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Diosmectite treatment reduces stress-induced visceral hypersensitivity but does not affect the gut microbiota in rats

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Abstract. – OBJECTIVE: To evaluate the effect of diosmectite on visceral hypersensitivity and intestinal transit in a rat chronic stress model and on the faecal microbiota.

MATERIALS AND METHODS: Wistar rats (175-225 g; n=10) were randomized into four groups: diosmectite/control, diosmectite/stress, vehicle/control, and vehicle/stress. Diosmectite (500 mg/kg, PO) was administered for five days for assessment of visceral hypersensitivity and intestinal transit and for three weeks for assessment of faecal microbiota. The stress procedure was a daily chronic passive water avoidance session. Intestinal transit was evaluated by faecal output in the hour following the last stress session. Visceral sensitivity in response to colorectal distension (CRD) was assessed at baseline and 30 min after the last stress session. In another group of rats, faecal material was collected before and after treatment with diosmectite or vehicle; genomic DNA was extracted and sequenced to characterize the faecal microbiota.

RESULTS: Under nonstressed conditions, diosmectite treatment did not modify intestinal transit compared to the vehicle group (p=0.33). After the stress procedure, a trend towards reduction in stress-induced stool production was observed with diosmectite compared to vehicle (6.3±1.1 *vs.* 4.9±1.2 respectively; p=0.38). In the control condition, the number of CRD-evoked abdominal contractions was 20±4 after diosmectite and 24±2 after vehicle (p=0.75). In the stressed condition, the number of contractions increased to 34.4±2.4 after vehicle (p<0.05 compared to control). Stress-related hypersensitivity was attenuated after diosmectite treatment (26.9±2.2; p<0.05 compared to vehicle). No relevant changes were observed in the faecal microbiotic profile after treatment with diosmectite or vehicle.

CONCLUSIONS: Diosmectite treatment attenuates stress-induced visceral hypersensitivity; this effect may contribute to the therapeutic effect of diosmectite in IBS in humans.

Key Words:
Diosmectite, Visceral hypersensitivity, Stress, Gut, Microbiota, Rat.

Introduction

Irritable bowel syndrome (IBS) is a functional bowel disorder affecting around 11% of people worldwide[1,2], in which abdominal pain or discomfort is associated with features of disordered defaecation[3-5]. Several pathophysiological mechanisms have been proposed to underly the development of IBS. These include increased visceral sensitivity in response to gut distension[6], damage to the intestinal epithelial barrier[7,8], low grade mucosal inflammation[9] and modifications to the gut microbiota[10-12]. Moreover, in patients with IBS, a positive correlation has been reported between increased permeability of the intestinal mucosa to large molecules and the severity of visceral pain[13,14].

Diosmectite is a natural clay used clinically in the treatment of acute diarrhoea[15,16], as well as in chronic bowel disorders such as IBS[17,18]. In acute infectious diarrhoea, diosmectite is thought to act as a mucus stabilizer. In this way, it may protect the gastrointestinal mucosa against microbial toxins, proinflammatory cytokines and bile salts. In addition, diosmectite has been shown to direct adsorb bacterial enterotoxins and viruses[19,20]. These mechanisms may contribute to beneficial effects on transit time, abdominal pain and intestinal permeability[15-17,21]. Moreover, it is also possible that diosmectite might influence the pathophysiological processes underlying chronic, non-infectious diarrheal disorders.

Several of the disturbances of intestinal function described in patients with IBS can be reproduced in experimental animals. For example, rats exposed to chronic stress develop visceral hypersensitivity and increased intestinal permeability[22-24]. Damage to the intestinal epithelial barrier may also lead to changes in the gut microbiota, thus activating mucosal immunity, leading to release of pro-inflammatory mediators which facilitate local inflammation and nerve sensitization[25-27]. It was thus

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of interest to evaluate whether diosmectite could modulate experimental visceral hypersensitivity in rats or modify the intestinal microbiota.

The specific objectives of this study were firstly to evaluate the effect of diosmectite on visceral hypersensitivity and intestinal transit time in the rat chronic stress model, and secondly to determine whether diosmectite affects the gut microbiota in rats.

Materials and Methods

Animals

Wistar rats aged 6-8 weeks and weighing 175-225 g were used. Animals were sourced from Janvier Labs (Le Genest-Saint-Isle, France) and housed individually in a temperature-controlled room (21±1°C), which was maintained on a 12/12-hour light/dark cycle. A 15-day acclimatization period was imposed between delivery of animals and the first treatment administration. Animals were given free access to water and food (Envigo laboratory pellets, Teklad Global Diet®; Gannat; France). The experimental protocols used were approved by the Local Animal Care and Use Committee of the French Agronomics Research Institute (Institut National de la Recherche Agronomique; INRA; Paris; France; approval N°APAFIS#5577-201606061639777). The test facility accreditation number for the use of laboratory animals is B3155513.

Overall Study Design

Six groups of ten Wistar rats were used (Table I). As in previous studies[28,29] using these experimental paradigms, female rats were used for evaluating intestinal transit and visceral hypersensitivity (Groups 1-4) and male rats for the evaluation of the gut microbiota (Groups 5 and 6). Groups 1 and 3 were treated with diosmectite for five days and Groups 2 and 4 with a matching vehicle. Groups 1 and 2 were exposed to a water avoidance stress (WAS) test and Groups 3 and 4 underwent a sham stress procedure. Groups 5 and 6 were used for evaluation of the gut microbiota. Group 5 was treated with vehicle for three weeks and Group 6 received diosmectite.

Test Product and Administration

Diosmectite powder was obtained from Ipsen SA, Dreux, France. Treatment was administered at a dose of 200 mg/kg/day by oral gavage suspended in 0.5 ml physiological saline (0.9% NaCl). This dose was based on the initial daily therapeutic human dose of 9-18 g. Control animals received 0.5 ml physiological saline only. The treatment period was five days for assessment of faecal output and visceral hypersensitivity and three weeks for evaluation of the faecal microbiota. In Groups 1-4, treatment was administered two hours before the WAS session. In Groups 5 and 6, individual rats were weighed each week to ensure that the dose was correctly adjusted to body weight.

Water Avoidance Stress Test

A chronic stress model[30] that has been previously shown to induce sustained visceral hyperalgesia was used. Briefly, rats were individually placed on a Plexiglass platform (6 cm × 6 cm and 14 cm high). The platform was mounted in the center of a Plexiglass tank (55 cm long × 37 cm wide × 26 cm high), which could be filled with water to within 1 cm of the top of the platform. In the experimental setting, the tank was filled with water at room temperature (25°C); in the control setting (no stress), the tank was left empty. Rats were placed on the platform for one hour and for five consecutive days. All stress sessions were performed at the same time of day (8:00 am and 10:00 am) to minimize any influence of circadian rhythms.

Table I. Diosmectite treatment in the WAS model.

Study Group	N	Treatment	Duration	Stress protocol	Evaluation
1.	10	Diosmectite	5 days	Yes	Intestinal transit Visceral sensitivity
2.	10	Vehicle	5 days	Yes	Intestinal transit Visceral sensitivity
3.	10	Diosmectite	5 days	No	Intestinal transit Visceral sensitivity
4.	10	Vehicle	5 days	No	Intestinal transit Visceral sensitivity
5.	10	Diosmectite	3 weeks	No	Microbiotic screen
6.	10	Vehicle	3 weeks	No	Microbiotic screen

Intestinal Transit

Intestinal transit was determined by faecal output. The number of faecal pellets was measured one hour after the end of the last WAS session on the fifth day.

Visceral Sensitivity (Electromyography)

Electromyographic (EMG) recordings were performed to assess visceral hypersensitivity in response to colorectal distension 20 min after the last WAS session, as previously described[28,29]. For recording, three groups of NiCr wire electrodes (Sandviken; Stockholm, Sweden) were surgically implanted into the abdominal external oblique muscle of female rats.

On the day of test, an arterial embolectomy catheter (Fogarty, Edwards Laboratories Inc., Santa Anna, CA, USA), was inserted into the colorectum to a depth of 1 cm from the anus and fixed at the base of the tail. The balloon was progressively inflated by steps of 0.4 mL, from 0 to 1.2 mL, each step lasting 5 min.

EMG recordings were performed five days after surgery. The electrical activity of the abdominal muscle was recorded and analyzed with Powerlab Chart 5 program from AD instruments (Colorado, CA, USA). The number of spike bursts (abdominal contractions) registered for each colorectal distension step was used as a visceral sensitivity index. Data are presented as the number of abdominal contractions/5 minutes.

Effect of Chronic Treatment of Diosmectite on the Gut Microbiota

Two groups of ten rats were studied in this experiment, one treated with diosmectite and the other receiving vehicle. Faecal material was obtained for assessment of the microbiota before the first treatment, following the last treatment three weeks later and following a further two-week washout period. In each case, a single stool per rat was collected from the anus. Genomic DNA was extracted from the collected faeces using a ZR faecal DNA MiniPrep Kit (Zymo Research; Irvine, CA, USA) and DNA concentrations were determined fluorometrically (Qubit® dsDNA HS assay, Invitrogen; Waltham, MA, USA).

Bacterial populations contained in the samples were determined using next generation high-throughput sequencing of variable regions (V3-V4) of the 16S rDNA bacterial gene (Illumina MiSeq System). Bioinformatic analysis was performed to classify organisms from a sample by taxonomic level (phylum, class, order, family, genus, and species).

Statistical Analysis

Continuous variables were expressed as mean values ± the standard error of the mean (SEM). The number of abdominal contractions and faecal output were compared between animals treated with diosmectite and controls using a one-way analysis of variance (ANOVA), followed by a *t*-test. A probability threshold of < 0.05 was considered as significant. Presentation of the faecal microbiota was purely descriptive. These data were presented as relative abundance cladograms. Data analysis was performed using SAS software (Cary, NC; USA), Version 16.1.

Results

Intestinal Transit

In unstressed rats, stool production during the hour following the beginning of the last sham WAS session was low (mean: 0.3 stools in the vehicle group and none in the diosmectite group; Table II). In contrast, faecal output was significantly increased ($p<0.05$) in stressed rats following both vehicle administration and diosmectite administration (Table II). In stressed rats, stool output following diosmectite administration was somewhat lower than after vehicle administration, but this difference was not statistically significant (Table II).

Visceral Hypersensitivity

The number of abdominal contractions increased with successive steps of colorectal distension (Figure 1). In unstressed rats, treatment with diosmectite did not modify the electromyographic response to colorectal distension observed in vehicle-treated rats.

In stressed rats administered vehicle, the number of abdominal contractions in response to colorectal distension was significantly increased at inflation volumes of 0.8 (30.1±2.5 *vs.* 19.8 contractions; $p<0.01$) and 1.2 ml (34.4±2.4 *vs.* 23.2±1.1; $p=0.0001$; Wilcoxon test) (Figure 1). After the treatment with diosmectite, this stress-related hy-

Table II. Intestinal transit.

	Vehicle	Diosmectite
Unstressed condition	0.3±0 3 pellets	None
Stressed condition	6.3±1.1 pellets	4.9±1.2 pellets

Data (mean ± SEM) are expressed as the number of faecal pellets collected during one hour after the fifth WAS session.

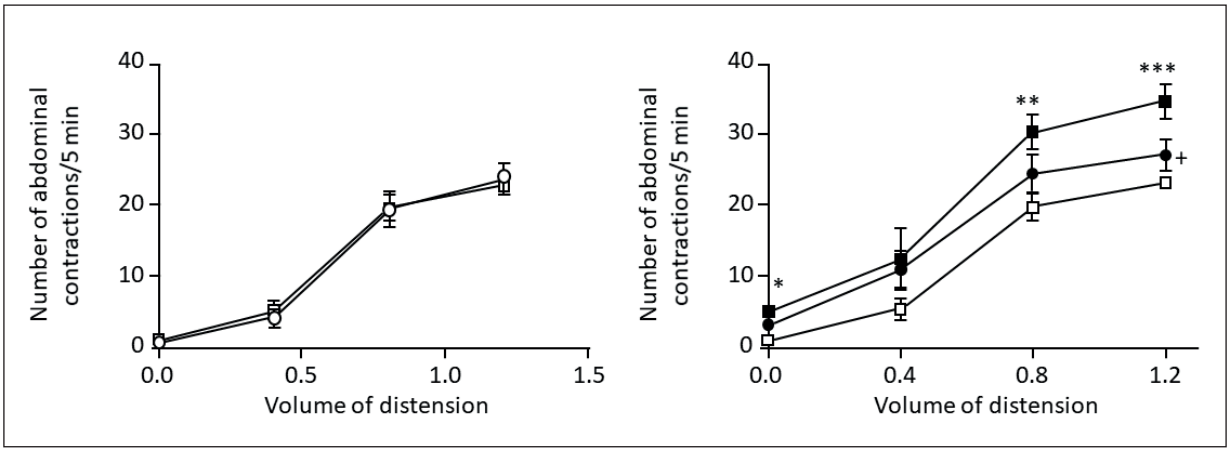

Figure 1. Effect of diosmectite treatment on visceral sensitivity in female rats. Data (mean ± SEM) are expressed as the mean number of abdominal contractions /5min with their standard errors.
○□: unstressed rats; ●■: stressed rats; □■: vehicle-treated; ○●: diosmectite-treated.
Differences between □ and ■ groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Difference between● and ■ groups: +$p < 0.05$.

persensitivity was attenuated, making this difference significant ($p<0.05$) at the highest level of colorectal distention (1.2 ml: 26.9±2.2 *vs.* 34.4±2.4) (Figure 1).

Evaluation of Faecal Microbiota

Before treatment, the microbiotic profiles of faecal samples were very similar in the two groups of rats (Figure 2). Following three weeks of treatment, the profiles were again comparable between groups (Figure 2). However, compared to baseline, some changes in the faecal microbiota were observed in both groups at the end of the three-week period. In particular, the abundance of both *Porphyromonadaceae* and *Lactobacillacea* families declined, whereas that of *Peptostreptococcaceae* and *Rikenellaceae* families increased (Figure 2).

Discussion

The objective of this pharmacological study in rats was to investigate the effect of diosmectite on gut mechanisms that might be relevant for the pathophysiology of IBS. The principal finding was that diosmectite reduces visceral hypersensitivity induced by stress. On the other hand, no effect of diosmectite was observed on the gut microbiota. A trend towards a reduction in the accelerated intestinal transit time observed following a repetitive session stress was also identified.

The principal effect of diosmectite observed in this study was the attenuation of visceral hypersensitivity. This hypersensitivity is a phenomenon that has been described extensively in human IBS[31] and may underly the abdominal pain, that is a key symptom of this condition[5]. Indeed, several antispasmodic agents that have been shown to be effective in the treatment of IBS have also been shown to prevent visceral hypersensitivity[28,32]. In contrast, diosmectite did not modify the visceral response to colorectal distension in the unstressed condition, suggesting that it does not influence the basal sensitivity of the colon. An important factor contributing to the development of visceral hypersensitivity in the clinical setting is chronic stress and the activation of the corticotropin-releasing factor signaling pathway[5]. In addition, local release of bacterially derived toxins within the gut lumen can stimulate the local release of certain cytokines which can sensitize the gut wall to nociceptive stimuli[33]. In the rats used for the present study, certain of these cytokines were also liberated[30] during the water-avoidance stress model. In this respect, the mechanism of action of diosmectite may involve physical protection of mucosa and reduced activation of proinflammatory cytokines[19], and these effects may contribute to the attenuation of visceral hypersensitivity observed in the present model.

The findings relating to potential effects of diosmectite on intestinal transit in stressed rats are difficult to interpret. A reduction in stress-induced stool output of around 20% compared to vehicle-treated animals was observed, but the difference was not statistically significant. It is possible that diosmectite had a modest effect on transit which the present study was insufficiently

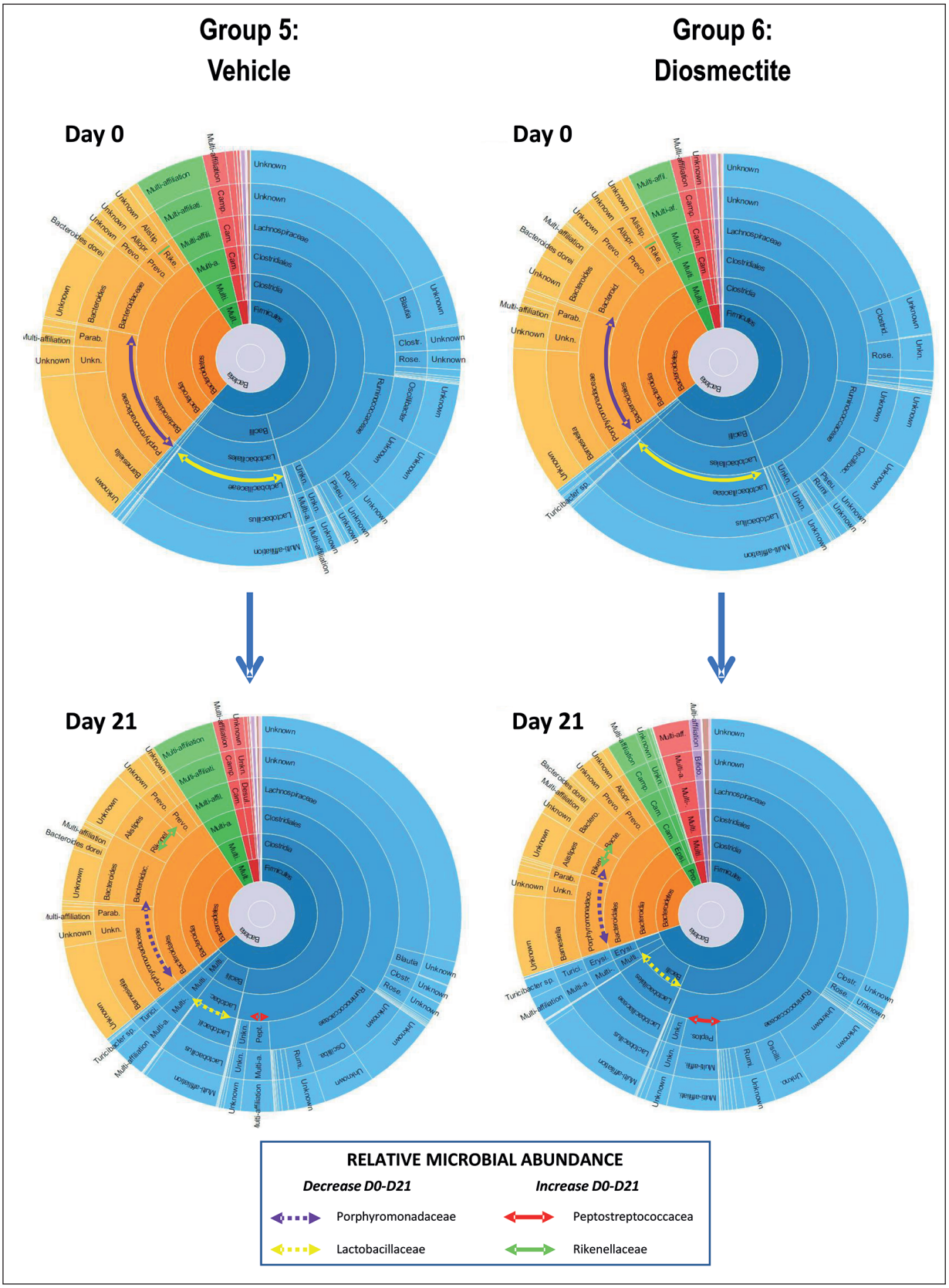

Figure 2. Relative microbial abundance at D0 and D21 in the two treatment groups. Treatment Group 5 received vehicle (0.5 mL water, PO) for three weeks. Treatment Group 6 received diosmectite (500 mg/kg, PO) for three weeks.

powered to demonstrate. In addition, stool output was rather low in this study, perhaps reflecting the relatively low number of stools passed by adult rats fed on a laboratory diet[34], and which may have limited the sensitivity of the test.

No major differences in the faecal microbiota were observed between animals treated with vehicle or diosmectite for 30 days. Although some changes in relative microbial abundance were observed over time, these were relatively small and similar in both groups. It is thus likely to represent normal physiological variability in the gut ecosystem, rather than a treatment effect alone. The present findings would indicate that, in rats, the effect of diosmectite on visceral hypersensitivity is not mediated by changes to the gut microbiota. In our study, it should be noted that the potential effect of diosmectite on the microbiota was evaluated in unstressed rats, and it is possible that diosmectite may modulate stress-induced changes in gut microbiota.

Numerous strands of evidence suggest that changes in the gut microbiota may influence visceral sensitivity to pain, and it has also been suggested that this phenomenon may play a role in the development or presentation of IBS[35,36]. A recent study[37], showing that diosmectite facilitates the formation of biofilms by probiotics, may indicate a possible mechanism for a potential effect of diosmectite on stress-induced perturbations of the gut microbiota. This study reported that *Bifidobacterium* and *Lactobacillus,* two bacterial strains found in the healthy intestinal microbiota, form biofilms on diosmectite microspheres efficiently *in vitro*. In stress-induced visceral hypersensitivity, diosmectite may, in this way, promote the survival of *Bifidobacterium* and *Lactobacillus* in the gut.

The findings of this experimental study may be relevant to those of clinical studies[17,18,38,39] on the effects of diosmectite in the treatment of IBS. Several studies have been performed, of which the most robust results to be a randomized, double-blind and placebo-controlled trial including 104 patients, which evaluated abdominal pain using a visual analogue scale over the course of the study. In the diosmectite group, but not the placebo group, a progressive reduction of pain ratings was observed, reaching around 40% after two months of treatment. Two other studies[18,38] have also reported reductions in pain scores following diosmectite treatment, although there were no placebo groups in these studies. The lack of effect of diosmectite in the present study on basal visceral sensitivity or on the gut microbiota is also consistent with the good tolerability of diosmectite reported in clinical trials, after both acute and long-term treatment[19,40].

However, our study presents certain limitations. For example, the effect of diosmectite on gut microbiota in basal conditions may depend on the rodent species used. In support of this, a recent study[37] demonstrated that oral administration of diosmectite in mice modifies the gut microbiota in favour of increased proportions of Firmicutes. In addition, the sample size was relatively small, which may have precluded the detection of a statistically significant reduction in intestinal transit. Finally, the role of diosmectite on gut microbiota also needs to be evaluated in stressed conditions, in order to determine whether treatment can correct a dysbiotic microbiota or facilitate the formation *in vivo* of a protective biofilm of specific bacteria known to help maintain gut homeostasis.

Conclusions

Treatment with diosmectite reduces stress-induced visceral hypersensitivity in rats independently from a direct effect on the gut microbiota, and this effect may contribute to the therapeutic effect of diosmectite in IBS in humans.

Conflict of Interest

NB is an employee of Ipsen CHC, Boulogne, France. HMF was an employee of this company at the time of the study. HE, CB and VT have no conflicts of interest to declare.

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