X-ray evaluation of intestinal dysmotility induced by *Eimeria pragensis* infection in C57BL/6 mice

A running head: COCCIDIOSIS INTESTINAL DYSMOTILITY

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This study was conducted to elucidate the intestinal dysmotility during coccidiosis. C57BL/6 male mice at seven weeks of age were inoculated with *Eimeria pragensis* sporulated oocysts (100 to 1,000 oocysts). The intestinal motility was evaluated by observing discharging time of barium sulfate (Ba₂SO₄) after oral administration (WITT: the whole intestinal transit time). The exact location of the dysmotility was analyzed by intermittent barium gastrography. Upper intestinal dysmotility was evaluated by charcoal propulsion study. Additionally, the occurrence of dysmotility was observed at different post-infection times (4, 7, and 14 days post-infection (d.p.i.)) and in infection-dose dependent manner (100, 300, and 1,000 oocysts). As the *E. pragensis* infected mice had significantly lower feed intake compared to the control group, we designed a feed apprehension study to evaluate the effect of low feed intake on the intestinal dysmotility. The WITT of infected mice at 7 d.p.i. was significantly longer (6 hr) than the uninfected mice (2.5 hr). Intestinal dysmotility was observed in the small intestine, caecum, and colorectum in the infected mice. Charcoal propulsion was slower in infected group (reaching to 40.4% of the whole small intestine) compared to control group (68.0%). The dysmotility was observed at the beginning of the patent period (7 d.p.i.) and subsided as the patency ended (14 d.p.i.). Mice with lower feed intake appeared to have similar intestinal motility as control mice. In summary, this study revealed the evidence of intestinal hypomotility during *E. pragensis* infection.

**KEY WORDS**: barium gastrography, coccidiosis, *Eimeria pragensis*, intestinal dysmotility, pan-enteric ileus
INTRODUCTION

Coccidiosis is a parasitic disease of the intestinal tract and is caused by infections of several members of the Protista phylum Apicomplexa that are characterized by the presence of an apical complex in their motile stages. All members belonging to this phylum are obligatorily parasitic. Various species of the Apicomplexan *Eimeria* are known to cause disease across a wide range of hosts. Invasion of the parasites into the epithelial cells and their subsequent destruction induce immune or inflammatory responses at the infected site. Eventually, these damages and responses result in clinical manifestations [1,2].

Recently, anecdotal reports have arisen of eimeriosis in cattle resulted in delayed intestinal motility. Some observation from field veterinarians claimed that coccidiosis cattle discharged smaller amount of feces despite having normal feed intake. These cattle were also reported to have bloat-like gas retention symptoms. Some veterinarians who observed this issue considered the administration of prokinetic agent to alleviate the symptoms. However, direct evidence and related mechanisms of delayed intestinal motility of cattle with eimeriosis has never been reported. Some work indicated a delay in intestinal motility. Altough, the study of cattle coccidiosis [5] reported a transient increase of nutrients apparent digestibility during clinical coccidiosis which might have reflected intestinal hypomotility, this work did not observe the intestinal motility directly. Another study of rabbits indicated that *Eimeria* infection could induce intestinal motility disturbance [8]. The disturbance, however, did not indicate a general delay in intestinal transit, because some parts of the intestine experienced faster motility and another part indicated slower motility.

As one of the cosmopolitan diseases of production animals, the existence of intestinal dysmotility by coccidiosis needs to be clarified. A control measure against intestinal dysmotility of coccidiosis needs to be proposed soon after the clarification of its clinical existence. The objective of this study is to find the evidence for intestinal dysmotility
occurrence during coccidiosis. For this purpose, we used murine *Eimeria* as a model for mammalian eimeriosis. To date, 15 species of murine *Eimeria* have been reported. This study used *Eimeria pragensis* that infects enterocytes in the caecum and colon of mice [18].

**MATERIALS AND METHODS**

*Experimental animal and parasite infection*

Animals used in this study were C57BL/6 male mice, and purchased from SLC Inc., Japan at six weeks of age. The animal experimentation was approved by the Animal Care and Use Committee, University of Miyazaki, No. 2016-037. The mice were housed in clean cages and provided with a standard diet and tap water *ad libitum* in an air-conditioned room (23 ± 1°C), under conventional conditions with a 12:12 hr, light: dark cycle. After one week of adaptation, mice were randomly assigned into different groups of treatments by receiving intragastric administration of sporulated *E. pragensis* oocysts for infected groups or distilled water for control groups. One day before oocyst excretion (at 6 days post-infection (d.p.i.)), the mice were separated into individual cages for feces collection.

*E. pragensis* has been maintained in our laboratory by oral passage every three months in C57BL/6 mice. Oocysts were purified and sporulated, as has been previously reported [17]. The infection of *E. pragensis* was confirmed by counting the number of oocysts per gram of feces (OPG) with the modified McMaster’s method, using a saturated salt solution with a specific gravity of 1.2. The animals’ condition was monitored by measuring feed intake, fecal output, body weight change, and clinical signs.

*The whole intestinal transit time of barium sulfate*

To investigate whether *E. pragensis* can induce intestinal dysmotility, the whole intestinal transit time (WITT) of barium sulfate was compared in *E. pragensis* infected and
uninfected mice. The WITT was defined as the transit time required for barium (Baritop®; Kaigen, Osaka, Japan; 2 g/ml) to be initially discharged in the feces after oral administration (0.3 ml/mice). Fecal excretions were monitored every 30 min for the discharge of white stained fecal droplet after the barium administration. For this evaluation, the infected group was inoculated with 1,000 sporulated *E. pragensis* oocysts. The observation was performed at 7 d.p.i.

Radiographic analysis of gastrointestinal motility

To identify the location of intestinal dysmotility, we measured gastrointestinal (GI) motility by contrast gastrography of barium sulfate of the infected group in comparison with the control group. The infected group was inoculated with 300 sporulated *E. pragensis* oocysts. Suspension (0.3 ml) of barium sulfate (Baritop®; Kaigen, Osaka, Japan; 2 g/ml) was administered to each mouse by oral gavage at 7 d.p.i., and then contrast gastrography was taken at 1, 2, 6, 12, 24, and 48 hr after barium administration. Transition of barium in the GI tract was analyzed semi-quantitatively from the images by assigning a compounded value to each region of the GI tract with the following parameters: percentage of the GI region filled with contrast (0–4); intensity of contrast (0–4); homogeneity of contrast (0–2); and sharpness of the GI region profile (0–2). Each of these parameters was scored and a sum (0–12 points) was calculated [9].

Gastrography imaging was performed with a Toshiba (Small animal exclusive line system VPX-120F: Toshiba medical supplies, Otawara, Japan) X-Ray apparatus (44 kV, 4.0 mA) and the images were recorded on digital x-ray apparatus (Regius Model 110: Konica Minolta Healthcare, Tokyo, Japan). Exposure time was adjusted to 20 ms and focus distance was manually fixed to 50 ± 1 cm. The mice were restrained in a prone position by placing them inside plastic tubes (adjustable, hand-made, and transparent). To avoid stress, animals
were immediately released after each shot (restraining lasted for less than 2 min).

Charcoal propulsion study

The upper gastrointestinal transition was evaluated by the charcoal propulsion study on the infected group which was inoculated with 300 sporulated *E. pragensis* oocysts in comparison with an uninfected control group. Each mouse was orally given 0.3 ml of charcoal meal (5% activated charcoal suspended in 10% aqueous Arabic gum) [4] at 7 d.p.i. The animals were killed 20 min later by cervical dislocation, and the extent of charcoal propulsion in the small intestine (the distance traveled by the charcoal from pylorus to the ileo-cecal junction) was measured.

Post-infection time dependency and infection-dose dependency study

The extensity of intestinal dysmotility was observed in the post-infection time dependent and the infection-dose dependent manner. In the post-infection time dependent study, the infected group was inoculated with 300 sporulated *E. pragensis* oocysts and then divided into three sub-groups of observation. The sub-groups were assigned as 4 d.p.i., 7 d.p.i., and 14 d.p.i. groups and intestinal motility was evaluated by contrast gastrography at 4, 7, and 14 d.p.i., respectively. Gastrographic observation was conducted at 6 and 12 hr after barium administration, and the data was analyzed semi-quantitatively as mentioned previously. In the infection-dose dependent study, the severity of intestinal dysmotility was evaluated with the different doses of infection. The infected group was divided into three sub-groups with different inoculation doses of sporulated *E. pragensis* oocysts. The sub-groups were assigned as Dose 100, Dose 300, and Dose 1,000 groups and inoculated with 100, 300, and 1,000 sporulated *E. pragensis* oocysts, respectively. Intestinal motility was evaluated semi-quantitatively at 7 d.p.i. by contrast gastrography with observation at 6 and 12 hr. after barium
administration.

Feed apprehension study

A fasting group was designed to be a sham group of mice that was inoculated with distilled water but had a limited feed intake, as little as 3 g per day, instead of an *ad libitum* feed intake. The limitation of feed intake was started at 6 d.p.i. Intestinal motility was evaluated at 7 d.p.i. by contrast gastrography with observation at 6 and 12 hr after barium administration. The intestinal motility of the fasting group was compared with those of the control group and the infected group with 300 sporulated *E. pragensis* oocysts.

Statistical analysis

The differences of mean values of independent two groups were analyzed using the Wilcoxon rank-sum test. The Kruskal-Wallis and pairwise Wilcoxon tests were used for the comparison of three or more groups and multiple two-pair comparisons between control group and individual treatment groups, respectively. A nominal significance level of 5% (α=0.05) was used for all statistical tests. All analyses were performed using the statistical program R (www.r-project.org). The results were expressed as the mean value with standard error of the mean.

RESULTS

In the WITT study, the *E. pragensis* infected mice required significantly longer WITT (approximately 6 hr) than the uninfected mice (approximately 2.5 hr) (Fig. 1).

The intermittent gastrography study revealed different patterns of barium transition in the infected and uninfected mice (Fig. 2). Apparently, the infected mice retained the barium longer in their GI tract than the uninfected mice.
Semi-quantitative analysis of barium transition revealed dysmotility in the different part of the intestinal tract in the infected mice. There was no difference of barium transition in the stomach between the infected and control groups (Fig. 3a), which indicated similar pattern of gastric emptying of both groups. The stomach was practically empty of barium content at 12 hr after barium administration in both groups. Accordingly, there was no delay in the filling of small intestine between the groups (Fig. 3b). However, a significantly higher amount of barium remained in the infected group compared to the control group at 6 hr, showing the delay of intestinal emptying in the infected group. Accordingly, the filling of barium in the caecum (Fig. 3c) was longer in the infected group as the maximum amount of barium was observed at 6 hr in the infected group that was 4 hr delay from the control group. Delay of the caecum emptying was apparent because all barium was expelled from the caecum less than 12 hr in the control group while the infected group retained a significant amount of barium for up to 48 hr after its administration. Significant delay of filling was also observed in the colorectum of the infected mice because significantly higher amount of barium was observed in the control group compared to the infected group at 2 hr after barium administration (Fig. 3d). There was no barium observed at 12 hr in the control group while barium was still observed in the colorectum at 24 hr in the infected group, indicating the potential delay of emptying of this organ in the infected group.

In the charcoal propulsion study, significantly slower propulsion was observed in the infected group than the control group. At 20 min after its administration, charcoal reached to 68.0% in average of the full length of the small intestine in the control group, while it reached to 40.4% in the infected group (Fig. 4a, b).

In the post-infection time dependent study, there was no difference of barium clearance in the stomach between the infected groups and the control group at 6 hr (Fig 5a). However, a significant amount of barium remained in the stomach at 12 hr in the 14 d.p.i.
group compared to the control group. In the small intestine, a significantly higher amount of barium was observed at 6 hr in the 7 d.p.i. group than the control group (Fig. 5b). Small intestine was practically empty of barium at 12 hr although two mice in the 14 d.p.i. group still had barium at 12 hr. At 6 hr in the caecum, a similar amount of barium was observed among the groups. Barium remained at 12 hr in all of the infected groups but not in the control group where a significantly higher amount was observed in the 7 d.p.i. than in the control group (Fig. 5c). The retention of barium was also observed in the colorectum at 12 hr, where a significantly higher amount was observed in the 7 d.p.i. than in the control group (Fig. 5d).

In the infection-dose dependent study, the amount of barium in the stomach was similar among the groups at 6 hr and 12 hr (Fig. 6a). In the small intestine, a significantly higher amount of barium was observed at 6 hr in the Dose 300 and Dose 1,000 groups compared to the control group (Fig. 6b). While the other groups had no barium left at 12 hr, the Dose 1,000 group still had a significant amount of barium remaining in the small intestine compared to the control group. There was no difference of barium amount at 6 hr in the caecum between the infected groups and the control group (Fig. 6c). However, all of the infected groups retained a significantly higher amount of barium in the caecum at 12 hr, while no barium was left in the control group. A similar amount of barium was observed among the groups in the colorectum at 6 hr, whereas significantly higher amount of barium was observed in the Dose 300 and the Dose 1,000 groups than in the control group at 12 hr (Fig. 6d).

Daily monitoring of feed intake revealed significantly lower feed intake from 6 to 9 d.p.i. in the infected group compared to the control group (Fig. 7a). However, the trend was inversed after 9 d.p.i. where the infected group had significantly higher feed intake compared to the control group. In the feed apprehension study, we observed a similar amount of barium among the groups in the stomach at 6 hr and 12 hr. (Fig. 7b). In the small intestine, the fasting
group and the control group had similar amount of barium at 6 hr, however, the infected group had a significantly higher amount of barium than the control group (Fig. 7c). No trace of barium was observed in the small intestine in all groups at 12 hr. Observation of the caecum and colorectum revealed a similar pattern among the groups at 6 hr (Fig. 7d, e). At 12 hr, however, there was no trace of barium in the control group in the caecum and colorectum, whereas significantly a higher amount of barium remained in the caecum and colorectum in the infected group compared to the control group. Although some barium also remained in the fasting group, the difference from the control group was not significant.

**DISCUSSION**

To investigate the delayed intestinal motility in \textit{E. pragensis} infected mice, it was decided to measure the Whole Intestinal Transit Time (WITT) before proceeding to further analysis. The significantly longer WITT observed in the infected mice indicated the occurrence of intestinal hypomotility. To evaluate intestinal hypomotility, the movement of luminal content or contractility of the GI tract can be observed [3]. However, measuring the intestinal contractility in mice is a tremendous challenge due to the need of invasive probe in relatively small size of mouse organs. Therefore, in this study, the luminal content movement was evaluated by intermittent x-ray observation which is suffice for the determination of the number and location of a retained radio-opaque marker [15,16].

The intermittent gastrography study revealed that the intestinal motility pattern of the control mice showed a similar pattern with previously reported one with ICR/CDI mice [9]. In both studies, the half-life of barium in the stomach was approximately 2 hr after its administration. Time required for the maximum filling of the small intestine, caecum, and colorectum were approximately 1 hr, 2 hr, and 6 hr, respectively (Fig 3b-e). The intestinal motility pattern of the infected mice was altered in the small intestine, caecum, and
The most prominent alteration was observed in the caecum of the infected mice as the time required for emptying of this organ was extended to more than 48 hr. Besides this, the caecum showed the delayed start of barium filling probably because the barium was retained longer in the small intestine and reached later to the caecum of the infected mice than the uninfected mice. The barium filling and emptying in the colorectum of the infected mice were altered in a similar fashion with those in the caecum.

As the main infection site of *E. pragensis* is in the caecum, it would be logical to consider the intestinal dysmotility to occur in the caecum. However, the intermittent gastrography study revealed the delayed empty of barium from the small intestine which indicated the hypomotility of the upper intestinal tract. The charcoal propulsion study confirmed this indication and furthermore indicated the occurrence of systemic alteration on GI motility.

To evaluate the onset of intestinal dysmotility occurrence in the course of *E. pragensis* infection, the intestinal motility was evaluated at the prepatent period (4 d.p.i.), during patency (7 d.p.i.), and after patent period (14 d.p.i.). The intestinal dysmotility in the *E. pragensis* infected mice was observed at the patency period, but not in the prepatent or after patent periods. Significant differences of the barium retention were observed between the control group and the 14 d.p.i. group in the stomach at 12 hr (Fig. 5a) and colorectum at 6 hr (Fig. 5d). However, the trace of barium in the stomach of the 14 d.p.i. was probably due to coprophagy which was also reported by another study using intermittent gastrography in mice [9]. This coprophagy behavior is natural for mice during distress [20] and most likely to be induced by significant increase of appetite during recovery of coccidiosis as observed in our study (Fig. 7a). The significant difference observed at the colorectum was hypermotility instead of hypomotility which could be induced by increased postprandial motility due to increase feed intake. From this finding, we deduced that the intestinal dysmotility might have
occurred in relation with the developmental stage of *E. pragensis* and subsequent damage resulting from its development.

The endogenous stage of *E. pragensis* life cycle starts when sporozoites penetrate to the enterocytes. The 1st generation of immature schizonts was principally located in the caecum and colon approximately at 18 hr post-infection and advanced into 4th generation schizonts at 6 d.p.i. [12]. The gametogony stage started as early as 6 d.p.i. and oocyst started to be discharged in the feces at 7 d.p.i. [23]. During the patent period of *E. pragensis*, considerable damage occurred to the intestine, especially to the caecum and colon as the primary infection site. The damage was reported as initial destruction of both cryptal and absorptive epithelial cells and submucosal edema followed by mucosal necrosis, ulcers, and localized granulomatous colitis [23]. This damage was also accompanied with gradual decrease (hypoplasia) of large intestinal goblet cells (cecum and colon) in association with the development of endogenous stages of parasite life cycle [22]. We predicted that these damages also subsequently alter the intestinal motility.

From the post-infection time dependent study, we proceeded to analyze infection-dose dependency of the occurrence of intestinal dysmotility. As mentioned previously, it was assumed that the dysmotility might have occurred due to the subsequent intestinal damage of *E. pragensis* development. Therefore, it was assumed that by increasing the infection dosage, a parallel increase of the dysmotility severity in the infected mice might be observed. In the Dose 100 group, the dysmotility was only observed in the caecum (Fig. 6c). In the Dose 300 group, the dysmotility was observed in the caecum, but also in the small intestine and colon. As the infection dose became higher in the Dose 1,000 group, the dysmotility occurred in the same sites with the Dose 300 group but for longer time in the small intestine. Accordingly, as the infection dose increased, the severity of intestinal motility became more prominent.

The delayed intestinal motility observed in the infected groups could be induced by
other factors such as restraint stress or fasting [13]. Concerning on restraint stress, a similar stress was applied to both infected and control groups in this study, and thus the restraint stress could be eliminated from the possible incurring factors in intestinal dysmotility. Accordingly, we proceeded to evaluate the effect of fasting and conducted the feed apprehension study to elucidate the consequence of low feed intake on intestinal dysmotility. In the infected groups, we observed that the decrease of feed intake was started one day before oocyst shedding (6 d.p.i.). This reduction of feed intake may result in slower intestinal motility as previously reported [13]. In this study, a fasting group was designed with limited feed intake, as low as half amount of daily feed intake of the control group and compared the intestinal motility of the infected and control groups. The transition of barium in the fasting group did not show significant difference from that of the control group. After eliminating restraint stress and fasting as a possible cause to intestinal hypomotility, it was deduced that the intestinal dysmotility in the infected group was induced by the infection of *E. pragensis* in the intestinal tissue.

The mechanism of intestinal dysmotility in *E. pragensis* is still under investigation. It was predicted that the intestinal dysmotility occurred because of the inflammatory reaction in the damaged intestinal tissue. Some reports indicated that inflammatory reaction in the intestine was capable of inducing intestinal hypomotility [6]. One possible key player is the activation of macrophages as modulator of GI motility. These macrophages formed a three-dimensional network within the muscle layers and produced a variety of mediators that could alter gut function [14]. It was reported that *E. vermiformis* infection in mice could induce Th-1 immune response [19]. If the Th-1 immune response could also be induced in *E. pragensis* infection, the intestinal dysmotility could be explained with similar mechanism of the post-operative ileus (POI) pathophysiology which was induced by Th-1 immune response [7, 21]. Mice with POI were observed to have depletion of goblet cells together with inflammation.
reaction [10] that was also observed in murine *E. vermiformis* [11] and *E. pragensis* infection [22].

In summary, it was revealed the evidence of intestinal hypomotility during coccidiosis. *E. pragensis* infection in the caecum of the mice induced hypomotility of the small intestine, caecum, and colorectum which could be observed from the onset of oocyst excretion and by dose-dependent manner. We proposed this newly observed clinical symptom in coccidiosis as coccidiosis intestinal dysmotility (CID). This finding is fundamental to re-evaluate the clinical manifestations of coccidiosis in another species.

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**REFERENCES**


**FIGURE LEGENDS**

Fig. 1. Whole intestinal transit time in the control group and the infected group. The infected group was inoculated with 1,000 sporulated *Eimeria pragensis* oocysts. Results are shown as mean + SEM (error bar). **p<0.01. n=5/group.
Fig. 2: Radiographic observation of gastrointestinal motility in the control group and the infected group. Barium was administered intragastrically (0.3 ml/mouse, 2 g/ml) on 7 d.p.i. and observations were performed at 1, 2, 6, 12, 24 and 48 hr after the administration. The infected mice were inoculated with 300 sporulated *Eimeria pragensis* oocysts. Scale bar: 2 cm.

Fig. 3: Semi-quantitative analysis of barium transition in the stomach (a), small intestine (b), caecum (c), and colorectum (d) of the infected group (square) and the control group (round). Barium was administered intragastrically (0.3 ml/mouse, 2 g/ml) on 7 d.p.i. and observations were performed at 1, 2, 6, 12, 24 and 48 hr after the administration. The infected mice were inoculated with 300 sporulated *Eimeria pragensis* oocysts. Results are shown as mean ± SEM (error bar). Statistical comparisons were made between groups at the same observation time at each site. *p<0.05, ***p<0.001. n=5/group.

Fig. 4: Evaluation of upper intestinal tract motility on 7 d.p.i. by charcoal propulsion. (a) Gastrointestinal transition of charcoal in mice belonging to the control group (upper) and infected group (lower). (b) Transition of charcoal 20 min after oral administration compared to total length of small intestine. The infected mice were inoculated with 300 sporulated *Eimeria pragensis* oocysts. Open triangle: stomach, closed triangle: Ileo-cecal junction. Results are shown as mean + SEM (error bar). **p<0.01. n=5/group.

Fig. 5: Semi-quantitative analysis of barium transition in the post-infection time dependent study of the stomach (a), small intestine (b), caecum (c), and colorectum (d). The infected mice were observed at 4 d.p.i. (before patency), 7 d.p.i. (start of patency) and 14 d.p.i. (end of patency). All mice in the infected groups were inoculated with 300 sporulated *Eimeria pragensis* oocysts. Barium was administered intragastrically (0.3 ml/mouse, 2 g/ml) and observations were performed at 6 and 12 hr after the administration. Results are shown as mean + SEM (error bar). *p<0.05, **p<0.01, ***p<0.001. n=5/group.

Fig. 6: Semi-quantitative analysis of barium transition in the infection-dose dependent study of the stomach (a), small intestine (b), caecum (c), and colorectum (d). Mice were infected with 3 different doses of sporulated *Eimeria pragensis* oocysts which were: 100 (Dose 100), 300 (Dose...
300), and 1,000 oocysts (Dose 1,000) per mouse. Barium was administered intragastrically (0.3 ml/mouse, 2 g/ml) on 7 d.p.i. and observations were performed at 6 and 12 hr after the administration. Results are shown as mean + SEM (error bar). *p<0.05, **p<0.01, ***p<0.001. n=5/group.

Fig. 7. Feed apprehension study in relation with intestinal dysmotility. Change in daily feed intake of the control and Infected group (a). Semi-quantitative analysis of barium transition in the stomach (b), small intestine (c), caecum (d), and colorectum (e) of the fasting group compared to the control and infected groups. The infected mice were inoculated with 300 sporulated *Eimeria pragensis* oocysts. The fasting group received 3 g of feed daily compared to ad lib in the control and infected groups. Barium was administered intragastrically (0.3 ml/mouse, 2 g/ml) on 7 d.p.i. and observations were performed at 6 and 12 hr after the administration. Results are shown as mean + SEM (error bar). *p<0.05, **p<0.01, ***p<0.001. n=5/group.
Fig. 1

![Graph showing time (hr) for control and infected conditions with a significant difference indicated by **.](image-url)
Fig. 2

Control

Infected

1 hr  2 hr  6 hr  12 hr  24 hr  48 hr
Fig. 3

(a) Radiographic index vs. Time (hr) for Control and Infected groups.

(b) Radiographic index vs. Time (hr) for Control and Infected groups.

(c) Radiographic index vs. Time (hr) for Control and Infected groups.

(d) Radiographic index vs. Time (hr) for Control and Infected groups.
Fig. 5

(a) Radiographic index
(b) Radiographic index
(c) Radiographic index
(d) Radiographic index