Feeding Activated Charcoal from Bark Containing Wood Vinegar Liquid (Nekka-Rich) Is Effective as Treatment for Cryptosporidiosis in Calves

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ABSTRACT

The effect of activated charcoal containing wood vinegar liquid (Nekka-Rich) on *Cryptosporidium parvum* was evaluated in vitro and in vivo. First, the adsorption of *C. parvum* by the activated charcoal component of Nekka-Rich was examined. When mixed, *C. parvum* oocysts were effectively adsorbed by activated charcoal. Next, the effect of the wood vinegar liquid component of Nekka-Rich was assessed. Wood vinegar liquid had an antiprotozoan activity against *C. parvum* oocysts. Finally, the anticryptosporidial effect of Nekka-Rich was evaluated in calves experimentally infected with *C. parvum*. Six neonatal calves at 7 d of age were orally infected with 1×10^5 oocysts of *C. parvum*. When diarrhea was observed (on d 3 after the experimental infection), 3 calves received a milk replacer containing 10 g of Nekka-Rich at 8-h intervals for 4 consecutive days, and 3 control calves were given a milk replacer without Nekka-Rich at 8-h intervals for 4 consecutive days. Calves fed milk with Nekka-Rich showed recovery from diarrhea 1 d after the start of treatment. Significantly less fecal excretion of *C. parvum* oocysts was observed 1 d after treatment in calves fed Nekka-Rich. On d 2 from the beginning of treatment with Nekka-Rich (on d 5 after the experimental infection), oocysts were not isolated from fecal samples. On the other hand, nontreated control calves developed severe diarrhea, and fecal excretion of oocysts was consistently observed. Calves in both groups were killed for collection of samples, which were subjected to scanning electron microscopy. Scanning electron microscopy revealed adherence of *C. parvum* oocysts to the intestinal epithelial surface of nontreated control calves, whereas a lack of *C. parvum* adhesion was observed in calves fed Nekka-Rich. These results suggest

that Nekka-Rich would be useful for the treatment of cryptosporidiosis in neonatal calves.

Key words: activated charcoal, cryptosporidiosis, diarrhea, wood vinegar liquid

INTRODUCTION

Cryptosporidium parvum is an intestinal protozoan parasite that causes diarrhea in both humans and domestic animals (Casemore et al., 1997; Anderson, 1998; Xiao et al., 2004). In livestock, *C. parvum* infection in calves is of clinical and economic concern for producers (Casemore et al., 1997). Because the feces of calves are a recognized source of *Cryptosporidium*, fecal contamination of drinking water is a potential means of transmission of this protozoan (Smith, 1998; Fayer et al., 2000). Thus, methods for controlling *C. parvum* infection in calves are needed not only to reduce the clinical and economic impacts on the producer, but also to alleviate environmental and public health concerns.

Activated charcoal is known as a universal adsorbent because it can bind with variety of molecules (Chandy and Sharma, 1998). It has been reported that activated charcoal is useful for the removal of bacteria and bacterial toxins, both in vitro and in vivo (Drucker et al., 1977; Pegues et al., 1979; Du et al., 1987; Gardiner et al., 1993). Recently, we have shown the usefulness of activated charcoal for removal of verotoxin-producing *Escherichia coli* and verotoxin (Naka et al., 2001). Others have reported that organic acids inhibit the growth of enteropathogenic bacteria (Anderson, 1992; Hsiao and Siebert, 1999; Nakai and Siebert, 2003) as well as the viability of *C. parvum* (Kniel et al., 2003). Nekka-Rich (Miyazaki-Midori Pharmaceuticals Inc., Miyazaki, Japan) is a product made by mixing activated charcoal and wood vinegar liquid that contains organic acids. The activated charcoal and wood vinegar liquid of Nekka-Rich are both obtained from the bark of evergreen oak (*Castanopsis cuspidata* and *Quercus acuta*) by carbonization.

Based on a recent study in our laboratory that demonstrated that Nekka-Rich effectively inhibits the infection

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of chickens with *Salmonella enterica* serovar Enteritidis (Watarai and Tana, 2005), we hypothesized that Nekka-Rich may be useful for the prevention of *C. parvum* infection in calves by inhibiting adsorption of oocysts and reducing the viability of oocysts. Therefore, we examined each component of Nekka-Rich for activity in vivo against *C. parvum* and evaluated the combined commercial product in neonatal calves experimentally infected with *C. parvum*. In addition, we also evaluated the ability of Nekka-Rich to inhibit intestinal colonization of *C. parvum* in neonatal calves experimentally infected with *C. parvum*.

MATERIALS AND METHODS

Materials and Parasites

Activated charcoal and wood vinegar liquid, which are obtained from the bark of evergreen oak (*C. cuspidata* and *Q. acuta*), and Nekka-Rich (a product made from these 2 components) were obtained from Miyazaki-Midori Pharmaceuticals Inc. In Japan, Nekka-Rich is approved as a feed additive. *Cryptosporidium parvum* oocysts were collected from feces of calves with cryptosporidiosis by sucrose flotation method as described previously (Uga et al., 2000). Purified oocysts were used for in vitro and in vivo experiments.

In Vitro Adsorption Test

This study was designed to test the usefulness of activated charcoal from the bark of an evergreen oak for *C. parvum* adsorption. *Cryptosporidium parvum* oocysts (2×10^4 oocysts) in 1 mL of PBS (pH 7.2) and activated charcoal from the bark of an evergreen oak (0, 1, 5, and 10 mg) were mixed together and incubated at 37°C for 1 h with gentle agitation. To remove the activated charcoal, the mixtures were passed through a membrane filter (Millipore, Bedford, MA; 10 µm pore size), which was rinsed with 10 mL of PBS. The filtrates were centrifuged at $1,200 \times g$ for 10 min, and the resulting supernatant was decanted. The remaining pellet of oocysts was suspended in PBS (100 µL) and the number of oocysts was counted 3 times in a hemocytometer.

In Vitro Effect of Wood Vinegar Liquid on *C. parvum* Oocyst

Cryptosporidium parvum oocysts (6×10^4 oocysts) were added to 1 mL of PBS containing 0, 0.5, 2.5, and 5.0% wood vinegar liquid and incubated at 37°C for 14 h with gentle agitation. After incubation, each mixture of oocysts was centrifuged at $1,200 \times g$ for 10 min and the resulting supernatant was removed as described above.

Oocysts were resuspended in 100 µL of PBS and counted 3 times in a hemocytometer.

Animals

Six male Holstein-Friesian calves that did not have natural infection with *C. parvum* were purchased at 3 d of age from 3 large local farms. Calves were housed individually in a biosafety level 2 isolation facility for large animals at Veterinary Teaching Hospital, School of Veterinary Medicine, Rakuno Gakuen University (Ebetsu, Japan). Calves were fed with a commercially available milk replacer (Scientific Feed Laboratory Co. Ltd., Tokyo, Japan) at intervals of 8 h for the duration of the experiment. They were maintained for the study in accordance with the guidelines of the Rakuno Gakuen University Committee on animal care and use.

Experimental *C. parvum* Infection in Calves and Administration of Nekka-Rich

Six calves were randomly assigned to 2 groups (3 calves/group). Physical contact between calves was prevented. All calves were inoculated orally with 1×10^5 *C. parvum* oocysts. At the onset of diarrhea, calves in the nontreated control group were given commercial milk replacer without Nekka-Rich, whereas calves in the Nekka-Rich-treated group received commercial milk replacer containing 10 g of Nekka-Rich at each feeding for 4 d. All feces were examined microscopically for the screening of *C. parvum* infection. The clinical response of each calf was noted throughout the experiment in terms of presence and severity of diarrhea and oocyst shedding in stool. The presence of diarrhea was based on fecal consistency score using the following index: 1 = normal feces; 2 = soft feces; 3 = mild diarrhea; 4 = severe watery diarrhea. A daily sample of feces was collected from each calf from d 0 until d 7, and these samples were examined for the presence of *C. parvum* oocysts by the sucrose flotation method. In brief, 5.0 g of feces was mixed with 30 mL of distilled water; then, the fecal suspensions were passed through a stainless steel mesh sieve to remove solids and centrifuged at $300 \times g$ for 10 min. Each resulting supernatant was decanted and the sediment was resuspended in 14 mL of sucrose solution (specific gravity: 1.2). The suspension was transferred to a 15-mL centrifuge tube and centrifuged at $300 \times g$ for 10 min. After centrifugation, the tube was left for 10 min. One drop of supernatant was then placed on a microscope slide, covered with an 18 × 18 mm coverslip, and examined at 1,000× magnification using a light microscope (Olympus Co., Tokyo, Japan). Fecal samples containing oocysts were categorized into 4 score groups as follows: 0: zero oocysts in all microscopic fields; 1: <10

oocysts in 30 microscopic fields; 2: ≥ 10 to < 300 oocysts in 30 microscopic fields; 3: ≥ 300 oocysts in 30 microscopic fields. Seven days after the experimental infection of *C. parvum*, all calves were humanely killed by excessive anesthetization in accordance with the guidelines of the Rakuno Gakuen University Committee on animal care and use, and sections of small intestines were collected to evaluate the degree of colonization of *C. parvum*.

Scanning Electron Microscopy

Scanning electron microscopy was used to evaluate the colonization of *C. parvum*. Tissue samples from mid duodenum, mid jejunum, and mid ileum were fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C and then postfixed with 0.2% osmium tetroxide in 0.1 M phosphate buffer at room temperature for 1 h. The tissue strips were dehydrated through a graded series of acetone, treated with *t*-butyl alcohol, dried in a freeze-drying apparatus (JFD-300, JEOL, Tokyo, Japan), coated with a 10-nm-thick gold layer in an ion coater (NL-OPC40, JEOL Datum Ltd., Tokyo, Japan), and examined with a scanning electron microscope (JSM600F, JEOL).

Statistical Analysis

Student's *t*-test was performed for statistical evaluation of in vitro results. Results are expressed as the arithmetic mean with the standard error of the mean (mean $\pm$ SEM). In addition, data were collected on the number of calves having diarrhea and the number of calves shedding oocysts for each treatment group for each day of data collection. These were compared between treatment groups by use of Fisher's exact test. The level of significance for all statistical tests was set at $P \leq 0.05$.

RESULTS

Adsorption Effect of Activated Charcoal from the Bark of an Evergreen Oak on *C. parvum*

As shown in Figure 1, activated charcoal from the bark of an evergreen oak caused a dose-dependent adsorption of *C. parvum* oocysts. That is to say, when *C. parvum* oocysts (2×10^4 oocysts) were incubated with 0, 1, 5, and 10 mg of activated charcoal at 37°C for 1 h, the number of oocysts was $1.89 \pm 0.11 \times 10^4$, $1.2 \pm 0.12 \times 10^4$ ($P < 0.0125$), $7.87 \pm 0.59 \times 10^3$ ($P < 0.001$), and $4.43 \pm 0.3 \times 10^3$ ($P < 0.0003$), respectively.

Effect of Wood Vinegar Liquid on Oocysts

As shown in Figure 2, the number of *C. parvum* oocysts was lowered in a dose-dependent manner by the addition

Figure 1. The capacity of activated charcoal to adsorb *Cryptosporidium parvum*. Data are expressed as mean $\pm$ SE of triplicate experiments. * = $P < 0.0125$, # = $P < 0.001$, @ = $P < 0.0003$ compared with control (0 mg) (Student's *t* test).

of wood vinegar liquid. On the other hand, the number of oocysts did not change when *C. parvum* was incubated with PBS without wood vinegar liquid.

Clinical Response of Calves after Challenge Exposure with *C. parvum* Oocysts

The clinical observations of calves after challenge with *C. parvum* oocysts and subsequent treatment with Nekka-Rich are shown in Table 1. All calves of the non-treated control group, when exposed to *C. parvum* oocysts, had mild (mean fecal consistency score 3.3) to

Figure 2. Effect of wood vinegar liquid on *Cryptosporidium parvum*. Data are expressed as mean $\pm$ SEM of triplicate experiments. * = $P < 0.0014$, # = $P < 0.0006$, @ = $P < 0.0004$ compared with control (0%) (Student's *t* test).

Table 1. Clinical response of calves to challenge exposure with *Cryptosporidium parvum* oocysts and treatment with Nekka-Rich on d 0 through 7

Group ¹	Calves with diarrhea ² /total calves, n/n							
	0	1	2	3	4	5	6	7
A	0/3 (1.0)	0/3 (1.0)	0/3 (1.0)	3/3 (3.3)	3/3 (3.3)	3/3 (3.7)	3/3 (3.3)	3/3 (3.3)
B	0/3 (1.0)	0/3 (1.0)	0/3 (1.0)	3/3 (3.3)	0/3* (2.0)	0/3* (2.0)	0/3* (2.0)	0/3* (2.0)

¹A = nontreated control calves; B = calves treated with Nekka-Rich.

²Data in parentheses are mean fecal consistency scores: 1 = normal feces; 2 = soft feces; 3 = mild diarrhea; 4 = severe watery diarrhea.

* $P = 0.05$ compared with group A (Fisher's exact test).

severe watery diarrhea (mean fecal consistency score 3.7) from postchallenge d 3 to 7 (the final day of experiment). However, there was a difference in fecal consistency score between the nontreated control group and the Nekka-Rich-treated group. Although calves of the treatment group developed mild diarrhea on postchallenge d 3 ($P = 1.0$), they had feces with a soft consistency within 1 d after feeding with commercial milk replacer containing Nekka-Rich (d 4: $P = 0.05$), and no overt diarrheal disease was observed for the period when milk replacer containing Nekka-Rich was administered (d 5, 6, and 7: $P = 0.05$).

Presence of *C. parvum* Oocysts in Feces

Table 2 shows the effect of Nekka-Rich-containing milk feeding on the suppression of *C. parvum* oocyst excretion in feces. On d 4 after challenge with *C. parvum* oocysts, the oocysts were isolated from fecal samples of all calves. However, there was a significant difference in fecal oocyst score between the nontreated control calves and calves treated with Nekka-Rich on d 5, 6, and 7 after challenge with *C. parvum* oocysts. The nontreated calves were excreting oocysts until the end of experiment period (d 7). On the other hand, no *C. parvum* oocysts were isolated from fecal samples of Nekka-Rich-fed

calves ($P = 0.05$) from postchallenge d 5 to 7, in contrast to the nontreated control calves.

Detection of *C. parvum* Adherence in Small Intestines of Calves

Figure 3 shows a scanning electron micrograph of ileal surfaces of nontreated control and Nekka-Rich-treated calves infected with *C. parvum* oocysts. The ileal section from nontreated control calves showed adherence of *C. parvum* on its surface. On the other hand, the ileal surface of Nekka-Rich-fed calves showed no *C. parvum* adhesion. Other intestinal sections such as duodenum and jejunum from calves treated with Nekka-Rich also revealed no adherent *C. parvum* oocysts along the intestinal epithelial surface, whereas adhering oocysts were present along the epithelial surface of the duodenum and jejunum of calves in the nontreated group (data not shown). These results are in accordance with those from *C. parvum* oocyst isolation shown in Table 2.

DISCUSSION

We showed that activated charcoal of Nekka-Rich has adsorption efficacy against *C. parvum*. In general, activated charcoal has a high adsorptive capacity although

Table 2. Isolation (on d 0 through 7) of *Cryptosporidium parvum* oocysts from feces of challenged calves treated with or without Nekka-Rich

Group ¹	<i>C. parvum</i> -positive calves ² /total calves							
	0	1	2	3	4	5	6	7
A	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	3/3 (1.0)	3/3 (2.0)	3/3 (2.0)	3/3 (2.0)
B	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	3/3* (1.0)	0/3* (0.0)	0/3* (0.0)	0/3* (0.0)

¹A = nontreated control calves; B = calves treated with Nekka-Rich.

²Data in parentheses show oocyst score: 0: zero oocysts in all microscopic field; 1: <10 oocysts in 30 microscopic fields; 2: ≥10 to <300 oocysts in 30 microscopic fields; 3: ≥300 oocysts in 30 microscopic fields.

* $P = 0.05$ compared with group A (Fisher's exact test).

Figure 3. Electron micrographs of representative ileal sections: A) villi from the ileum of a nontreated calf showing adherent *Cryptosporidium parvum* oocysts (arrow); B) villi from the ileum of a calf treated with Nekka-Rich, showing the absence of adherent *C. parvum* oocysts. Bar = 10 μ m.

it tends to be nonselective (Wicks et al., 1980; Chandy and Sharma, 1998). The adsorptive capacity of activated charcoal depends on its pore size. For removal of relatively large materials, an activated charcoal having larger pores is needed, whereas small substances need small pores (Chandy and Sharma, 1998). In this study, *C. parvum* oocysts were effectively adsorbed by activated charcoal from the bark of an evergreen oak, which suggests that this activated charcoal possessed pores of sufficient diameter for *C. parvum* oocysts. Indeed, the existence of various pores of 10 μ m or less in diameter was confirmed in the activated charcoal from the bark of an evergreen oak (unpublished data). More recently, we reported that activated charcoal from the bark of an

evergreen oak has low binding capacity to *Enterococcus faecium*, which is smaller than *C. parvum* (Watarai and Tana, 2005). This suggests that oral administration of this activated charcoal could reduce *C. parvum* oocysts while minimizing the removal of normal bacterial flora in the intestinal tract.

We also found that the number of *C. parvum* oocysts was decreased by treatment with wood vinegar liquid. This suggests that wood vinegar liquid from the bark of an evergreen oak contains compounds with disinfectant activity against *C. parvum* oocysts. Wood vinegar liquid contains organic acids, which have been shown by others to inhibit the growth of enteropathogenic microbes (Anderson, 1992; Hsiao and Siebert, 1999; Nakai and Siebert, 2003) and to reduce the viability of *C. parvum* (Kniel, 2003). Thus, we believe the organic acids are responsible for the inhibitory effect on *C. parvum*.

Our data showed the significant therapeutic effect of Nekka-Rich-containing milk replacer in calves experimentally infected with *C. parvum*. Feeding of milk replacer with Nekka-Rich reduced the severity and duration of diarrhea induced by *C. parvum* infection. In addition, isolation of *C. parvum* oocysts from feces was decreased by Nekka-Rich-containing milk replacer feeding. In fact, no oocysts were isolated 2 d after Nekka-Rich feeding begun. Furthermore, intestinal epithelial cells of calves treated with Nekka-Rich showed no adherent *C. parvum* oocysts on their surfaces, whereas epithelial cells of calves in the nontreated group showed strong *C. parvum* adhesion. These results indicate that *C. parvum* colonization in the intestines of calves infected with *C. parvum* oocysts can be entirely cleaned by feeding of Nekka-Rich and that calves infected with *C. parvum* can be cured by the administration of Nekka-Rich.

In this study, we showed that Nekka-Rich has a protective effect against *C. parvum*-infected calves. It has been shown that probiotics (microbes that beneficially affect the host by improving its intestinal microbial balance) such as bifidobacteria and lactic acid bacteria, are useful in treating and preventing various intestinal infections and diarrhea caused by pathogenic bacteria (Gorbach, 1990; Abe et al., 1995; Bernet-Camard et al., 1997; Bomba et al., 1999; Naidu et al., 1999; Heyman, 2000; Vold et al., 2000) and *C. parvum* (Alak et al., 1997, 1999). Previously, we showed that wood vinegar liquid from the bark of an evergreen oak, which is present in Nekka-Rich, has a stimulatory effect on the growth of *E. faecium* and *Bifidobacterium thermophilum*, both of which act as probiotics (Watarai and Tana, 2005). Thus, the growth-stimulating effect of wood vinegar liquid from the bark of an evergreen oak against probiotics would also be related to the removal effect of Nekka-Rich against *C. parvum*-infected calves.

A new method that inhibits the infection of *C. parvum* oocysts in calves would clearly be valuable. We have found that *C. parvum* colonization of mucosal surfaces and excretion of the oocysts in feces are effectively inhibited by administration of Nekka-Rich. This can be explained by the fact that activated charcoal of Nekka-Rich showed effectiveness for removal of *C. parvum* oocysts, and also that wood vinegar liquid from the bark of an evergreen oak has a disinfectant effect against *C. parvum* oocysts. The use of Nekka-Rich as an additive would be effective in preventing *C. parvum* infection in calves, thereby facilitating eradication of the disease. Additionally, inhibition of oocyst shedding by feeding of Nekka-Rich would have a significant impact on controlling contamination of the environment and potential waterborne spread of disease.

CONCLUSIONS

Cryptosporidium parvum oocysts were adsorbed, in a dose-dependent manner, by activated charcoal of Nekka-Rich. In addition, wood vinegar liquid included in Nekka-Rich showed activity against *C. parvum* oocysts. It was shown that Nekka-Rich has an anticryptosporidial effect in calves. Although nontreated control calves showed severe diarrhea and fecal excretion of oocysts after infection with *C. parvum* oocysts, those fed milk replacer with Nekka-Rich showed recovery from diarrhea 1 d after the start of treatment, and no shedding of oocysts occurred on 2 d from the beginning of Nekka-Rich treatment. Thus, we have very promising preliminary data supporting the anticryptosporidial efficacy of Nekka-Rich in reducing or eliminating *C. parvum* infection in calves.

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