



Toltrazuril and diclazuril: comparative evaluation of anti-coccidial drugs using a murine model

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ABSTRACT. Intestinal coccidiosis caused by *Eimeria* protozoan species is an economically important disease, especially in poultry and cattle. Anti-coccidial drugs commonly used for controlling coccidiosis are toltrazuril (TTZ) and diclazuril (DCZ). In this study, the efficacies of TTZ and DCZ were compared using a murine model, and the effect of these treatments on the induction of acquired resistance was evaluated. Male C57BL/6J mice were inoculated with 1,000 sporulated *E. vermiformis* oocysts and treated with TTZ or DCZ. The recommended TTZ dose for cattle (15 mg/kg) completely prevented oocyte excretion. But, mice required 5 mg/kg of DCZ, which is five times the recommended dose for cattle, to reduce oocyte excretion. In *E. vermiformis* re-infection, TTZ (15 mg/kg) and DCZ (5 mg/kg) treatments did not interfere with the development of acquired resistance. Bodyweight gain was significantly higher in the TTZ-treated group than in the control (untreated/infected) group and the DCZ-treated group, and no significant difference in bodyweight gain was observed between the TTZ-treated group and the healthy (uninfected/untreated) group. Analysis of T lymphocyte subsets in the spleen and mesenteric lymph nodes indicated that the relative populations of CD4⁺ and CD8⁺ T cells were reduced in the DCZ-treated and control (untreated/infected) groups, suggesting there was immunosuppression during the infection. However, no reductions in T cell populations were observed in the TTZ-treated group. The results indicated that an optimal anti-coccidial drug is one that can completely break the parasite life cycle in the host animal.

KEYWORDS: body-weight gain, diclazuril, *Eimeria vermiformis*, host immunity, toltrazuril

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Intestinal coccidiosis caused by parasites of the genus *Eimeria* [16, 31] is an extremely serious disease in livestock animals owing to the huge economic losses suffered [3, 4]. Control of the disease using anti-coccidial drugs is frequently necessary to prevent the prevalence of coccidia, especially in highly intensive farming systems with high stocking rates and the frequent relocation of young animals [15, 18]. Drugs used to control coccidiosis include amprolium, nicarbazin, and sulfonamides [9, 13, 14]. Owing to the extensive usage of some anticoccidial products, drug resistance has developed, and thus, toltrazuril (TTZ) and diclazuril (DCZ) are most commonly used to control coccidiosis at present [23, 24, 38]. Both TTZ and DCZ belong to the triazine chemical class, but they have different pharmacodynamic and pharmacokinetic properties [24, 38].

Reportedly, TTZ and DCZ have different activity mechanisms and are effective at different stages of *Eimeria* infections [16, 20, 36, 37]. TTZ affects the perinuclear space, mitochondria, and endoplasmic reticulum of the parasites inhibits all phases of differentiation, including nuclear division [21], and is effective against all life stages of *Eimeria* [39]. DCZ, in contrast, does not impact the early phase of differentiation, such as nuclear division in schizonts and microgamonts, but interrupts later phases, thereby preventing the further development of the parasites [38]. In addition, DCZ is effective against particular stages of *Eimeria* spp., such as the first-generation meront, second-generation meront, and gamont stages [17, 38].

During its first encounter with *Eimeria*, the host body develops acquired immunity. The expected effects of TTZ and DCZ involve killing of the parasite but also allowing the development of acquired immunity against subsequent infections [23, 37]. The efficacies

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of TTZ and DCZ have been mainly assessed in trials using either TTZ [1, 20, 21] or DCZ [5, 6, 26] or comparisons of the using the two drugs to treat animals in the field [22, 23]. Owing to the difficulty in excluding unrelated factors that affect the onset of coccidiosis and the induction of immune responses in field animals, there is a lack of accurate comparative studies regarding the anti-coccidial effects of TTZ and DCZ and their effects on the development of acquired immunity in re-infections. In this study, the efficacy of TTZ and DCZ treatment and the effect of the treatments on the induction of protective immunity in re-infections were evaluated using a murine coccidiosis model.

MATERIALS AND METHODS

Experimental animals and parasite infection

Male C57BL/6J mice, 7–8 weeks old, approximately 25 g in body weight (BW), were purchased from SLC Inc. (Hamamatsu, Japan). All animal experiments were conducted in accordance with the ethical guidelines for animal experimentation of the University of Miyazaki (2017-038). The mice were housed in clean cages and fed with a standard diet and tap water *ad libitum*. They were kept under conventional conditions with a 12/12 hr light/dark cycle in an air-conditioned room ($23 \pm 3^\circ\text{C}$).

E. vermiformis was maintained in our laboratory by oral passage every 3 months in C57BL/6 mice. Oocytes were purified and sporulated as has been previously reported [28]. Mice in the infected groups were orally inoculated with 1,000 sporulated oocytes of *E. vermiformis*. Fresh feces were individually collected every 24 hr from day 7 post primary infection until the samples tested negative for oocytes. Mice in single-infection and re-infection control group were untreated/infected, and those in the healthy group were untreated/uninfected. Excepting those in the single-infection control group, the mice were re-inoculated with 1,000 sporulated oocytes 21 days after the first inoculation, and feces were collected from day 29 until the samples tested negative for oocytes. Infection with *E. vermiformis* was confirmed by the number of oocysts per gram of feces (OPG) using a modified McMaster's method [19]. Then, the number of oocytes excreted per day (OPD) was calculated by multiplying the OPG value by the total weight of feces excreted in 24 hr.

Anti-coccidial drug preparation

TTZ (Baycox, Bayer, Greenfield, IN, USA) and DCZ (Vecoxan, Elanco, Leverkusen, Germany) were diluted with distilled water (DW) to different doses (TTZ: 3 mg/kg, 15 mg/kg, and 75 mg/kg BW and DCZ: 0.2 mg/kg, 1 mg/kg, and 5 mg/kg BW), which were administrated to the mice orally on day 3 after inoculation. DW was administrated to the control (untreated/infected) group.

Body weight gain

BW was measured for each mouse individually on day 0 and day 37. The BW gain of each mouse was calculated, based on the average BW gain compared with the healthy (untreated/uninfected) group.

Flow cytometry

The spleens and mesenteric lymph nodes (MLNs) were collected on days 10, 21, 24, and 28, and cells were suspended in phosphate buffer saline (PBS) (Fujifilm Wako Pure Chemical Co., Tokyo, Japan) supplemented with 0.5% bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan) and 0.05% sodium aside (Fujifilm Wako Pure Chemical Co.) (BSA-PBS). Viable cells ranging in concentration from 1×10^5 to 1×10^6 were specified and counted by trypan blue exclusion test and incubated with fluorescently labeled monoclonal antibodies at 4°C for 1 hr. Anti-CD4 labeled FITC (1:400 dilution, GK1.5, Biolegend, San Diego, CA, USA) and anti-CD8 labeled PE (1:100 dilution, 53–6.7, Biolegend) antibodies were applied. The stained cells were washed three times with BSA-PBS and re-suspended in BSA-PBS containing propodeum iodide ($1 \mu\text{g}/\text{mL}$; Sigma-Aldrich, St. Louis, MO, USA). The relative immunofluorescence intensities were determined by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Data were analyzed using the statistical package R for windows (www.r-project.org). Multiple comparisons using the Tukey HSD and Paired Wilcoxon (F) tests were used to determine significant differences between the experimental groups. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effective treatment dose of TTZ and DCZ against *E. vermiformis* infection

To evaluate the appropriate dosages of TTZ and DCZ treatments for *E. vermiformis* infection, 35 mice were divided into three groups: TTZ-treated ($n=15$), DCZ-treated ($n=15$), and control (untreated/infected) groups ($n=5$). Each treatment group was divided into three subgroups for testing the different doses (Table 1). Oocyst excretions were not observed in the TTZ-treated group treated with 15 mg/kg or 75 mg/kg doses, whereas mice treated with 3 mg/kg of TTZ only showed significant reduction in OPD compared to the control (untreated/infected) group (Fig. 1a). Mice treated with doses of 0.2 mg/kg and 1 mg/kg DCZ did not show a notable decrease

Table 1. Effective doses of toltrazuril (TTZ) and diclazuril (DCZ) for treating *Eimeria vermiformis* infections in different treatment groups

Group	Number of mice	Dose
TTZ	5	3 mg/kg
	5	15 mg/kg
	5	75 mg/kg
DCZ	5	0.2 mg/kg
	5	1 mg/kg
	5	5 mg/kg
Control (untreated/infected)	5	(–)

in oocyte excretion, and their OPD remained at the same level as that of the control (untreated/infected) group (Fig. 1b). Only the 5 mg/kg DCZ-treated mice showed significantly decreased OPD in comparison to the control (untreated/infected) group (Fig. 1b). In addition, 15 mg/kg and 45 mg/kg doses of DCZ were tested to evaluate the appropriate dose (data not shown); however, these doses did not elicit a sufficient anti-coccidial effect, even when a 45 mg/kg dose of the undiluted solution was administered to mice.

Effect of TTZ and DCZ treatment on the induction of acquired resistance

To determine the effects of TTZ and DCZ treatment on the induction of acquired resistance against re-infection, 25 mice were divided into five groups (Table 2). TTZ (15 mg/kg)-treated, DCZ (5 mg/kg)-treated, and re-infection control (untreated/infected) groups were inoculated with *E. vermiformis* oocysts twice: on days 0 and 21. Mice in the single-infection control (untreated/infected) group were only inoculated with oocysts on day 21. The healthy (uninfected/untreated) group was not inoculated with the oocysts at all and not treated with any drug. The TTZ- and DCZ-treated groups were treated on day 3. After the second inoculation with oocysts, oocyte excretion was not observed in any of the re-infection groups, i.e., TTZ-treated, DCZ-treated, and re-infection control (untreated/

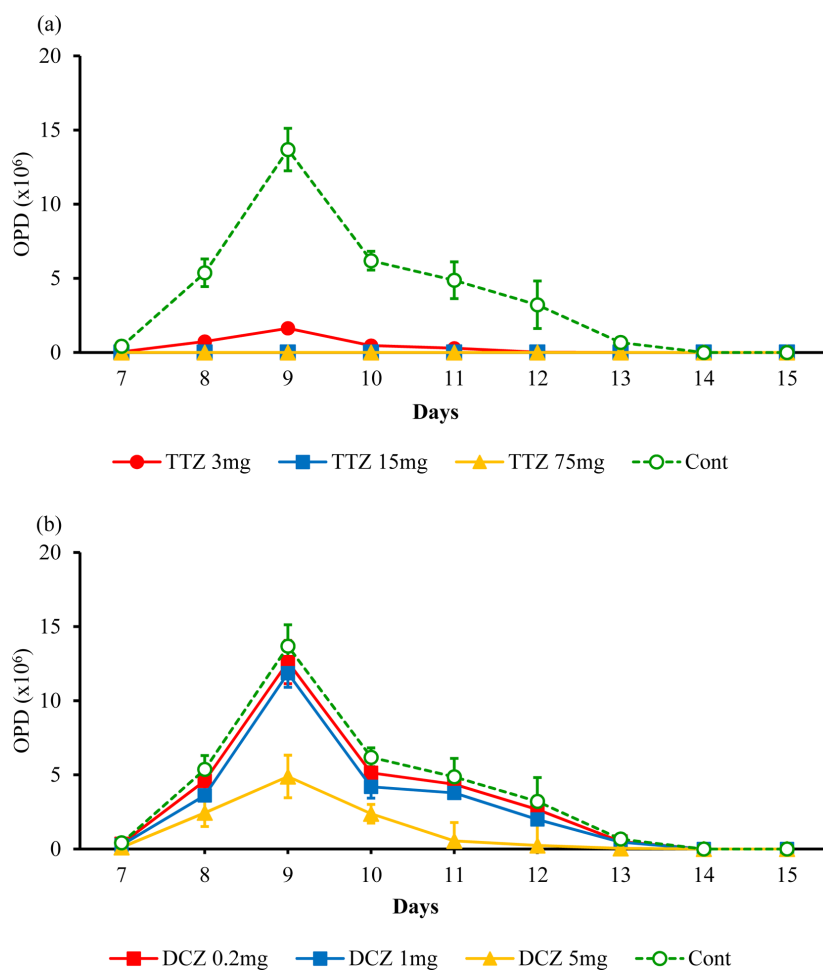


Fig. 1. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment on oocytes excreted per day (OPD) in *Eimeria vermiformis*-infected mice. Mice were orally inoculated with 1,000 sporulated oocysts of *E. vermiformis* on day 0 and orally treated with several doses of TTZ (a) and DCZ (b) on day 3. Oocyte count and identification were performed using a modified McMaster's method. Results are shown as mean \pm SEM, * P <0.05, n=5/group.

Table 2. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment on the induction of acquired resistance to *Eimeria vermiformis* infection in different treatment groups

Group	Number of mice	Infection	Dose	Treatment
TTZ	5	Day 0, 21	15 mg/kg	Day 3
DCZ	5	Day 0, 21	5 mg/kg	Day 3
Re-infection control	5	Day 0, 21	(-)	(-)
Single infection control	5	Day 21	(-)	(-)
Healthy	5	(-)	(-)	(-)

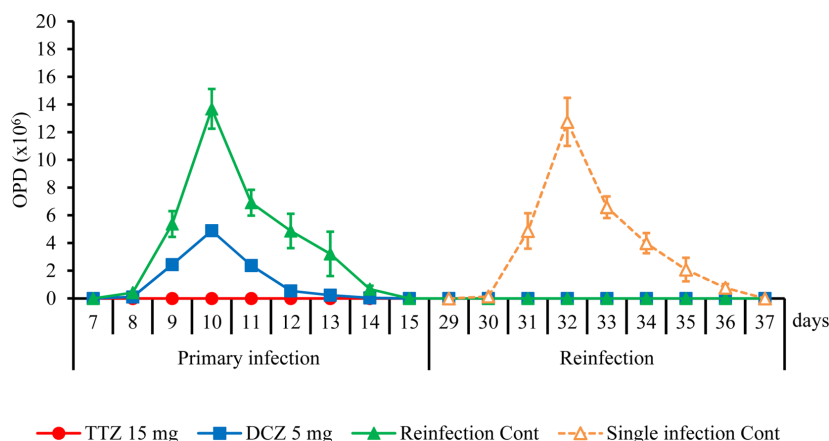


Fig. 2. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment against *Eimeria vermiformis* re-infection. Mice were orally inoculated twice with 1,000 sporulated oocytes of *E. vermiformis* on days 0 and 21 and orally treated with TTZ at 15 mg/kg and DCZ at 5 mg/kg on day 3. The single-infection group was infected once with 1,000 sporulated *E. vermiformis* oocytes. Oocyte count and identification were performed using a modified McMaster's method. Results are shown as mean \pm SEM, * P <0.05, n =5/group.

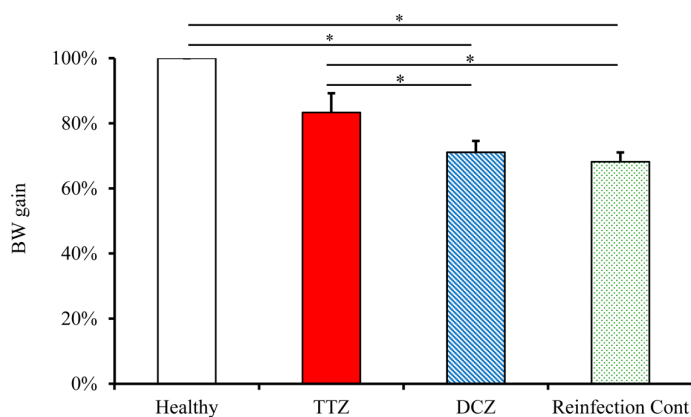


Fig. 3. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment on body weight (BW) gain in *Eimeria vermiformis*-infected mice. Mice were orally inoculated twice with 1,000 sporulated oocytes of *E. vermiformis* on days 0 and 21 and orally treated with TTZ at 15 mg/kg and DCZ at 5 mg/kg on day 3. BW gain in each group was assessed during the whole period of infection. The mean percentage BW gain in the healthy (uninfected/untreated) control was considered to be 100%. Results are shown as mean \pm SEM, * P <0.05, n =5/group.

infected) groups, although oocyst excretion was observed in the single-infection control (untreated/infected) group, which indicated the second infection was successful (Fig. 2).

Effect of TTZ and DCZ treatment on body weight gain

To evaluate the effectiveness of TTZ and DCZ treatments on BW gain in mice infected with *E. vermiformis*, BW was assessed during the whole period of infection in the TTZ-treated, DCZ-treated, and control (untreated/infected) groups (Fig. 3). The control (untreated/infected) groups showed a significant decrease in BW gain in comparison to the healthy (uninfected/untreated) group. There was no significant difference in BW gain between the DCZ-treated and control (untreated/infected) groups, while the TTZ-treated group showed a significantly higher BW gain than the DCZ-treated and control (untreated/infected) groups. In addition, no significant changes were observed in the TTZ-treated compared with the healthy (uninfected/untreated) groups.

Effects of TTZ and DCZ treatment on host T cell immunity

To evaluate the effects of TTZ and DCZ treatment on the immune response to *E. vermiformis* infection, 52 mice were divided into four groups (Table 3). TTZ (15 mg/kg) and DCZ (5 mg/kg) were administered on day 3. TTZ-treated, DCZ-treated, and re-infection control (untreated/infected) groups were infected twice, on day 0 and 21, and the healthy (uninfected/untreated) group was untreated and uninfected. T cell subsets from the spleen and MLNs were analyzed on days 10, 21, 24, and 28 (Fig. 4). On days 10 and 21, the DCZ-treated and re-infection control (untreated/infected) groups showed significantly fewer CD4⁺ and CD8⁺ cells in the spleen

Table 3. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment on host T cell immunity in different treatment groups

Group	Number of mice	Time of infection	Time of sacrifice	Dose
TTZ	16	Day 0, 21	Day 10, 21, 24, 28	15 mg/kg
DCZ	16	Day 0, 21	Day 10, 21, 24, 28	5 mg/kg
Re-infection control	16	Day 0, 21	Day 10, 21, 24, 28	(-)
Healthy	4	(-)	Day 28	(-)

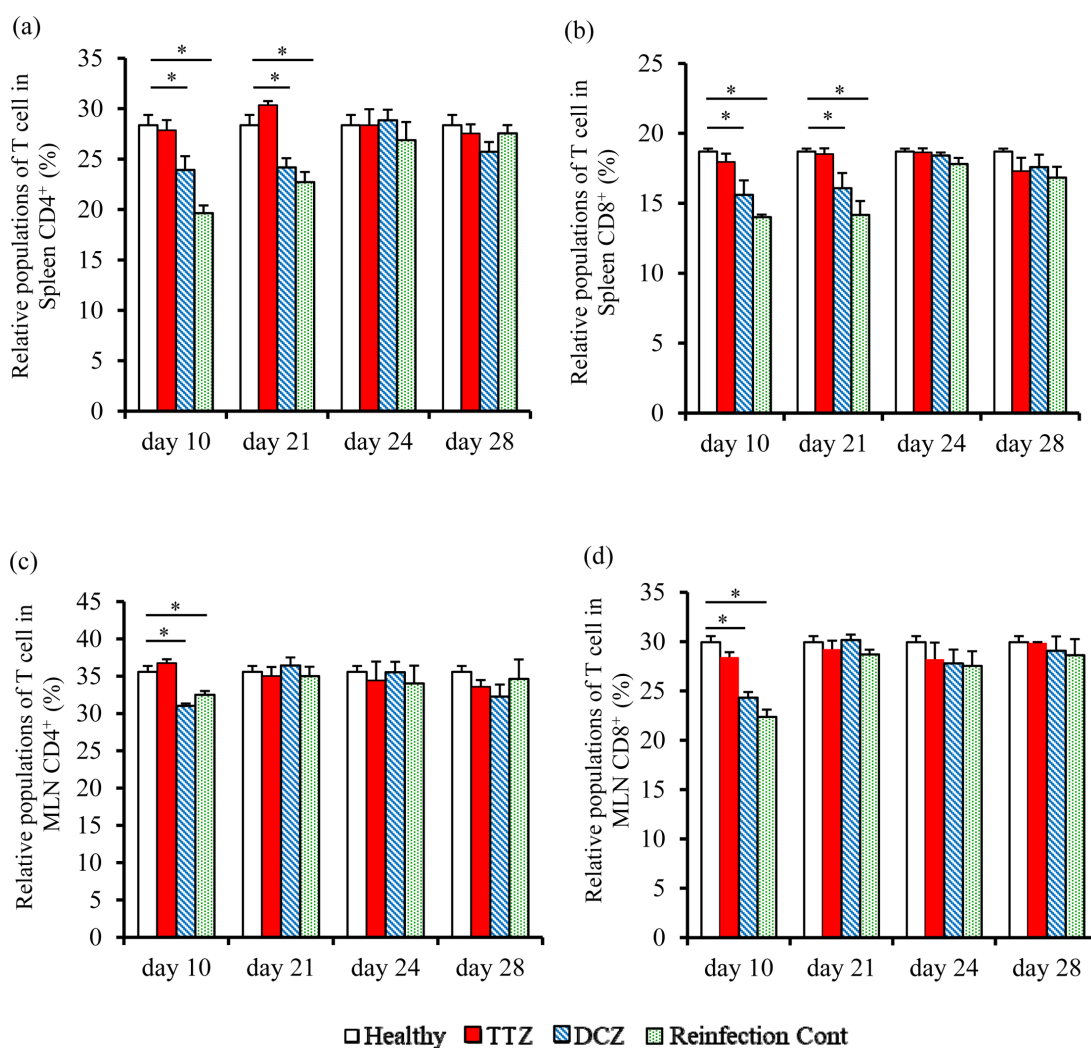


Fig. 4. Effect of toltrazuril (TTZ) and diclazuril (DCZ) treatment on splenic and mesenteric lymph node T cell subsets in *Eimeria vermiformis*-infected mice. Mice were inoculated twice with 1,000 sporulated *E. vermiformis* oocysts on days 0 and 21 and orally treated with TTZ at 15 mg/kg and DCZ at 5 mg/kg on day 3. Mice were sacrificed on days 10, 21, 24, and 28, and spleens and MLNs were harvested. The populations of CD4⁺ (a) and CD8⁺ cells (b) in the spleen and the populations of CD4⁺ (c) and CD8⁺ cells (d) in MLNs were analyzed by flow cytometry. Results are shown as mean \pm SEM, * P <0.05, n=4/group.

than the healthy (uninfected/untreated) control group. However, T cell subsets in the spleen were maintained at the same level in the TTZ-treated group as in the healthy (uninfected/untreated) group on both day 10 and day 21. In the MLNs, significantly fewer CD4⁺ and CD8⁺ cells were observed on day 10 in the DCZ-treated and control (untreated/infected) groups than in the healthy (uninfected/untreated) group, but there was no significant difference in the TTZ-treated group (Fig. 4c, 4d).

DISCUSSION

Intestinal coccidiosis is an economically important disease of domestic animals such as poultry, cattle, sheep, and pigs [16, 31]. In this study, oocyte excretion of *E. vermiformis* was completely prevented when mice were treated with a 15 mg/kg dose of TTZ, which is the recommended dose for cattle [12], even though it has been reported that *E. kriegsmanni*, murine *Eimeria*, showed resistance to TTZ treatment at the same dose as that used in our study [8, 34]. Our findings showed that the effects of DCZ were dose-dependent over the range of doses tested (0.2, 1, 5, 15, or 45 mg/kg), and 45 mg/kg of DCZ was the most effective dose for reducing oocyst excretion. However, as a result of ethical considerations regarding overdose treatment in animals, 5 mg/kg of DCZ was applied as the highest treatment dose. DCZ has been reported to be an effective agent for controlling coccidiosis in calves when administered at 1 mg/kg [5, 36]. Whereas it was reported that higher than the recommended dose of DCZ was required to control coccidiosis in lambs [36, 37]. Considering these facts, the effectiveness of DCZ may be affected by the host and/or *Eimeria* species, and DCZ on its own is not effective enough for the elimination of murine *Eimeria* infections.

Various studies have been conducted to understand the immune responses induced by *Eimeria* infection [27, 32]. On first encounter with *Eimeria* spp., the host develops acquired resistance, which protects the animals against subsequent heavy challenges [2]. The host immune response consists of two interacting systems: the innate and acquired immune responses [35]. The innate immune response is the first line of defense against infection and involves nonspecific proinflammatory responses [27, 32], while the acquired immune response is the second line of defense and protects animals from subsequent re-infections with the same pathogen [7]. In cases of *Eimeria* re-infection, the acquired immune response is likely to play a dominant role in controlling the disease [27, 32].

Hesketh *et al.* [29] reported that the acquired immune response is highly effective in controlling *E. vermiformis* infections. Mice developed protective immunity against re-infection within 2 to 3 weeks after primary infection, and this prevented subsequent infections and the onset of coccidiosis. The observations in the current study showed that, despite not receiving any treatment, mice did not excrete oocysts after re-infection, suggesting that acquired resistance to *E. vermiformis* infection developed during the primary infection. In addition, the TTZ and DCZ treatments did not affect the induction of protective immunity against *E. vermiformis*. These results support previous reports that TTZ and DCZ can terminate ongoing *Eimeria* infections without interfering with the establishment of protective immunity against re-infections in domestic animals [10, 23, 33].

Coccidia infection reduces food intake and decreases the digestion of nutrients [15], resulting in a lower BW gain in infected animals. In this study, there were no significant differences in BW gain between the TTZ-treated and healthy (uninfected/untreated) group, while the DCZ-treated and re-infection control (untreated/infected) groups had significantly less BW gain compared with those in the healthy (uninfected/untreated) group. Therefore, for *Eimeria*-infected domestic animals, TTZ treatment, but not DCZ treatment, can help to improve productivity by maintaining BW gain.

The results of oocyte excretion in re-infection suggested that TTZ and DCZ treatment on day 3 did not interfere with the induction of protective immunity against *E. vermiformis*. Previous studies have indicated that CD4⁺ and CD8⁺ T cells are involved in the development of an immune response against *E. vermiformis* infections [25, 30]. Therefore, T cell subsets were assessed in detail to investigate the effects of TTZ and DCZ treatment on the immune system. Unexpectedly, the populations of CD4⁺ and CD8⁺ T cells in the DCZ-treated and control (untreated/infected) groups significantly decreased on days 10 and 21 in the spleen and on day 10 in the MLNs, even though there was no impact on the populations of T cell subsets in the TTZ-treated group compared to the healthy (uninfected/untreated) group. Our results suggest that immune system activities are reduced because of the decreased T lymphocyte population during primary infection with *E. vermiformis*.

The decline in T cell population sizes may attenuate the immune system; therefore, the host would be at risk of contracting other infectious diseases. *Eimeria* infections have been reported to predispose cattle to bovine hemorrhagic enteritis (HE) caused by *Clostridium perfringens* by providing growth advantages to the bacteria [11]. The mechanism by which *C. perfringens* induced HE is associated with *Eimeria* infection is still not clear. However, the depression of host T cell immunity by *Eimeria* infection might contribute to the development of HE. Thus, the ability of TTZ treatment to control the parasite load during a primary infection and avoid a decline in T cell populations in host animals would be considered advantageous.

In this study, we found that a single 15 mg/kg dose of TTZ was the most effective treatment for eliminating parasites and preventing oocyte excretion by *E. vermiformis*-infected mice. Neither TTZ nor DCZ treatment affected the development of acquired resistance to the infection. However, DCZ efficacy may be affected by the host and/or *Eimeria* species. Oocyst excretion represents the completion of the parasite life cycle, and this event could affect the host T cell response/capacity and BW gain. Based on these immunological and metabolic parameters, an optimal anti-coccidial drug would completely stop the parasite life cycle in the host animal.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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