Evaluation of the Ability of Di-tri-octahedral Smectite to Adhere to Clostridium difficile Toxins and Clostridium perfringens Enterotoxin In Vitro

J. S. Weese, DVM, DVSc; N. M. Cote, DMV, DVSc;* and R. V. G. deGannes, DVM

Di-tri-octahedral smectite was effective in neutralizing Clostridium difficile toxins A and B as well as Clostridium perfringens enterotoxin in vitro. However, di-tri-octahedral smectite did not inhibit the growth of Clostridium difficile and Clostridium perfringens, nor did it interfere with the activity of metronidazole. Di-tri-octahedral smectite may be a useful option for the treatment of clostridial colitis in horses. Authors’ addresses: Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (Weese, Cote); deGannes Equine Veterinary Services, 191 Western Avenue, P.O. Box 143, Schomberg, Ontario, Canada L0G 1T0 (deGannes). © 2002 AAEP.

*Presenter

1. Introduction

Primary or secondary enterocolitis is a common problem seen in adult horses and foals in practice. Various pathogens, such as Salmonella sp,1 cyathostomes,2 and more recently Clostridium difficile and Clostridium perfringens,3–5 have been implicated. C. difficile produces at least five toxins6; however, the effects of only toxin A and toxin B are well understood. Toxin A is a potent enterotoxin with slight cytotoxic activity,7 whereas toxin B is a potent cytotoxin.6 Clinical signs of equine C. difficile associated disease (CDAD) can be variable and range from mild enteritis to fulminant necrotizing hemorrhagic enteritis. C. perfringens has been associated with enterocolitis in multiple species including the horse.8 Strains of C. perfringens are differentiated into five types based on the production of four major toxins.8 A number of other toxins can be produced, including C. perfringens enterotoxin and β-2 toxin.8 C. perfringens enterotoxin (CPE) is a cytotoxic enterotoxin that has recently been associated with disease in horses.9 Treatment of equine clostridial enterocolitis is mainly supportive and includes fluid therapy, anti-inflammatory agents, and oncotic support. Metronidazole and bacitracin are also widely used. Despite aggressive treatment, the mortality rate and treatment costs can be high.

Infectious diarrhea is accompanied by an impairment of the structure and function of the intestinal barrier. Various topical digestive preparations such as clays have been shown to protect the intestinal mucosa from infectious diarrhea.10 Di-tri-octahedral (DTO) smectite, a natural hydrated aluminomagnesium silicate of lamellar structure, which binds to digestive mucus11,12 and increases resistance to bacterial damage,13 has been shown to increase water and electrolyte absorption in rabbit...
intestinal loops in the presence of *Escherichia coli* infection. In experimental studies, DTO smectite has been shown to be effective in the prevention of *E. coli* in calves and rabbits. Campylobacter jejuni enteritis in mice, *Campylobacter jejuni* enteritis in calves, and cholera toxin activity in dogs, and rotavirus enteritis in calves, as well as affecting the activities of other substances, including bile salts and T-2 mycotoxin in the intestinal tract. Furthermore, DTO smectite has also been shown to fully restore the barrier properties of human intestinal cell monolayers after exposure to tumor necrosis factor (TNF)-α in an in vitro model. One of the most valuable properties of DTO smectite is the ability to efficiently absorb substances in the intestinal tract, particularly positively charged organic cations, such as endotoxins, exotoxins, and organic vapors. In humans, the use of DTO smectite during rehydration therapy has been shown to shorten the course of acute diarrhea inambulatory infants and may reduce the occurrence of prolonged diarrhea. In another study, a significantly shorter duration of diarrhea and decrease in number of stools was reported. Recently, DTO smectite was demonstrated to prevent development of lincomycin-associated colitis in horses. The reason for this protective effect was not evaluated; however, it was suggested that DTO smectite binds to the clostridial toxin and either inactivates it or prevents its absorption.

*C. difficile* and *C. perfringens* are common causes of diarrhea in adult horses and foals; however, no studies have been performed evaluating DTO smectite for the prevention or treatment of clostridial disease in horses. The purpose of this study was to determine whether a commercially available DTO smectite product was able to bind to *C. difficile* toxins A and B as well as *C. perfringens* enterotoxin, inhibit clostridial growth, and inhibit the effects of metronidazole in vitro.

2. Materials and Methods

Clostridial Toxin Binding Assay

A toxigenic, equine-origin strain of *C. difficile* (WCD4) was inoculated into 50-ml brain-heart infusion (BHI) broth and incubated at 37°C for 7 days, at which point the presence of *C. difficile* toxins A and/or B were detected in the broth using a commercial enzyme-linked immunosorbent assays (ELISA). Culture tubes were centrifuged at 4400 g for 10 min, and the supernatant was passed through a 0.2-μm syringe filter to obtain a sterile filtrate containing bacterial toxins. Serial dilutions of diocahedral smectite, from 1:2 to 1:256, were performed in phosphate buffered saline (PBS, pH 7.4). One milliliter of bacterial toxin supernatant was added to 1 ml of each dilution, vortexed, and incubated at 37°C for 1 h. Tubes were then centrifuged and the supernatant was tested for the presence of toxins through ELISA. The intensity of the color reaction, indicating the presence of toxins, was scored from 0 to 4+

Evaluation of Inhibition of *C. difficile* and *C. perfringens*

Smectite was sterilized in dry heat at 160°C for 2 h, and serial dilutions from 1:2 to 1:128 2002 were performed in PBS. *C. difficile* was inoculated onto pre-reduced brucella blood agar and incubated anaerobically for 48 h. A MacFarland 3.0 suspension was then prepared in PBS. *C. difficile* suspension (200 μl) was added to 5 ml of BHI broth and 1 ml of smectite dilution. A positive control consisted of PBS instead of a smectite dilution. Tubes were vortexed and incubated for 24 h at 37°C and read visually for the presence or absence of bacterial growth, indicated by increasing turbidity of the suspension. Confirmation of the presence of absence of bacterial growth and confirmation that contamination of the samples did not occur was performed by inoculating 100 μl of each tube onto pre-reduced blood agar and incubating anaerobically at 37°C for 48 h. Testing was performed in triplicate and was repeated for *C. perfringens*.

Evaluation of the Ability of Smectite to Inhibit the Effects of Metronidazole

In vitro, 1 ml of 5 mg/ml metronidazole was added to 1 ml of sterile smectite dilution from 1:2 to 1:256 and incubated for 1 h at room temperature. Tubes were then centrifuged, and 1 ml of supernatant was added to 5 ml of brain-heart infusion broth and 200 μl of a MacFarland 3.0 suspension of *C. perfringens*. A positive control consisting of PBS and a negative control consisting of metronidazole combined with PBS were used. Tubes were incubated at 37°C for 24 h and read as described above.

3. Results

Clostridial Toxin Binding Assay

The mean semiquantitative toxin score of the positive control (toxin supernatant plus PBS) was 3.7 for *C. difficile* toxins and 3.3 for CPE. This is consistent with the intensity of reaction that can be seen in clinical cases. Neither *C. difficile* toxins nor CPE were detected in any sample from 1:2 to 1:16. For *C. difficile* toxins, the degree of reaction of dilutions 1:32 to 1:256 was less than that of the control for two of the replicates, whereas dilutions 1:32 through 1:128 were of a lesser reaction for the third. For
CPE, the degree of reaction of dilutions 1:32 through 1:128 was less than the positive control for two of the replicates and 1:32 through 1:64 for the third. There was no difference between filtered and unfiltered supernatant, indicating that the negative toxin results were caused by binding of toxin rather than interference with the ELISA.

Evaluation of the Inhibition of C. difficile and C. perfringens

Both C. difficile and C. perfringens grew in the presence of all smectite dilutions. Quantitative culture and the control. Therefore, growth of C. difficile and C. perfringens was not inhibited.

Evaluation of the Inhibition of Metronidazole

No bacterial growth was present in any of the tubes containing metronidazole that had been incubated with smectite. Growth was present in the positive control, indicating that there was no effect of smectite on the bactericidal activity of metronidazole.

4. Discussion

This study demonstrated that DTO smectite is able to bind to C. difficile toxins A and B and C. perfringens enterotoxin in vitro, without affecting clostridial growth or the activity of metronidazole. These findings are compatible with a previous study, which looked at the neutralization effect of DTO smectite on 10 toxigenic C. difficile as well as 8 enterotoxigenic Bacteroides fragilis. A minimalization of the cytopathic effect of C. difficile toxin B on McCoy cell lines by DTO smectite was observed. Neutralization of the toxic effects of B. fragilis enterotoxins was also found.23 These effects may be the result of the excess negative charge at its surface, which attracts positively charged organic cations, such as endotoxins and exotoxins. For the same reason, a study has shown that smectite delays the absorption of basic drugs but does not alter the absorption kinetics of acidic drugs.19 In the present study, no direct inhibition of metronidazole, an acidic drug, was identified.

This study has demonstrated that DTO smectite has the ability to bind to C. difficile toxins A and B and C. perfringens enterotoxin in vitro. This suggests that DTO smectite may be useful for the treatment or prevention of clostridial colitis in horses. In addition to the inhibitory effects on clostridial toxins, it was noteworthy that binding of metronidazole was not reported. This is important because metronidazole is widely used for the treatment of clostridial colitis. This study suggests that there is no contraindication to the combined use of DTO smectite and metronidazole. A commercial DTO smectite product is currently available for use in horses. Label recommendations suggest an initial dose of 3 lbs, followed by 1 lb every 6–8 h. Ecke et al. reported that horses with experimentally induced colitis passed 5.15 ± 0.92 mg/kg/d of feces, corresponding to approximately 56 ± 10 l/d for a 454 kg (1000 lb) horse.24 Assuming homogenous mixing, 56 l/d of diarrhea and a dose of 3 lbs followed by 1 lb every 6 h, an estimated dilution of 1:17 would be present in the first 24 h, followed by 1:30 during subsequent 24-h periods. This is within the range that resulted in partial binding of clostridial toxins. Therefore, it is plausible that the effects demonstrated in vitro would be present in vivo. Whereas further in vivo testing is required, it is possible that DTO smectite will be a safe and effective option for the treatment and prevention of clostridial colitis. Research evaluating the effects of DTO smectite on other intestinal pathogens is also indicated.

References and Footnotes


"Bio-Sponge™, Platinum Performance, Inc. Buellton, CA 93427
"Clostridial difficile TOX A/B Test TechLab Inc, Blacksburg, VA.
"Clostridium perfringens enterotoxin test. TechLab Inc, Blacksburg, VA.

Proceedings of the Annual Convention of the AAEP 2002