General Articles

Evaluation of *in vitro* properties of di-tri-octahedral smectite on clostridial toxins and growth

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Summary

Reasons for performing study: **Clostridial colitis and endotoxaemia of intestinal origin are significant causes of morbidity and mortality in horses. Intestinal adsorbents are available for treatment of these conditions; however, little information exists supporting their use.**

Objectives: **To evaluate the ability of di-tri-octahedral smectite to bind to *Clostridium difficile* toxins A and B, *C. perfringens* enterotoxin and endotoxin, inhibit clostridial growth and the actions of metronidazole *in vitro*.**

Methods: ***Clostridium difficile*** **toxins, *C. perfringens* enterotoxin and endotoxin were mixed with serial dilutions of di-tri-octahedral smectite, then tested for the presence of clostridial toxins or endotoxin using commercial tests. Serial dilutions of smectite were tested for the ability to inhibit growth of *C. perfringens* in culture broth, and to interfere with the effect of metronidazole on growth of *C. perfringens* in culture broth.**

Results: ***Clostridium difficile*** **toxins A and B, and *C. perfringens* enterotoxin were completely bound at dilutions of 1:2 to 1:16. Partial binding of *C. difficile* toxins occurred at dilutions up to 1:256 while partial binding of *C. perfringens* enterotoxin occurred up to a dilution of 1:128. Greater than 99% binding of endotoxin occurred with dilutions 1:2 to 1:32. No inhibition of growth of *C. difficile* or *C. perfringens* was present at any dilution, and there was no effect on the action of metronidazole.**

Conclusions: **Di-tri-octahedral smectite possesses the ability to bind *C. difficile* toxins A and B, *C. perfringens* enterotoxin and endotoxin *in vivo* while having no effect on bacterial growth or the action of metronidazole.**

Potential relevance: ***In vivo*** **studies are required to determine whether di-tri-octahedral smectite might be a useful adjunctive treatment of clostridial colitis and endotoxaemia in horses.**

Introduction

Enterocolitis and endotoxemia are of considerable importance in equine medicine. Some of the clinical and pathological changes encountered in cases of equine colitis may be the result of, in part, the effects of a variety of bacterial toxins including enterotoxins, cytotoxins and endotoxin. The presence of *C. difficile* toxins A and/or B and *C. perfringens* enterotoxin (CPE) in faeces has been associated with enterocolitis in horses and other species (Jones *et al.* 1987; Lyerly *et al.* 1998; Donaldson and Palmer 1999; Weese *et al.* 2001a,b), and these toxins can be detected via ELISAs. Endotoxin (lipopolysaccharide) is a component of the outer membrane of Gram-negative bacteria and is released during cell lysis (Moore and Barton 1998). Endotoxaemia may occur in cases of equine colitis of any aetiology from absorption of endotoxin normally present in the intestinal lumen because of compromise of the intestinal mucosal barrier. Endotoxaemia of intestinal origin can also occur following carbohydrate overload or intestinal accident (McClure *et al.* 1982; Pickersgill and Marr 1998).

Di-tri-octahedral (DTO) smectite is a natural hydrated aluminomagnesium silicate that possesses a number of *in vitro* and *in vivo* properties that could be useful in the treatment of colitis or endotoxaemia of intestinal origin in horses. In experimental studies, DTO smectite has been shown to prevent *Escherichia coli* enteritis in calves and rabbits (Rateau *et al.* 1982), *Cambylobacter jejuni* enteritis in mice (Droy *et al.* 1985), cholera toxin activity in dogs (Fioramonti *et al.* 1987a) and rotavirus enteritis in calves (Droy-Lefaix *et al.* 1986). It has also been shown to affect the activities of bile salts and t-2 mycotoxin in the intestinal tract (Fioramonti *et al.* 1987b; More *et al.* 1987), increase water and electrolyte absorption in rabbit intestinal loops in the presence of *E. coli* infection (Rateau *et al.* 1982) and restore barrier properties of human intestinal cell monolayers after exposure to tumor necrosis factor-α (Mahraui *et al.* 1997). Many of these beneficial effects are thought to result from the ability of DTO smectite to adsorb substances efficiently in the intestinal tract, particularly positively charged organic cations such as endotoxins, exotoxins, and organic vapours (Albengres *et al.* 1985). In man, the use of DTO smectite during rehydration therapy has been shown to shorten the course of acute diarrhoea and reduce the occurrence of prolonged diarrheoa in infants (Madkour *et al.* 1993; Lexomboon *et al.* 1994; Guarino *et al.* 2001; Narkeviviute *et al.* 2002).

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A commercial product containing DTO smectite (Bio-sponge)[1] is marketed as an intestinal adsorbent for use in chronic and acute colitis in mature horses and for the prevention and treatment of rotaviral and *Clostridium difficile*-associated enteritis in foals. Anecdotal success has been reported; however, there has been little scientific evaluation of its use for the treatment or prevention of equine enteric disease. A preliminary study reported that administration of DTO smectite prevented the development of lincomycin-induced colitis in 4 horses while 4 nontreated horses died or were subjected to euthanasia due to severe colitis (Herthel 1998). The authors speculated that DTO smectite prevented overgrowth of clostridial organisms or prevented absorption of clostridial toxins; however, none of these effects were investigated further. The purpose of this descriptive study was to determine whether a commercially available DTO smectite product (Bio-sponge)[1] was able to bind to *Clostridium difficile* toxins A and B, *Clostridium perfringens* enterotoxin and endotoxin, and inhibit clostridial growth. Further, because metronidazole is widely used for the treatment of clostridial and idiopathic colitis, the effect of DTO smectite on metronidazole *in vitro* was also evaluated.

Materials and methods

Clostridial toxin binding assay

A toxigenic, equine-origin strain of *Clostridium 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 was detected in the broth using a commercial ELISA (*Clostridium difficile* TOX A/B Test)[2]. Culture tubes were centrifuged at 4400 ***g*** for 10 mins and the supernatant passed through a 0.2 µm syringe filter to obtain a sterile filtrate containing bacterial toxins. Serial dilution of DTO smectite, from 1:2 to 1:256, was performed in phosphate buffered saline (PBS, pH 7.4). One ml 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 tested for the presence of toxins via ELISA. The intensity of the color reaction, indicating the presence of toxins, was scored from 0–4+ by a blinded observer based on manufacturer's instructions. Positive (toxin supernatant without smectite) and negative (uninoculated BHI broth) controls were used. Testing was performed in triplicate.

To ensure that negative toxin results were not due to inhibition of the assay by residual smectite in the supernatant following centrifugation, a comparison of results between the supernatant and supernatant passed through a 0.2 µm filter was performed.

Testing was repeated for *C. perfringens* enterotoxin (CPE) using an equine-origin enterotoxigenic strain of *C. perfringens* (WCP23) and a commercial ELISA for the detection of CPE (*Clostridium perfringens* Enterotoxin Test)[2]. Procedures for CPE testing were identical to that used for *C. difficile* toxins.

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

Smectite was sterilised in dry heat at 160°C for 2 h and serial dilutions from 1:2 to 1:256 were performed in sterile PBS. Aliquots of each dilution were inoculated onto blood agar plates that were incubated aerobically and anaeobically at 37°C for 48 h to confirm sterility. *Clostridium difficile* WCD4 was inoculated onto prereduced brucella blood agar and incubated anaerobically for 48 h. A MacFarland 3.0 suspension of *C. difficile* was then prepared in 2 ml PBS. *C. difficile* suspension (200 µl) was added to 5 ml BHI broth and 1 ml 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 as indicated by increasing turbidity of the suspension. Confirmation of the presence or absence of bacterial growth, and confirmation that contamination of the samples did not occur was performed by inoculating 100 µl of each tube onto prereduced 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

One ml of 5 mg/ml metronidazole was added to 1 ml sterile smectite dilution, from 1:2 to 1:256, and incubated for 1 h at room temperature. Tubes were then centrifuged and 1 ml supernatant was added to 5 ml brain-heart infusion broth and 200 µl MacFarland 3.0 suspension of a metronidazole-sensitive strain 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.

Evaluation of endotoxin adsorption

One ml of each smectite dilution was inoculated with 25 endotoxin units (EU) of endotoxin (Control Standard Endotoxin *E. coli* 055:B5)[3]. Tubes were vortexed and incubated at 37°C for 1 h. One ml sterile water with 25 EU endotoxin was used as a positive control and 1 ml sterile endotoxin-free water as a negative control. After incubation, tubes were centrifuged at 4400 ***g*** for 5 mins and the supernatant tested for the presence of endotoxin using a qualitative limulus amebocyte lysate (LAL) assay (Limulus Amebocyte Lysate Endosafe)[3] with a sensitivity of 0.25 EU/ml. One hundred µl of supernatant was added to a tube containing 100 µl assay reagent which was then incubated at 37°C for 1 h. Results were read visually. A positive result was indicated by a firm clot, which maintained its integrity when inverted. Based on the sensitivity of the test and initial endotoxin concentration, a negative result indicated a clearance of ≥99% of endotoxin. Testing was performed in triplicate. All testing was performed using endotoxin-free tubes and pipettes.

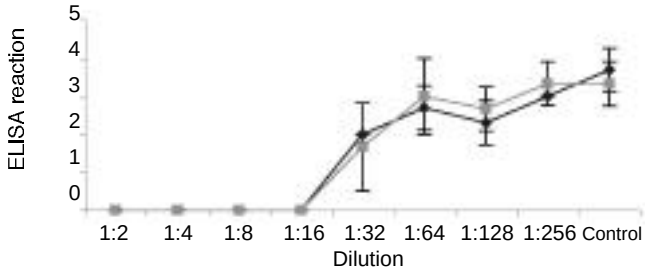

Fig 1: Effect of different dilutions of di-tri-octahedral smectite on semiquantitatively assessed ELISA reaction for Clostridium difficile *toxins A and/or B, and* Clostridium perfringens *enterotoxin (mean ± s.d.).* ◆ = C. difficile *toxins;* ■ = C. perfringens *enterotoxin.*

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 (J.S. Weese, unpublished data). Neither *C. difficile* toxins nor CPE were detected in any sample from 1:2 to 1:16 (Fig 1).

For *C. difficile* toxins, the degree of reaction of dilutions 1:32 to 1:256 was less than that of the control for 2 of the replicates, while dilutions 1:32 to 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 2 of the replicates and 1:32 to 1:64 for the third (Fig 1). There was no difference between filtered and unfiltered supernatant, indicating that the negative toxin results were due to binding of toxin rather than interference with the ELISA by residual smectite particles.

Evaluation of the inhibition of C. difficile *and* C. perfringens

Both *C. difficile* and *C. perfringens* grew in the presence of all smectite dilutions.

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.

Evaluation of endotoxin adsorption

The qualititative endotoxin assay reaction was negative for all replicates of dilutions 1:2 to 1:32, indicating removal of at least 99% of endotoxin. The endotoxin assay was positive for dilutions 1:64 to 1:256.

Discussion

This study demonstrated that DTO smectite binds *in vitro* to *C. difficile* toxins A and B, *C. perfringens* enterotoxin and endotoxin, without affecting clostridial growth or the activity of metronidazole. These findings are consistent with a previous study, which reported a minimalisation of the cytopathic effect of *C. difficile* toxin B on McCoy cell lines and neutralisation of the effects of *B. fragilis* enterotoxins by DTO smectite (Martirosian *et al*. 1998).

The effects reported in this study are thought to have occurred as a result of adsorption of bacterial toxins. The surface of DTO smectite is negatively charged (Albengres *et al.* 1985), which may permit attraction and binding with positively charged organic cations, such as endotoxins and exotoxins. The lack of inhibition of the effect of metronidazole may be because metronidazole is an acidic drug as it has been reported that smectite affects absorption of basic drugs but does not alter the absorption kinetics of acidic drugs (Albengres *et al.* 1985). This is an important finding as metronidazole is commonly used for the treatment of clostridial and idiopathic colitis (Jang *et al.* 1997; McGorum *et al.* 1998) and these results suggest that co-administration of DTO smectite and metronidazole would not be antagonistic.

A commercial DTO smectite product is currently available for use in horses. Label recommendations suggest an initial dose of 1.4 kg followed by 454 g every 6–8 h. Ecke *et al.* (1998) reported that horses with experimentally-induced colitis passed 5.15 ± 0.92 ml/kg/day of faeces, corresponding to approximately 56 ± 10 l/day for a 454 kg horse. Assuming homogenous mixing, an admittedly questionable assumption, production of 56 l/day of diarrhoea and a dose of DTO smectite of 1.4 kg followed by 454 g 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 and endotoxin. Therefore, it is plausible that the effects demonstrated *in vitro* would be present *in vivo.*

Di-tri-octahedral smectite may be a useful adjunctive treatment in cases of equine colitis. It may also have a role in prevention of endotoxaemia associated with intestinal injury and carbohydrate overload. Adverse effects have not been reported in human studies (Madkour *et al.* 1993; Guarino *et al.* 2001; Narkeviviute *et al.* 2002). One drawback is the large volume that must be administered, particularly with the initial dose that is recommended for the commercial equine product. Frequent passage of a nasogastric tube is necessary, which may be difficult in some situations. Regardless, clinical trials evaluating the efficacy of DTO smectite *in vivo* are indicated to determine whether the *in vitro* effects demonstrated here and elsewhere are clinically relevant in horses.

Manufacturers' addresses

[1]Platinum Performance Inc., Buelton, California, USA.
[2]TechLab Inc., Blacksburg, Virginia, USA.
[3]Charles River Endosafe, Charleston, South Carolina, USA.

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