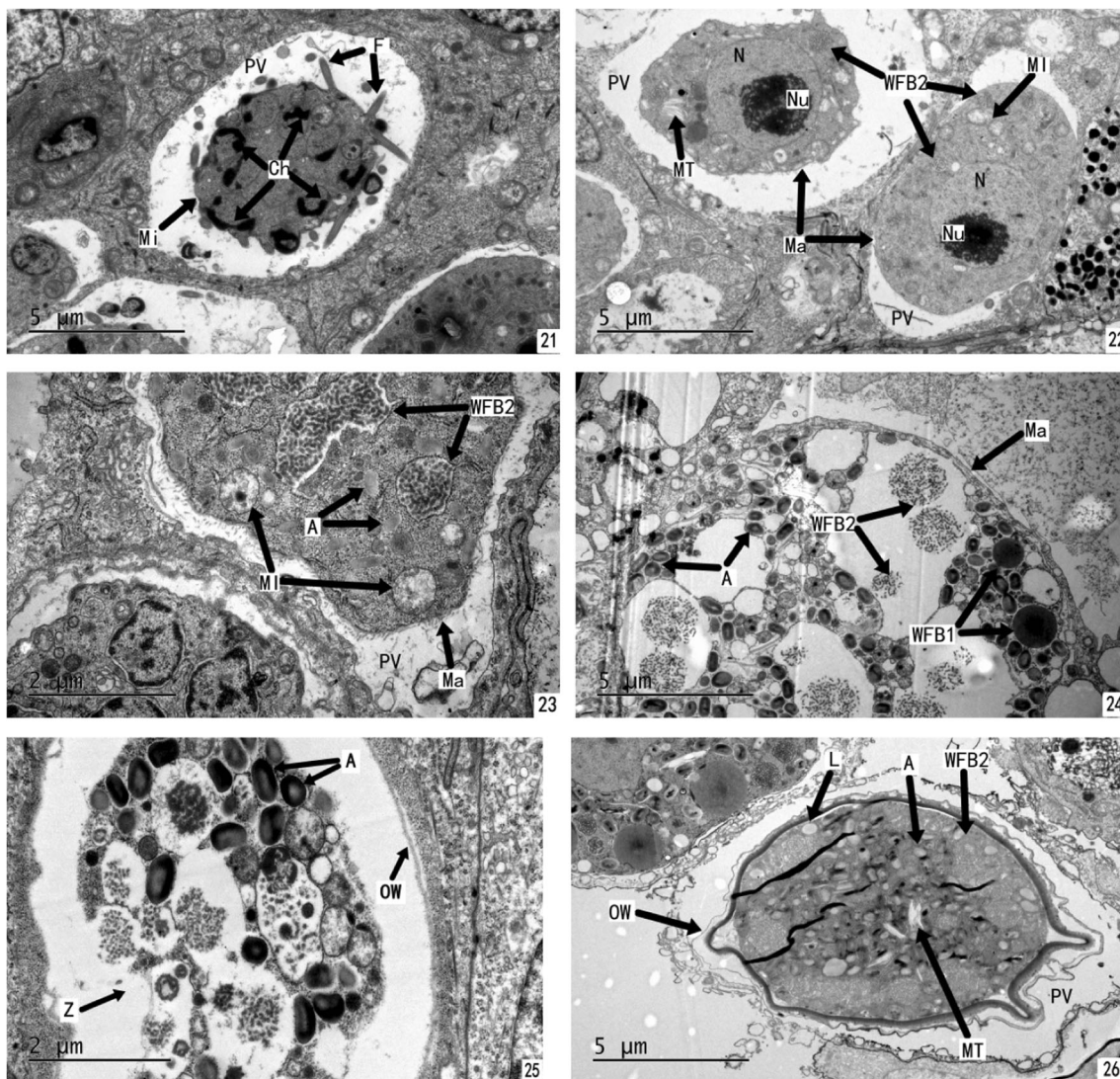


to support the various life activities (Kita et al. 2003). Besides, mitochondria are related to the programmed cell death (Makiuchi and Nozaki 2014) and the early apoptosis of parasites (Zhou et al. 2010a). Therefore, mitochondria are prone to be disturbed in the process of drug action (Danforth et al. 1997; Daszak et al. 1991; Mehlhorn et al. 1984). In this review, we also observed that the mitochondria were swelling and presented the vacuolization in the respective stages of *E. tenella*. In addition, we discerned the merozoite chromatin heterochromatinization, which was more obvious in microgamonts. This phenomenon is often observed in some apoptotic cells or parasites (Jiménez-Ruiz et al. 2010).

In the medicated chickens, the early nuclear divisions of microgamonts appeared to be unaffected, but the subsequent

nuclear evagination hampered the microgamete maturation and resulted in the degeneration of microgamonts. This is in agreement with that previously reported for diclazuril in *E. tenella* (Verheyen et al. 1988). In the course of macrogametogenesis, the WFB2 is firstly formed and subsequently the WFB1 formation (Ferguson et al. 1977a; Pittilo and Ball 1979). The WFB2 underwent the apparent alterations in both the number and the size with the AZL action. Moreover, partial WFB2 were fused and lead to the cytoplasm vacuolization in the macrogamonts; however, the WFB1 were less affected, as the similar effect with triazinones on *E. tenella* (Mehlhorn et al. 1984).

Although Zhou et al. (2006) thought that the WFB2 play a vital role in oocyst wall-forming process and the WFB1 may limit the oocyst wall formation, most of researchers believed



**Fig. 10–26 (continued)** The formation of microgametes was inhibited obviously (Fig. 21, treated at 140 h p.i. and killed at 156 h p.i.). Developing macrogamont with the irregular outer member and blurred subcellular organelles (Fig. 22, treated at 116 h p.i. and killed at 132 h p. i.). Developing macrogamont (macrogamete) with the fused WFB2 and swelling mitochondria (Fig. 23, treated at 128 h p.i. and killed at 144 h p.

i.). Developing macrogamont (macrogamete) with the disintegrated WFB2 and large vacuoles in the cytoplasm (Fig. 24, treated at 140 h p.i. and killed at 156 h p.i.). Necrotic and degenerating zygote with irregular oocyst wall formation (Fig. 25, treated at 152 h p.i. and killed at 168 h p.i.). Developing oocyst with the shrinking oocyst wall (Fig. 26, Treated at 152h p.i. and killed at 168h p.i.). For abbreviations, see Fig. 1

that both WFB1 and WFB2 are the major components of the oocyst wall formation. After the macrogamet fertilization, the WFB1 and WFB2 move to the plasma member and the oocyst wall formation is begun: First of all, the granules from WFB1 form the outer layer of the oocyst wall; afterward, the WFB2 form the inner layer (Ferguson et al. 1977b; Michael 1978; Pittilo and Ball 1980). In medicated chickens, nevertheless, the aberrant WFB2 further impeded the formation of the oocyst wall, as have found in previous studies (Ball et al. 1985, 1987; Verheyen et al. 1989). Even if the fertilization took place, the zygote progressed the complete degeneration owing to the malformed oocyst wall. In addition, it was uncertain that individual oocyst wall shrinking in the AZL group was due to the AZL action or inappropriate treatment (Ferguson et al. 2003).

It has been suggested that the same anticoccidial (Verheyen et al. 1988, 1989) or chemically related anticoccidials (Chappel et al. 1974; Ryley et al. 1974) have a different site or stages of action in different *Eimeria* species. As expected, the chemically related anticoccidials may have a different effect on the same *Eimeria* species, such that the outer membrane blister and the perinuclear space enlargement of merozoites and microgamonts caused by AZL were not found in diclazuril-treated *E. tenella*. Although the specific mode of action of AZL is unknown, above findings provide some clues for further exploring the mechanism of action of AZL.

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#### Compliance with ethical standards

**Ethical approval** All procedures were conducted according to the guidelines of the Animal Ethics Committee of the Faculty of Veterinary Medicine and Institutional Animal Care and Use Committee of China.

**Conflict of interest** The authors declare that they have no competing interests.

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