



Proposal for effective treatment of Shiga toxin-producing *Escherichia coli* infection in mice



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ABSTRACT

Previously, we reported that minocycline, kanamycin and norfloxacin improved the survival rate in the E32511 model that we developed (FEMS Immunol Med Microbiol 26, 101–108, 1999), but fosfomycin did not. In this study, we investigated the effectiveness of azithromycin (AZM) against Stx2d-producing EHEC O91:H21 strain B2F1 or Stx2c-producing *Escherichia coli* strain E32511 treated with mitomycin C *in vivo*. Recently, we reported the effectiveness of AZM in our model and AZM strongly inhibited the release of Stx2c from E32511 *in vitro* (PLOS ONE e58959, 2013). However, it was very difficult to completely eliminate E32511 in the mouse feces by treatment with AZM alone. In this report, only AZM or Daio effectively promoted survival of mice infected with B2F1 compared to untreated mice. Furthermore, Daio inhibited the colonization of GFP-expressing B2F1 in the mouse intestine. Similarly, a combination of AZM and Daio in the E32511-infected mice reduced E32511 in the mouse feces and significantly improved survival.

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1. Introduction

A large outbreak of Shiga toxin (Stx)-producing enteroaggregative *Escherichia coli* (EAEC) O104:H4 occurred in northern Germany. Of the outbreak, at least 810 developed hemolytic uremic syndrome (HUS), resulting in more than 54 deaths in 2011 [1,2]. 30% of HUS patients showed encephalopathy. In Japan, an outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O111 infection occurred in 2011. A total of 181 people suffered from diarrhea, and surprisingly, encephalopathy developed in 21 (62%) of the HUS patients [3]. Central nervous system (CNS) dysfunction is an important predictive factor for HUS and mortality. Previously, we developed a mouse model of CNS disorders by oral infection with the Stx2c-producing EHEC O157: H–, resulting in damage to the endothelial cells of capillaries and to nerve fibers in the cerebral cortex and spinal cord of the infected mice [4].

All members of the Stx family are comprised of 1A and 5B subunit proteins [5]. The A subunit is an N-glycosidase, that

removes adenine from nucleotide No. 4324 of 28S RNA of the 60S ribosomal subunit [6]. Stx produced by *Shigella dysenteriae* is identical to Stx1 [7]. The amino acid sequence identity between the A subunits of Stx1 and Stx2 has been reported to be only 55% [8]. Each B subunit of Stx1 and Stx2 has a high affinity to the glycosphingolipid globotriaosylceramide (Gb3 or CD77), which is present in some eukaryotic cells [9].

In 1996, a large outbreak of *E. coli* O157:H7 infection occurred among schoolchildren in Sakai City, Osaka, Japan. 9492 patients suffered from diarrhea and hemorrhagic colitis, 121 patients developed HUS, and 3 children died. After the outbreak in Japan, the treatment guidelines for EHEC infection were made by the Ministry of Public Welfare. The administration of antimicrobial agents, fosfomycin (FOM), kanamycin (KM) and norfloxacin (NFLX) was recommended in the said guidelines. Also, Ikeda K et al. reported that FOM was the most frequently administered, and that its administration within 3 days of illness reduced the risk of HUS [10]. We previously reported the therapeutic effects of the antimicrobial agents FOM, minocycline (MINO), KM and NFLX in our EHEC O157:H– (strain E32511/HSC) infected mouse model [11]. Results showed that MINO, KM and NFLX were significantly effective but FOM was not [11]. Zhang et al. reported that gnotobiotic piglets,

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when treated with a pediatric dose of AZM after oral infection with the Stx1- and Stx2-producing EHEC O157:H7 strain 933W, were able to survive and had little or no brain hemorrhage compared with a ciprofloxacin (CPFX)-treated group [12]. Recently, we also reported the effectiveness of azithromycin (AZM) in our model, and AZM strongly inhibited the release of Stx2c from E32511 *in vitro* [13]. Similarly, we confirmed the effectiveness of AZM in our E32511 model with oral inoculation of E32511 [13]. We also used a streptomycin (SM)-treated mouse model of infection with the highly virulent Stx2d-producing EHEC O91:H21 strain B2F1 (B2F1 model, see methods). The 50% lethal dose (LD₅₀) of the SM-resistant strain B2F1, when fed to SM-treated mice, was less than 10 bacteria [14].

In the current study, we developed a new treatment combining AZM and the Chinese medicine Daio. Daio is a traditional medicine from China frequently used for the treatment of constipation, inflammation and cancer [15]. Daio has active substances, such as anthranoids and anthraquinone glycosides, which have laxative and purgative effects, thus is able to increase the movement of the intestine [16,17]. Through this mechanism, we hypothesize that Daio will accelerate the shedding of bacteria, including the toxin, in the feces.

2. Materials and methods

2.1. Ethics statement

Animal experiments were carried out in strict accordance with the recommendations in Guidelines for Proper Conduct of Animal Experiments of Science Council of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kyushu University, Japan (Permit Number: A23-141-1). All surgical procedures were performed under sevoflurane anesthesia, and all efforts were made to minimize suffering.

2.2. Bacterial strains

The Stx2c-producing *E. coli* O157:H– strain E32511/HSC was used in this study [4]. The Stx2d-producing *E. coli* O91:H21 strain B2F1 was isolated from a human [18] and was a gift from Professor Alison O'Brien (Uniformed Services University of the Health Sciences, USA). Streptomycin (SM)- and mitomycin C (MMC)-resistant E32511/HSC were grown in nutrient agar supplemented with 100 µg/mL of SM (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.5 µg/mL of MMC (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). SM-resistant B2F1 was prepared as above. *E. coli* K-12, which is resistant to SM and MMC, was used as a negative control. E32511/HSC, B2F1 and *E. coli* K-12 were grown with shaking for 17 h in Luria–Bertani broth (LB broth) at 37 °C. The bacterial pellets were then harvested by centrifugation at 6000× *g* for 25 min at 4 °C before being washed twice with phosphate-buffer saline (PBS) by centrifugation (6000× *g* for 20 min at 4 °C). Finally, bacterial pellets were suspended in PBS and the appropriate number of CFUs was given orally to the mice.

E. coli O91:H21 strain B2F1 expressing pGFP was prepared as follows. The pGFPuv vector (100 ng; BD Biosciences, San Jose, CA, USA) was transformed into the competent *E. coli* O91:H21 strain B2F1. Competent cells were prepared using rubidium chloride as previously described [19]. Reactions were plated on an L-agar plate containing ampicillin (100 µg/mL), and transformed colonies were identified by fluorescence when exposed to UV light.

2.3. Animals

Four week-old female ICR outbred mice purchased from Japan SLC, Inc. (Shizuoka, Japan) were used for all *in vivo* experiments in

this study. They were housed in cages with a 12 h light–dark cycle and free access to laboratory food and distilled water.

2.4. B2F1 model

We used previously developed SM-resistant B2F1 and GFP-B2F1 bacteria as B2F1 and GFP-B2F1 models [14]. The mice were given SM-containing drinking water (5 g/L) *ad libitum*. On day 3 of SM treatment, mice were completely deprived of food for 12 h prior to bacterial inoculation. The mice were infected with 0.5 mL of bacterial suspension containing 10² CFU of B2F1 or 10⁴ CFU of GFP-B2F1, the dose required in order to cause 100% death in mice, using a sterile disposable feeding needle passed into the stomach by orogastric inoculation. In this model, the following neurological signs and symptoms were observed: weight loss, flaccid paralysis, and tremor culminating in death within 6–12 days after infection.

2.5. E32511 model

For the *in vivo* experiments, the mice were divided into groups of 5 or 6. In this study, as our late stage mouse model, we used a mouse model orally infected with E32511/HSC and i.p. injected with MMC [4]. Briefly, all mice were given SM-containing drinking water (5 g/L) *ad libitum* to reduce the level of their normal intestinal flora [20]. On day 3 of SM treatment, all the mice were completely deprived of food for 12 h until bacterial inoculation. Each mouse was infected with 0.5 mL of bacterial suspension using a sterile disposable feeding needle (diameter, 1.9 mm; length, 38 mm, Fuchigami Co., Ltd., Nara, Japan) attached to a syringe passed into the stomach by orogastric inoculation. Subsequently, they were injected i.p. with 2.5 µg/g MMC. We reported that 90% of the mice infected with 5 × 10¹⁰ CFU of E32511 orally plus i.p. injection of MMC developed weight loss, flaccid paralysis and tremor, culminating in death [4]. In this study, the bacterial suspension of E32511 was adjusted to 1 × 10¹¹ CFU in PBS and caused 100% death. After the completion of treatment, they were housed according to the treatment groups and given food and SM-containing drinking water (5 g/L) *ad libitum*. The mice were observed twice daily for 2 weeks, for survival and/or signs of illness. We collected the mouse feces, plated, and counted the total bacterial load. Log-rank and χ^2 tests were used to evaluate the survival rate of the different treatment groups. Statistical significance was set at $p < 0.05$.

2.6. *In vivo* colonization of GFP-expressing B2F1 in the mouse colon tract

The colonization sites of SM-resistant-GFP-expressing B2F1 (GFP-B2F1) in the intestinal tract were evaluated in the SM-treated mouse model. Four-week-old female ICR mice were divided into three groups. The mice were given SM-containing drinking water (5 g/L) *ad libitum* to reduce the level of their normal intestinal flora. On day 3 of SM treatment, all the mice were completely deprived of food for 12 h until bacterial inoculation. Each group was infected with 0.5 mL of bacterial suspension using a sterile disposable feeding needle. 10⁴ CFU of GFP-B2F1 were administered orally, and PBS was orally administered as a negative control. The treatment group was treated with 300 µg/g Daio in distilled water once a day for 5 days starting from the day after inoculation with GFP-B2F1. The Daio-treated group consisted of seven mice, and three mice were used as negative controls. Eight and 10 after treatment, three mice from each group were sacrificed to observe the colonization by GFP-B2F1. Mice were sacrificed after being anesthetized with an i.p. injection of a mixture of 2,2,2-tribromoethanol and 2-methyl-2-butanol, after which the colon was excised and cut into 1 cm pieces. Before cutting, the colon was washed to remove fecal material by

forcing sterile PBS through the bowel lumen. Then the colon was cut in the longitudinal plane and put into 35 mm glass-based dishes (Iwaki & Co., Ltd., Tokyo, Japan), upside down. Immediately, the images of colon mucosa layer were captured with a Keyence HS All-in-one Fluorescence Microscope BZ-9000E, with 20× magnification. GFP expression was analyzed using BZ-II Analyzer BZ-H2AE/BZ-H1ME/BZ-H1RE/BZ-H2TLE/BZ-H1CE software (Keyence). Simultaneously, bacterial counting was done by homogenizing the colon and the ileum in PBS using a Multi Bead Shocker (Yasui Kikai Corp., Osaka, Japan), followed by serial dilution and plating on L-agar plates containing 100 µg/mL of SM, and the number of CFU/mouse colon and ileum were counted. The strain of selected colonies was confirmed by slide-agglutination with anti-O91 serum (Denka Seiken Co., Ltd, Tokyo, Japan).

2.7. Statistical analysis

Data were analyzed with SPSS Statistics 19.0 (IBM Japan, Tokyo, Japan) and Delta Graph 6vJ Win (Red Rock Software, Inc. Salt Lake City, UT, USA) was used to draw the graphs. The significance of the survival rate from each treatment group was compared using a Kaplan–Meier test followed by a pair-wise comparison of Log Rank (Mantel–Cox) test, and Student's *t*-test was used to show the significant values between negative control, positive control and treatment groups. Significance was set at a value of $p < 0.05$ in all cases.

3. Results

3.1. The effects of AZM or Daio in the B2F1 model

We investigated the effectiveness of treatment with AZM or Daio alone using the B2F1 model. To cause death in 100% of mice infected with B2F1, WT B2F1 bacteria and GFP-expressing B2F1 bacteria required a dose of 10^2 CFU and 10^4 CFU, respectively. AZM was given orally at a dose of 25 µg/g at 24 h after infection as a single dose or once a day for three days in the B2F1 model. The mice treated with these doses showed significantly improved survival compared to the untreated mice ($p < 0.01$) (Fig. 1A).

Next, we investigated the effectiveness of Daio in the GFP-B2F1 model only. In *in vitro* experiments, Daio had no antibacterial activity against either the B2F1 or the GFP-B2F1 strains (data not shown). Daio administered at a concentration of 300 µg/g at 24 h after infection once a day for 5 consecutive days was found to significantly enhance the survival of treated mice compared with those to which the same concentration of Daio was administered once a day for 3 days or with the untreated group (Fig. 1B).

3.2. In vivo colonization of GFP-expressing B2F1 in the mouse colon tract

To confirm the effectiveness of Daio, we counted the number of GFP-B2F1 cells in the tissues of the mouse colon and ileum. One day after infection with GFP-B2F1, mice were treated with 300 µg/g Daio once a day for 5 consecutive days. The mice were sacrificed and the bacteria were harvested from the tissues of the colon and the ileum at days 5, 8 and 10 after treatment. After washing the feces out, the intestines were homogenized and GFP-B2F1 cells were enumerated. Results showed that there was a significant decrease in the number of GFP-B2F1 in the tissues of the colon and the ileum of infected mice at days 8 and 10 after treatment (Fig. 2A). To confirm the colonization of the colon by the GFP-B2F1 strain, the mice infected with GFP-B2F1 were sacrificed and the colon tissue was washed with PBS to remove the feces without attempting to remove the bacteria that adhered to the mucosa of the colon. The colon was then observed using fluorescence and light microscopy. The mucosal epithelium of the colon of the mice treated with Daio and that of the untreated mice were observed under a fluorescence microscope. In the Daio-treated group, no GFP-B2F1 was observed on day 10 (Fig. 2B; Daio-treated, day 10, down), whereas many GFP-B2F1 were observed in the untreated group at day 10 (Fig. 2B; untreated, day 10, down). On day 8, the colonization by B2F1 of the Daio-treated mouse colon was almost the same as that of the untreated mouse colon (Fig. 2B; untreated and Daio-treated, day 8, down). Furthermore, under the light microscope, the shape of the mucosal epithelium of the colon of Daio-treated mice was regular on day 10 after treatment (Fig. 2B; Daio-treated, up). In the untreated group, the mucosal epithelium of the colon was irregularly shaped on day 10 (Fig. 2B; untreated, day 10, up). Based on our results, we conclude that a single dose of 25 µg of AZM and 300 µg/g Daio are effective in treating B2F1-infected mice.

3.3. Effects of AZM combined with Daio in the E32511 model

Then, we determined the extent of the effectiveness of AZM by combining it with Daio in the E32511 model. Before the combination treatment, we confirmed whether Daio alone was effective in the E32511 model. Treatment with Daio (300 µg/g) alone, once or twice, was not effective in this model (Table 1, Nos. 2–5). Additionally, treating mice with AZM (200 µg/g) 6 h after inoculation of E3211 was not effective (Table 1, No. 6). We administered a combination of AZM (200 µg/g) 6 h after infection with Daio 300 µg/g once 2 h after infection, and it was not effective in this mouse model (Table 1, No. 7). Subsequently, we tested a combination of

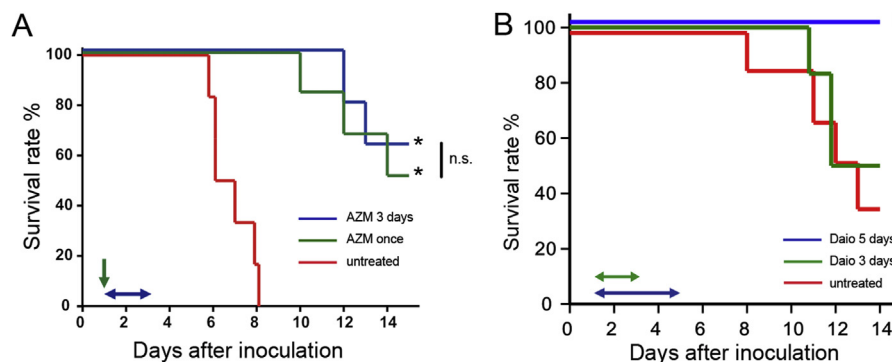


Fig. 1. Effects of AZM or Daio in the B2F1 model. (A) 25 µg/g AZM, once a day for 3 consecutive days after infection were significantly more effective than no treatment, with a significant effect on survival (AZM 25 µg/g single dose vs. untreated, $p = 0.001$; AZM 25 µg/g 3 consecutive days vs. untreated, $p = 0.001$). $*p < 0.05$. (B) 300 µg/g Daio, once a day for 5 consecutive days after infection was significantly more effective than no treatment (Daio 5 days vs. untreated, $p = 0.018$), while 300 µg/g Daio, once a day for 3 days after infection was not effective compared to untreated; (Daio 3 days vs. untreated, $p > 0.05$). $*p < 0.05$.

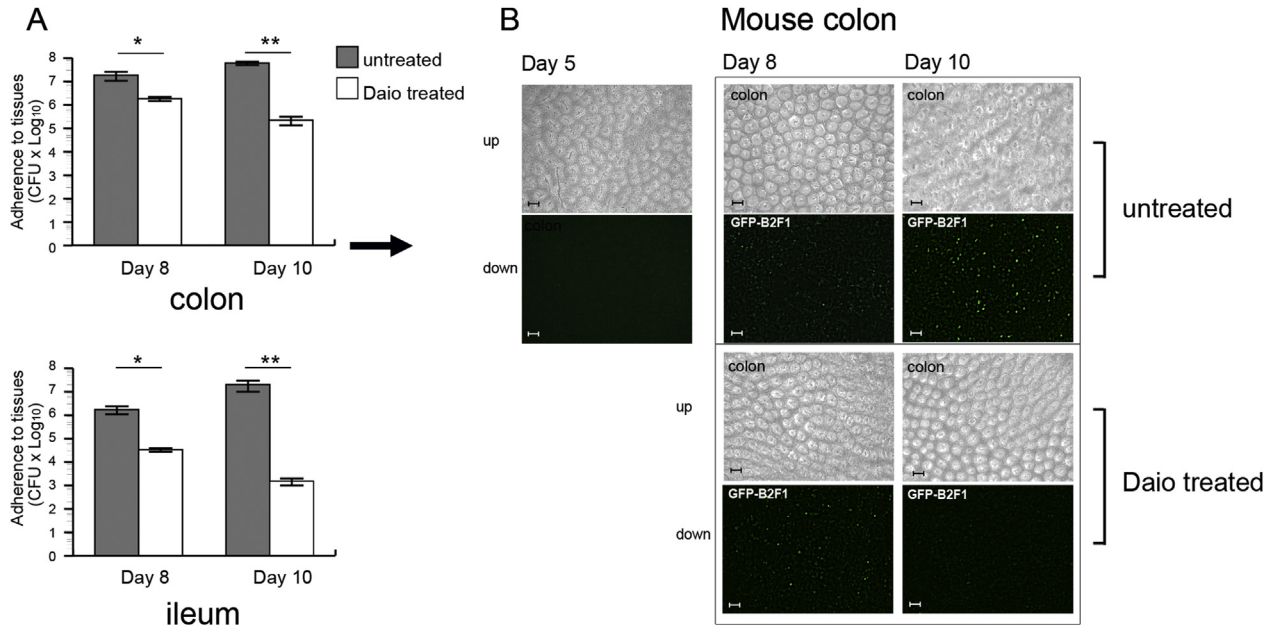


Fig. 2. *In vivo* colonization of GFP-expressing B2F1 in the mouse colon tract. (A) The number of bacteria on days 8 and 10 after infection was significantly decreased in the colon of GFP-B2F1-infected mice by administration of 300 $\mu\text{g/g}$ Daio once a day for 5 consecutive days (day 8 bacteria in colon of Daio vs. untreated, $p = 0.020$; day 10 bacteria in colon of Daio vs. untreated, $p < 0.0001$) and in the small intestine of GFP-B2F1-infected mice on days 8 and 10 after infection after the administration of 300 $\mu\text{g/g}$ Daio once a day for 5 consecutive days (day 8 bacteria in the small intestine of Daio vs. untreated, $p = 0.010$; day 10 bacteria in the small intestine of Daio vs. untreated, $p < 0.0001$). Student's *t* test, $*p < 0.05$; $**p < 0.001$. (B) GFP-B2F1 colonization in the mouse colon. An increase in green spot distribution (representing colonization by bacteria) was observed in the untreated group compared with the Daio-treated group at days 8 and 10 after treatment. (Scale bars = 50 μm). Data were collected from at least 3 out of the 7 mice per day, from two independent experiments for each treatment group.

Table 1
Effect of Daio, AZM and combination treatment of AZM and Daio on the lethality of the E32511 model.

No. of experimental design	Treatment of Daio and/or AZM	Time line after infection			No. of death/experimental total no.
		2 h	6 h	24 h	
1	No treatment				5/5
2	Daio once	Daio			5/5
3	Daio once		Daio		5/5
4	Daio once			Daio	5/5
5.	Daio twice	Daio		Daio	5/5
6	AZM once		AZM		3/5
7	Combination of AZM and Daio	Daio	AZM		3/5
8	Combination of AZM and Daio	Daio	AZM	Daio	1/5*

AZM (200 $\mu\text{g/g}$) at 6 h after infection with Daio treatment (300 $\mu\text{g/g}$) twice, 2 h and 24 h after infection. This was significantly effective (Table 1, No. 8). This combination of AZM and a double dose of Daio improved the survival period compared with the group treated with the combination of AZM and a single dose of Daio (Fig. 3A). Furthermore, the bacterial count in the feces significantly decreased compared with that of the mice treated with AZM alone (Fig. 3B). We were, therefore, able to develop a new treatment, comprising of a combination of AZM and Daio, which is effective in the E32511 model.

4. Discussion

In this study we report four significant results. 1) The macrolide antibiotic AZM or Daio was strongly effective in the B2F1 model. 2) In the GFP-B2F1 model, treatment with the Chinese medicine Daio alone was found to inhibit the colonization by GFP-B2F1 in the

mouse intestine. 3) We developed a new therapy using a combination of AZM and Daio in the E32511 model. Because it was very difficult to completely eliminate gram-negative bacteria, such as *E. coli* E32511, in the mouse feces by treatment with AZM alone, we were able to show that a combination of AZM and Daio reduced E32511 in the mouse feces.

Recently, Zhang et al. reported that a pediatric dose (10 $\mu\text{g/kg}$) of AZM was effective in the piglet model orally infected with Stx2-producing *E. coli*, and that *recA* mutants treated with the phage-inducing agent MMC and a sub-inhibitory concentration of CPFY failed to produce Stx2 [12]. We also previously reported that MINO, KM and NFLX improved the survival rate in our E32511 model, but FOM did not [11]. Other antimicrobial agents induced the production of Stx2c including FOM, NLFX, KM, ofloxacin (OFLX), and ciprofloxacin (CPFY) by 1.5-, 12.5-, 2.25-, 12.5- and 100-fold over control, respectively [13].

In this report, the treatment with the Chinese medicine Daio alone was found to be effective in the GFP-B2F1 model. It was very difficult to completely eliminate gram-negative bacteria, such as *E. coli* E32511, in the mouse feces by treatment with AZM alone. We developed a new therapy using a combination of AZM and Daio in the E32511 model. The combination of AZM and Daio reduced E32511 in the mouse feces.

To determine the extent of the effectiveness of AZM, we used a Chinese medicine, Daio. Oi et al. reported that several purified components of Daio effectively inhibited the ADP-ribosyl transferase activity of cholera toxin [21]. In this study, Daio used in the B2F1 and GFP-B2F1 models may wash out B2F1 with an increase in bowel movements, because the mice treated with Daio had a lot of diarrhea (data not shown). Some components from Daio might also inhibit the colonization of the mouse colon by B2F1. This is the effect that we expected to observe, because we assumed that the pathogen could be cleared out from the gut. In addition, there are still many active substances in Daio, for example, rhein, which has

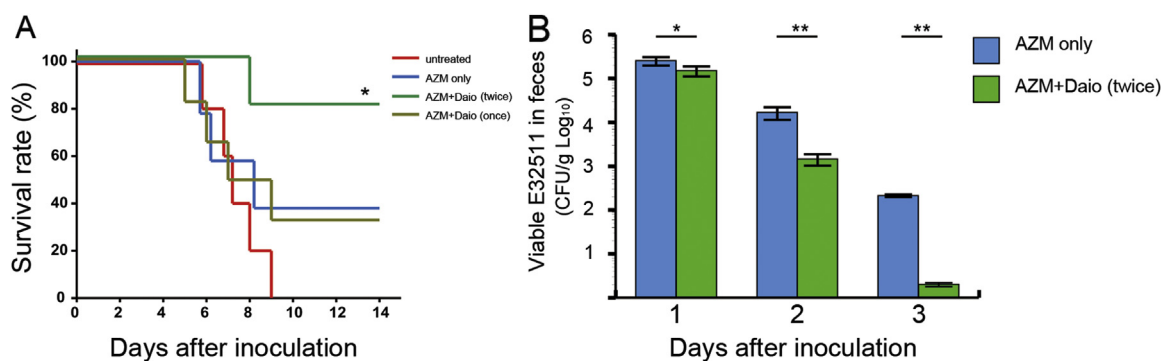


Fig. 3. Combination treatment with AZM and Daio in the E32511 model. (A) A single dose of 200 $\mu\text{g/g}$ AZM 6 h after infection, combined with 300 $\mu\text{g/g}$ Daio (twice) 2 h and 24 h after infection, but not 200 $\mu\text{g/g}$ AZM alone 6 h after infection or 200 $\mu\text{g/g}$ AZM 6 h after infection combined with 300 $\mu\text{g/g}$ Daio (once) 2 h after infection, had a statistically significant effect on the survival curve 200 $\mu\text{g/g}$ AZM 6 h after infection combined with Daio (twice) vs. untreated, $p = 0.009$; 200 $\mu\text{g/g}$ AZM only and 200 $\mu\text{g/g}$ AZM 6 h after infection combined with Daio (once) vs. untreated, $p > 0.05$. (B) Viable E32511 decreased significantly in feces of E32511-infected mice treated with 200 $\mu\text{g/g}$ AZM 6 h after infection combined with 300 $\mu\text{g/g}$ Daio (twice) 2 h and 24 h after compared with treatment with 200 $\mu\text{g/g}$ AZM alone 6 h after infection (day 1 AZM combination Daio vs. AZM alone, $p = 0.022$; day 2 AZM combination Daio vs. AZM alone, $p = 0.001$ and day 3 AZM combination Daio vs. AZM alone, $p < 0.0001$). Student's *t* test, * $p < 0.05$; ** $p < 0.001$.

potent activity against *Bacteroides fragilis* and *Candida albicans*, gram-positive bacteria and acid-fast bacteria, but has weak activity against *E. coli* [22]. In accordance with our *in vitro* experiments, we checked the MIC of Daio against E32511 and showed that this medicine did not have any antibacterial activity (data not shown). Furthermore, it has been reported that Daio has an anti-inflammatory effect by inhibiting the metabolism of arachidonic acid to prostaglandins E2 and F2 and thromboxane *in vitro* [23,24]. Our results showed that the administration of a single dose of 200 $\mu\text{g/g}$ AZM 6 h after inoculation with the bacteria was not effective in improving the survival rates of infected mice, but when combined with Daio once a day for two days, the survival rates improved and the number of viable bacteria in the feces significantly decreased. Antibiotic use during *E. coli* O157:H7 infections has been reported to be associated with a higher rate of subsequent HUS and should be avoided [25]. However, in the biggest outbreak of EHEC O104:H4 in Germany, treatment with AZM was associated with a lower frequency of long-term STEC carrier status [26]. We then investigated the effectiveness of Daio treatment alone using the GFP-B2F1 model, infected with 10^4 CFU of GFP-B2F1. Our results showed that Daio effectively promoted survival after EHEC infection compared with the untreated mice. The number of bacteria that colonized the colon and small intestine decreased 3 and 5 days after treatment demonstrating that Daio prevents the colonization of bacteria in the colon and small intestine.

It was very difficult to completely eliminate gram-negative bacteria like *E. coli* E32511 in the mouse feces by treatment with AZM only. However, we showed that a combination of AZM and Daio could reduce E32511 in the mouse feces. Furthermore, only Daio prevented the colonization of the mouse colon by B2F1. These results, however, merit further and thorough investigations.

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