A Macroporous Magnesium Oxide-Templated Carbon Adsorbs Shiga Toxins and Type III Secretory Proteins in Enterohemorrhagic Escherichia coli, Which Attenuates Virulence

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Enterohemorrhagic Escherichia coli (EHEC) is one of the most common foodborne pathogens. However, no drug that prevents the severe complications caused by this bacterium has been approved yet. This study showed that a macroporous magnesium oxide (MgO)-templated carbon material (MgOC150) adsorbs Shiga toxins, and Type III secretory EspA/EspB proteins responsible for EHEC pathogenesis, and decreases the extracellular levels of these proteins. On the other hand, this material did not affect the growth of EHEC.

Citrobacter rodentium traditionally used to estimate Type III secretion system-associated virulence in mice is highly virulent. The survival period of infected mice was prolonged when MgOC150 was administered. This adsorbent disturbed neither mammalian cells nor normal intestinal bacteria, such as Enterococcus hirae, Lactobacillus acidophilus, and Lactobacillus casei. In contrast, MgOC150 adsorbed antimicrobial agents, including β-lactams, quinolones, tetracyclines, and trimethoprim/sulfamethoxazole. However, fosfomycin and amikacin were not adsorbed. Thus, MgOC150 can be used with fosfomycin and amikacin to treat infections. MgOC150 is used for industrial purposes, such as an electrode catalyst, a bioelectrode, and enzyme immobilization. The study proposed another potential application of MgOC150, assisting anti-EHEC chemotherapy.

Keywords: bacterial pathogenesis, antimicrobial resistance, porous carbon, virulence, enterohemorrhagic Escherichia coli, Shiga toxin, antimicrobial chemotherapy, Type III secretion system

INTRODUCTION

Shiga toxin-producing Escherichia coli (STEC) are a group of foodborne pathogens that can cause severe bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), which may lead to acute kidney failure, and neurological issues, such as acute encephalopathy (Tarr et al., 2005). Food poisoning caused by STEC is an infectious disease that affects more than
2.8 million people in 21 countries annually (Majowicz et al., 2014). STEC is known to produce two genetically distinct Shiga toxins named Stx1 and Stx2. Stx1 and Stx2 proteins bind to its receptor, globotriaosylceramide Gb3, localized on the host cell membrane, such as renal glomerular endothelial cells, resulting in cell death induction by inhibiting protein synthesis within host cells, which are closely associated with HUS development (Lingwood et al., 1987; Romer et al., 2007). The cytotoxicity of Shiga toxins depends on the Gb3 receptor because Gb3-deficient cells have very low susceptibility to these toxins (Shin et al., 2009). Shiga toxins also bind to the globotriaosylceramide Gb4 receptor to a lesser extent, and its receptor is involved in the induction of host cell death (Lingwood et al., 1987; Samuel et al., 1990). Enterohemorrhagic E. coli (EHEC) is the most important subgroup of STEC. In addition to Shiga toxins, EHEC produces effector proteins, which are responsible for the pathogenicity of this bacterium (Nataro and Kaper, 1998; Croxon and Finlay, 2010). Effector proteins are secreted via a protein transport machinery termed the Type III secretion system, and induce the formation of attaching and effacing (A/E) lesions in intestinal epithelial cells (Galan and Wolf-Watz, 2006). A/E lesions are characterized by the attachment of bacteria to the host cell membrane via the interaction of intimin and its receptor, the destruction of gut epithelial microvilli, and actin polymerization in the host cells (Kenny et al., 1997).

Antibiotics are commonly used to treat bacterial infections. However, the efficacy of antibiotic treatment for this infectious disease is controversial because in vitro experiments suggested that some antibiotics promote the release of Shiga toxins, which might increase the risk of HUS development (Wong et al., 2000; Safdar et al., 2002). Some alternative methods to prevent severe complications including HUS have been proposed, such as Gb3 analogs, recombinant antibodies that target Shiga toxins, effector proteins or intimin, and synthetic small organic molecules that inhibit Gb3 or Stx2 production (Nakao et al., 1999; Yamagami et al., 2001; Trachtman et al., 2003; Rasko et al., 2008; Yu et al., 2011; Ruano-Gallego et al., 2019). However, these molecules have not yet been approved as therapeutics.

Adsorbent is a term for materials that can trap certain chemical substances, and it is typically used to remove process contaminants in industries. Several adsorbents are also used as oral medicines. Colesteryamine strong ion exchange resin is an approved medicine for the treatment of the hypercholesterolemia and the pruritus that often occurs during liver failure (Scaldaferri et al., 2013; Hegade et al., 2015). This drug acts as a bile acid sequestrant in the gastrointestinal tract. Activated charcoal is another type of adsorbent, and it can be used for the treatment of acute poisoning and the removal of bodily wastes associated with certain cardiovascular diseases (Zellner et al., 2019). AST120 (Kremezin) has been approved as an oral activated charcoal medicine to treat progressive chronic kidney disease as it removes the uremic toxin precursors produced by gut microorganisms (Niwa, 2011). In addition, a pilot study in clinical trial showed that a dietary supplement with an activated charcoal material reduced trimethyl amine concentration in urine from individuals with trimethylaminuria, and alleviated the symptoms of this disease (Yamazaki et al., 2004). However, no adsorbent medicine is currently approved for treatment of infectious diseases.

Porous carbon can adsorb non-polar organic molecules that fit its internal pore. Activated charcoal is the most well-known substrate of porous carbons, and it is made from carbon substrates, such as coals, coconut shells, and phenolic resins, and pores with various sizes can be generated in the activation process. Magnesium oxide (MgO)-templated carbon (MgOC) is another recently industrialized porous carbon. Its pore is produced by a method that is distinct from conventional activation (Inagaki et al., 2004). The pore template is formed by incorporating the MgO molecule generated during the pyrolysis of an Mg-containing organic substrate into a carbon matrix, and the pore is produced by removing the MgO molecule. This method enables the production of a more uniform size of pores than the activation method. Then, the resulting pores highly adsorb a targeted size molecule, although the adsorption of untargeted size molecules may be limited (Morishita et al., 2010).

This study aimed to establish a strategy to adsorb the Shiga toxins and Type III secretory proteins responsible for EHEC pathogenicity without disturbing host cells and beneficial bacteria in the host. In this study, we found that one MgOC material with an average pore size of 150 nm could adsorb both Shiga toxins and Type III secretory proteins produced by EHEC without impairing bacterial growth, including EHEC and several normal intestinal bacteria. Mice infected with bacteria exhibited an extended survival when MgOC was administered. Herein, we propose a potential option to treat EHEC/STEC infections.

**MATERIALS AND METHODS**

**Bacterial Strains, Host Cells, Culture Conditions, and Materials**

EHEC O157:H7 Sakai, Citrobacter rodentium DBS100, Enterococcus hirae ATCC9790RF, Lactobacillus acidophilus ATCC4356, and Lactobacillus casei ATCC393 strains were used. EHEC and C. rodentium were cultured in Luria-Bertani (LB) medium unless otherwise indicated. E. hirae was cultured in Brain Heart Infusion (BHI) medium. L. acidophilus and L. casei were cultured in De Man, Rogosa, and Sharpe (MRS) medium. Bacteria were cultured in glass tubes at 37°C. Caco-2 (ATCC HTB-37) cells and Vero cells, and human kidney cells (HTB-44) were cultured in Dulbecco's modified Eagle medium (DMEM) and Eagle's minimal essential medium (EMEM), respectively containing 10% HyClone FetalClone III serum (HyClone Laboratories, Inc., Logan, UT, United States) at 37°C and in an atmosphere of 5% CO2. The MgOC material with a pore size of 150 nm, named MgOCiso, was obtained from Toyobo (Osaka, Japan). The activated charcoal material (made from coconut shell) was obtained from Nacalai Tesque (Kyoto, Japan). The purified Shiga toxin protein and lysozyme were obtained from ATCC.
**Shiga Toxin Assay**

To estimate the amounts of Shiga toxins (Stx1 and Stx2), latex agglutination reagents (Denka Seiken Co. Ltd., Tokyo, Japan) were used. EHEC strains were cultured with shaking to the early stationary phase in Mueller–Hinton medium, and their supernatants were separated by centrifugation at 13,000 g. Cell pellets were resuspended in EzBacYeastCrusher containing 60 mg/l lysozyme (ATTO, Tokyo, Japan) to extract intracellular proteins, and diluted into phosphate-buffered saline (PBS). These cell lysates (0.63 ng) and culture supernatants were serially diluted in 96-well round bottomed plates containing PBS, and an equal volume of latex suspension sensitized with the Stx1 or Stx2 antibody was then added. After incubation for 14 h at 4°C, titers were determined. The titers are presented as the reciprocal of the dilution of the last well before agglutinations were observed. To test the adsorption of Stx1 and Stx2 to MgOC\textsubscript{150} and activated charcoal, the cell-free culture supernatant from EHEC was incubated with and without these materials for 2 h at 4°C. The cell-free culture supernatant was prepared by centrifugation at 13,000 g and passing through a membrane filter (pore size: 0.22 μm). After removal of porous materials by centrifugation at 15,000 g, Stx1 and Stx2 in the supernatant were assayed using latex agglutination reagents. To test the adsorption of purified Shiga toxin to MgOC\textsubscript{150}, the toxin (0.4 μg) was incubated with and without MgOC\textsubscript{150} for 2 h at 4°C. Non-adsorbed toxin in the supernatant was quantified in a Bio-Rad protein assay according to the Bradford method (Bio-Rad Laboratories, Hercules, CA, United States). The cytotoxicity of Shiga toxins in Vero cells and HTB-44 was measured. Bacteria-free culture supernatants were 10-fold diluted into DMEM and EMEM, respectively containing 10% HyClone FetalClone III serum, and added to cultured Vero and HTB-44 cells in 96-well plates. As a control, a 10-fold diluted Mueller–Hinton medium in DMEM and EMEM containing 10% HyClone FetalClone III serum was added to host cells. After incubation for 48 h, the cell viabilities were determined as previously described with CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI, United States; Hirakawa et al., 2021). Cell viabilities were represented as relative light units (RLUs) by their ratios (%) to the RLU of the control sample.

**Western Blotting**

EHEC strains were cultured with shaking to an early stationary phase in Dulbecco's modified Eagle medium (DMEM). Secreted proteins were precipitated from the supernatants using 10% trichloroacetic acid (TCA) and were dissolved in a Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, United States). Bovine serum albumin (BSA) was used as a loading control and was added to the secreted protein samples prior to the precipitation with TCA. Intracellular proteins were resuspended in 50 mM phosphate buffer containing 8 M urea and then lysed by sonication. EspA and EspB were detected with their antisera, as previously described (Hirakawa et al., 2020a,b). To test the adsorption of EspA and EspB to MgOC\textsubscript{150} cell-free culture supernatant prepared by centrifugation of EHEC culture at 13,000 g and passing through a membrane filter was incubated with and without MgOC\textsubscript{150} for 2 h at 4°C. After removal of the MgOC\textsubscript{150} material by centrifugation at 15,000 g, EspA and EspB in the supernatant was measured by western blotting.

**Citrobacter rodentium Infection in Mice**

Three-week-old female C3H/HeJ mice were obtained from CLEA Japan (Tokyo, Japan). MgOC\textsubscript{150} was orally administered with a feed during the experiment as performed in previous studies used AST-120, an approved porous carbon medicine (Yang et al., 2017; Nakada et al., 2019). In those studies, 5 to 8% (w/w) of AST-120 was contained in a feed. For this reason, we supplied 7.5% (w/w) of MgOC\textsubscript{150}. The mice were housed for 7 days before infection (N=5 control mice for non-infection and mice bled without MgOC\textsubscript{150} for infection, N=6 mice bled with MgOC\textsubscript{150} for infection). C. rodentium DBS100 was cultured overnight in LB medium. Bacterial cells were resuspended in fresh LB medium at a concentration of 1 x 10\textsuperscript{9} CFU/ml, and 200 μl bacterial suspension (2 x 10\textsuperscript{8} CFU) was orally administered. As a control group, 200 μl bacteria-free LB medium was inoculated into mice. To measure the survival rates and body weight, mice were monitored daily for 21 days.

**Cytotoxicity Assays**

To test the toxicity of MgOC\textsubscript{150} in host cells, we used Caco-2 cells. MgOC\textsubscript{150} was added to cultured Caco-2 cells. After incubation for 24 h, the cell viabilities were determined with CellTiter-Glo Luminescent Cell Viability Assay. The cell viabilities were represented as relative light units (RLUs) by their ratios (%) to RLU of the sample incubated without MgOC\textsubscript{150}.

**Adsorption Assays for Antimicrobial Agents**

To estimate the capability of MgOC\textsubscript{150} to adsorb antimicrobial agents, 1.25 mg aztreonam, ciprofloxacin, minocycline, trimethoprim, and sulfamethoxazole, 0.68 mg rifampicin, or 5 mg fosfomycin and amikacin in 5 ml aqueous solution were incubated with and without 30 mg MgOC\textsubscript{150} for 2 h. Drug amounts were calculated as described previously (Hirakawa et al., 2020a).

**Statistical Analyses**

We used the Gehan–Breslow–Wilcoxon tests for mouse survival experiments and the unpaired t-tests for mouse body weight measurement, cytotoxicity assays, and adsorption experiments, and then determined p-values using GraphPad Prism version 6.00.

**RESULTS**

**Macroporous Carbon MgOC\textsubscript{150} Adsorbs Both Stx1 and Stx2 Shiga Toxins and Decreases the Extracellular Levels of These Toxins**

We aimed to find materials that adsorb proteins responsible for EHEC pathogenicity and attenuate the virulence of this...
bacterium. For this purpose, we used one MgOC material (MgOC\textsubscript{150}) with an average pore size of 150 nm because this size is predicted to highly adsorb protein molecules that are more than 50,000 Da (Funabashi et al., 2017). First, we tested the capability of MgOC\textsubscript{150} to adsorb the Shiga toxins produced by EHEC. The bacteria-free supernatant from an EHEC culture was incubated with MgOC\textsubscript{150}, and Stx1 and Stx2 titers after MgOC\textsubscript{150} removal were measured in latex agglutination assays. The agglutination titers of Stx1 and Stx2 from the EHEC supernatant were 64 and 128, respectively. However, no agglutination of Stx1 and Stx2 in the supernatant when incubated with 30 mg MgOC\textsubscript{150} was observed (Table 1). To compare the capability of MgOC\textsubscript{150} with ordinal activated charcoal, the bacteria-free supernatant was incubated with 30 mg activated charcoal made from coconut shells. The agglutination titers of Stx1 and Stx2 were then measured. These titers were the same as those in supernatants incubated without the activated charcoal (Table 1). These observations indicated that MgOC\textsubscript{150}, not activated charcoal, highly adsorbs Shiga toxins. To estimate the adsorption affinity of MgOC\textsubscript{150} to Shiga toxins, a purified Shiga toxin standard was incubated with different amounts of MgOC\textsubscript{150}. More than 60% of the toxin (0.4 μg) in a solution was adsorbed when incubated with at least 0.04 mg of MgOC\textsubscript{150}, and more than 95% of the toxin was adsorbed when incubated with 0.2 mg of MgOC\textsubscript{150} (Figure 1A). We also tested the ability of MgOC\textsubscript{150} to adsorb lysozyme, a small-sized protein. Its predicted molecular size is approximately 14,300 Da thus, the ability of MgOC\textsubscript{150} to adsorb this protein molecule may be low. As predicted, more than 90% of the lysozyme protein (50 μg) remained in a solution even after being incubated with 25 mg of MgOC\textsubscript{150} (Figure 1B). We next measured the levels of Shiga toxins in bacterial cultures. EHEC was cultured with MgOC\textsubscript{150}, and agglutination titers were then determined. No agglutinations of Stx1 and Stx2 in the EHEC supernatant cultured with 30 mg MgOC\textsubscript{150} were observed, whereas there was no apparent difference in these titers between the cell lysates from strains cultured with and without MgOC\textsubscript{150} (Table 2). Bacterial colony-forming units (CFUs) were essentially the same when cultured with and without MgOC\textsubscript{150} (data not shown). Therefore, MgOC\textsubscript{150} could reduce extracellular Stx1 and Stx2 levels in EHEC without suppressing bacterial growth. These results indicated that MgOC\textsubscript{150} adsorbs the Stx1 and Stx2 secreted by EHEC and decreases the extracellular Stx1 and Stx2 levels but does not impair Shiga toxin production and EHEC growth.

Shiga toxins cause damage to human renal cells (Kiyokawa et al., 1998). We tested whether the removal of Shiga toxins by MgOC\textsubscript{150} treatment reduces toxicity to human HTB-44 renal cells. The addition of a bacteria-free supernatant from an EHEC culture killed approximately 80% of HTB-44 cells, whereas

**TABLE 1 |** Shiga toxin titers after adsorption by porous carbons.

| Porous carbon materials\* | Stx1 | Stx2 |
|--------------------------|------|------|
| None                     | 64   | 128  |
| MgOC\textsubscript{150}  | <2   | <2   |
| Activated charcoal       | 64   | 128  |

\*30 mg of MgOC\textsubscript{150} or ordinal activated charcoal (from coconut shells) was added into 5 ml of the bacteria-free supernatant and incubated. Shiga toxin titers were determined after removal of MgOC\textsubscript{150} and the activated charcoal material.
70% of the host cells survived when incubated with EHEC supernatants cultured with 30 mg MgOC₁₅₀ (Figure 2). The cytotoxicity of Shiga toxins is also commonly evaluated in Vero cells as alternative cells (Konowalchuk et al., 1977). The toxicity of EHEC supernatants cultured with and without MgOC₁₅₀ to Vero cells was also examined. The addition of the supernatant killed approximately 85% of Vero cells. Similar to HTB-44 cells, the cytotoxicity of the supernatant cultured with 30 mg MgOC₁₅₀ was modest (58% of the cells survived; Figure 2). These results suggested that MgOC₁₅₀ decreases Shiga toxin-associated cytotoxicity.

**MgOC₁₅₀ Also Adsorbs Type III Secretory Proteins, Including EspA and EspB, and Decreases the Levels of These Proteins**

Type III secretory proteins are the other subset of proteins required for EHEC virulence. EspB is a member of these proteins, and it is required to translocate other effector proteins into host epithelial cells. This protein also has an effector activity (Taylor et al., 1999; Kodama et al., 2002). The EspB level in EHEC supernatants and cell extracts cultured with and without MgOC₁₅₀ was measured. The addition of at least 2 mg MgOC₁₅₀ reduced the EspB level in the supernatant (Figure 3A). In contrast, the EspB level in the cell extract was not reduced even when 30 mg MgOC₁₅₀ was present (Figure 3B). The capability of MgOC₁₅₀ to adsorb the EspB protein was also assessed. The EHEC supernatant was incubated with MgOC₁₅₀, and the EspB level in the supernatant was measured after MgOC₁₅₀ removal. No residual EspB protein was observed in the supernatant after incubation with at least 2 mg MgOC₁₅₀ (Figure 3C). In addition to EspB, the adsorption and extracellular accumulation of EspA another protein secreted via the Type III secretion system were examined. MgOC₁₅₀ also adsorbed the EspA protein and reduced its extracellular level (Figures 4A,B).

**MgOC₁₅₀ Attenuates the Virulence of Citrobacter Rodentium in Mice**

To assess the in vivo effectiveness of MgOC₁₅₀ that attenuates bacterial virulence, a murine intestinal infection model with *C. rodentium* was used. *C. rodentium* is a natural pathogen of mice, and it causes typical diarrheal symptoms (Mundy et al., 2005). This pathogen produces one subset of orthologous Type III secretory proteins including EspA and EspB, but does not have genes that encode Shiga toxins. For this reason, *C. rodentium* is used to evaluate Type III secretion system-associated virulence in mice. To treat mice with MgOC₁₅₀, it was administered with their feed. The mice did not adequately consume the MgOC₁₅₀-containing diet on the first few days, but they consumed the diet normally after that. Thus, the mice were pretreated with MgOC₁₅₀ for 7 days before infection and the treatment was maintained until the end of experiments. The *C. rodentium* DBS100 strain that is highly virulent in C3H/HeJ mice was used. A decrease in body weight was observed after 4 days of infection in non-treated mice, and all mice died within 9 days post-infection (Figures 5A,B). When MgOC₁₅₀ was administered with the feed, the mice infected with *C. rodentium* exhibited no body weight decrease until 11 days post-infection, and mice survived significantly longer (Figures 5A,B). The median duration of survival could be prolonged up to 12.5 days post-infection. These results suggested that MgOC₁₅₀ administration reduces the virulence of *C. rodentium* in mice. No significant difference in weight gain between non-infected control mice fed with a regular diet and a MgOC₁₅₀-containing diet was observed (Figure 5C).

**MgOC₁₅₀ Does Not Disturb Host Cells and Some Species of the Normal Intestinal Flora, Such as Enterococcus and Lactobacillus**

We used Caco-2 cells to test the cytotoxicity of MgOC₁₅₀ in human intestinal epithelial cells. The host cells were incubated...
FIGURE 3 | Determination of EspB levels. (A) EspB levels in the EHEC supernatant cultured with and without MgOC150. (B) EspB levels in EHEC whole-cell extracts cultured with and without MgOC150. (C) The EHEC supernatant was incubated with and without MgOC150 for 2h, and EspB levels were estimated after MgOC150 removal. Proteins including EspB were separated by SDS-PAGE. EspB was visualized by Western blotting with EspB antiserum. For loading control (LC), BSA was visualized by CBB stain. Locations of molecular mass standards (in kilodaltons) are shown on the left.

FIGURE 4 | Determination of EspA levels. (A) EspA levels in the EHEC supernatant cultured with and without MgOC150. (B) The EHEC supernatant was incubated with and without MgOC150 for 2h, and EspA levels were estimated after MgOC150 removal. Proteins including EspA were separated by SDS-PAGE. EspA was visualized by Western blotting with EspA antiserum. For loading control (LC), BSA was visualized by CBB stain. Locations of molecular mass standards (in kilodaltons) are shown on the left.
with MgOC₁₅₀ for 24h. MgOC₁₅₀ did not exhibit cytotoxicity because no significant reduction of viable cell numbers was observed after incubation with MgOC₁₅₀ (Figure 6A). When MgOC₁₅₀ is orally administered, it may disturb normal bacterial flora in the intestinal tract by adsorbing some beneficial compounds and/or bacteria. To test this hypothesis, the growth of Enterococcus hirae, Lactobacillus acidophilus, and Lactobacillus casei, which are commonly isolated from the normal intestine, was examined after being cultured with and without MgOC₁₅₀ for 8 and 24 h. When the bacterial strains were cultured without MgOC₁₅₀, the CFUs of E. hirae, L. acidophilus, and L. casei reached 8.2 × 10⁸, 2.4 × 10⁶ and 4.6 × 10⁹ after 8 h, and 1.0 × 10⁸, 1.8 × 10⁶ and 9.6 × 10⁹ after 24 h, respectively (Figures 6B–D). The CFUs of these bacteria reached similar values even when cultured with 30 mg MgOC₁₅₀ (8.0 × 10⁸, 1.5 × 10⁶, and 3.5 × 10⁹ after 8 h, and 1.0 × 10⁸, 1.8 × 10⁶, and 9.0 × 10⁹ after 24 h, respectively). These results indicated that MgOC₁₅₀ does not disturb E. hirae, L. acidophilus, and L. casei.

**MgOC₁₅₀ Does Not Adsorb Fosfomycin and Amikacin, and these Drugs Are Still Active in the Presence of MgOC₁₅₀**

MgOC₁₅₀ may adsorb some antimicrobial agents. This property may impair a combination therapy that uses MgOC₁₅₀ and an antimicrobial agent. We tested whether MgOC₁₅₀ adsorbs antimicrobial agents that are generally active in EHEC. Each antimicrobial agent was incubated with and without 30 mg MgOC₁₅₀ for 2 h and the amount of each agent in the supernatant was measured after MgOC₁₅₀ removal. Undesirably, aztreonam, ciprofloxacin, rifampicin, minocycline, trimethoprim, and sulfamethoxazole were highly adsorbed by MgOC₁₅₀, as these amounts after incubation with MgOC₁₅₀ were lower than 20% compared to the MgOC₁₅₀-free control (Figure 7A). In contrast, fosfomycin and amikacin retained more than 80% even after incubation with MgOC₁₅₀ (Figure 7A). The *in vitro* activities of fosfomycin and amikacin in EHEC were examined when cultured with MgOC₁₅₀. Bacterial cell deaths were observed as a significant reduction in bacterial CFUs was found when cultured with 32 μg/ml fosfomycin and 64 μg/ml amikacin. Similar reductions in CFUs were also observed even when MgOC₁₅₀ was present (Figure 7B). Therefore, MgOC₁₅₀ does not impair the antimicrobial activities of fosfomycin and aminoglycosides, such as amikacin.

**DISCUSSION**

Some molecules that attenuate the virulence of EHEC have been proposed [recently reviewed in Muhlen and Dersch, 2020]. However, no clinically approved method that absolutely prevents severe complications, including HUS, has been established yet. This study showed that a macroporous MgOC particle MgOC₁₅₀ adsorbed both Shiga toxins and Type III secretory proteins EspA/EspB and reduced the extracellular levels of these proteins responsible for EHEC pathogenesis (Tables 1, 2; Figures 1–4), whereas it impaired neither host nor several members of normal intestinal flora (Figures 5, 6).
MgOC_{150} is used for industrial purposes, such as an electrode catalyst, a bioelectrode, and enzyme immobilization (Tsujimura et al., 2014; Funabashi et al., 2017; Mazurenko et al., 2018). Herein, we propose another benefit of MgOC_{150} for the development of anti-EHEC/STEC chemotherapy. Previous in vitro research has shown that an activated charcoal material adsorbed the Shiga toxins produced by EHEC (Naka et al., 2001). However, it also undesirably adsorbed beneficial intestinal bacterial cells. This study used another activated charcoal material that showed no adsorption of Shiga toxins. We suggest that MgOC_{150} is superior to these conventional activated charcoals.

Although MgOC_{150} adsorbs certain molecules in a non-specific manner, it is predicted to highly adsorb protein molecules that are more than 50,000 Da (Funabashi et al., 2017). Shiga toxins are released into the extracellular space as free protein complexes consisting of a monomer A subunit protein and pentamer B subunit proteins (Le Nours et al., 2013). Their whole molecular sizes are estimated at 70,000 Da. Therefore, the effective adsorption of Shiga toxins by MgOC_{150} is reasonable while the ability to adsorb lysozyme, a small-sized protein, is relatively low. In contrast, the monomer sizes of EspA and EspB are approximately 21,000 and 33,000 Da, respectively. These are much smaller than the average pore sizes in MgOC_{150}. EspA and EspB proteins form heteroprotein complexes with other protein members composing the Type III secretion system and plasma membrane proteins of host cells when EHEC attaches to the host cells. EspA forms a polymeric filamentous structure and builds a bridge between bacteria and host cell surfaces when it attaches to the EscF needle protein (Wilson et al., 2001). EspB is delivered via EspA and then forms a pore structure with the EspD protein on the host cell membrane (Ide et al., 2001). This protein also enters the host cells, where it binds to several host proteins, such as α-catenin and myosin (Kodama et al., 2002; Iizumi et al., 2007).

In the in vivo experiment used the alternative C. rodentium and mice, MgOC_{150} attenuated the virulence of this bacterium in mice (Figure 5). C. rodentium is commonly used to evaluate virulence associated with the Type III secretion system in the intestine of mice. Therefore, we believe that EspA and EspB proteins are adsorbed by MgOC_{150} in vivo. This in vitro study showed the adsorption of these proteins in the host cell-free bacterial supernatant (Figures 3, 4). The oligomeric states of EspA and EspB proteins in the bacterial supernatant are unknown when host cells are absent. Some studies showed that the EspB protein could bind...
to EspA and EspD proteins in a solution even when host cells are absent (Hartland et al., 2000; Ide et al., 2001; Yip et al., 2005). If EspA and EspB form a complex with EspD, this protein complex may fit the pores of MgOC_{150}.

EspA and EspB expression is induced by the indole produced by some enteric bacteria (Hirakawa et al., 2009). We previously found that AST-120, an oral adsorbent, eliminated the effect by adsorbing the indole molecule (Hirakawa et al., 2020a). MgOC_{150} may not eliminate the indole effect because addition of MgOC_{150} decreased neither EspA nor EspB expression. In contrast to MgOC_{150}, the average pore size of AST-120 is approximately 2nm, and this pore size is suitable for highly adsorbing the indole molecule, although it may be too small to adsorb EspA and EspB proteins. This may support the difference in adsorption capabilities of MgOC_{150} and AST-120 for the indole molecule.

However, MgOC_{150} adsorbed antimicrobial agents such β-lactams and quinolones, which are commonly used to treat bacterial infections, despite the fact that the sizes of these antimicrobial molecules are much smaller than the pore size of MgOC_{150} (Figure 7A). The pore of MgOC_{150} is produced from an Mg-containing template substrate. If some hydroxyl groups derived from this substrate molecule remain even after the pyrolysis process, a significant amount of a small antimicrobial compound may be bound by this hydroxyl group. Therefore,
improving the method of pore production may be important to minimize the adsorption of small antimicrobial compounds. In contrast, the adsorption capability for fosfomycin and aminoglycosides was relatively low (Figure 7A). For this reason, MgOC$_{150}$ did not impair activities of these drugs (Figure 7B). The use of antibiotics is controversial for the treatment of STEC infections because the release of Shiga toxins is promoted by antibiotics-induced bacterial cell lysis (Wong et al., 2000; Safdar et al., 2002). Antibiotics could be effective for non-STEM, such as enteropathogenic E. coli, which do not produce Shiga toxins, but still produce Type III secretory proteins. MgOC$_{150}$ may offer a benefit by assisting conventional fosfomycin and aminoglycoside therapy to treat non-STEM infections.

We note that our in vivo study has certain limitations. The effectiveness of MgOC$_{150}$ to attenuate the cytotoxicity associated with Shiga toxins was validated ex vivo by using vero and human kidney epithelial cells (Figure 2). However, we used the C. rodentium DBS100 strain, which does not produce Shiga toxins, for the in vivo experiment (Figure 5). Mice were pretreated with MgOC$_{150}$ before infection for the technical reason mentioned above. Therefore, the results of our in vivo experiment show a prophylactic value for this adsorbent, although the exact mechanism of this in vivo prophylaxis has not been discerned. The therapeutic utility of MgOC$_{150}$ and in vivo adsorption of Shiga toxins need to be addressed in the future. In addition, the safety of this adsorbent must be extensively validated for medical applications. We provided evidence that the administration of MgOC$_{150}$ impairs neither mice nor human intestinal epithelial cells (Figures 5, 6). Although the current study showed that MgOC$_{150}$ does not disturb several species of normal intestinal flora, a comprehensive study would be necessary to fully understand the impact on gut microbiome. However, the idea of using microporous adsorbent may open the door to develop an anti-EHEC/STEM therapy.

### DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

### ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Experimental Animal Research of Gunma University (The approval number: 19-094).

### AUTHOR CONTRIBUTIONS

HH, KS, MU, WK, and HT designed the research and wrote the manuscript. HH, KS, WK, and HT analyzed the data. HH, KS, and AT performed the experiments. All authors contributed to the article and approved the submitted version.

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### REFERENCES

Croxon, M. A., and Finlay, B. B. (2010). Molecular mechanisms of Escherichia coli pathogenicity. Nat. Rev. Microbiol. 8, 26–38. doi: 10.1038/nrmicro2265

Funabashi, H., Takeuchi, S., and Tsujimura, S. (2017). Hierarchical meso/macroporous carbon fabricated from dual MgO templates for direct electron transfer enzymatic electrodes. Sci. Rep. 7:45147. doi: 10.1038/srep45147

Galan, J. E., and Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. Nature 444, 567–573. doi: 10.1038/nature05372

Harland, E. L., Daniell, S. I., Delahay, R. M., Neves, B. C., Wallis, T., Shaw, R. K., et al. (2000). The type III protein translocation system of enteropathogenic Escherichia coli involves EspA–EspB protein interactions. Mol. Microbiol. 35, 1483–1492. PMID: 10760148

Hegade, V. S., Kendrick, S. F., and Jones, D. E. (2015). Drug treatment of pruritus in liver diseases. Clin. Med. 15, 351–357. doi: 10.7861/clinmedicine.15-4-351

Hirakawa, H., Kodama, T., Takumi-Kobayashi, A., Honda, T., and Yamaguchi, A. (2009). Secreted indole serves as a signal for type III secretion system translocators in enterohaemorrhagic Escherichia coli O157:H7. Microbiology 155, 541–550. doi: 10.1099/mic.0.02620-0

Hirakawa, H., Suzue, K., Takita, A., Awazu, C., Kurushima, J., and Tomita, H. (2020b). Roles of the Tol-Pal system in the type III secretion system and flagella-mediated virulence in enterohaemorrhagic Escherichia coli. Sci. Rep. 10:15173. doi: 10.1038/s41598-020-72412-w

Hirakawa, H., Uchida, A., Uchida, M., Kaneko, Y., Kakishima, Y., Tanimoto, K., et al. (2021). Adsorption of phenazines produced by pseudomonas aeruginosa using AST-120 decreases pycocyanin-associated cytotoxicity. Antibiotics 10:434. doi: 10.3390/antibiotics10040434

Hirakawa, H., Uchida, M., Kurabayashi, K., Nishijima, F., Takita, A., and Tomita, H. (2020a). In vitro activity of AST-120 that suppresses indole signaling in Escherichia coli, which attenuates drug tolerance and virulence. PLoS One 15:e0232461. doi: 10.1371/journal.pone.0232461

Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner, H., and Schmidt, M. A. (2001). Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic Escherichia coli. Cell. Microbiol. 3, 669–679. doi: 10.1046/j.1462-5822.2001.00146.x

Iizumi, Y., Sagara, H., Kabe, Y., Azuma, M., Kume, K., Ogawa, M., et al. (2007). The enteropathogenic E. coli effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. Cell Host Microbe 2, 383–392. doi: 10.1016/j.chom.2007.09.012

Inagaki, M., Kobayashi, S., Kojin, F., Tanaka, N., Morishita, T., and Tryba, B. (2004). Pore structure of carbons coated on ceramic particles. Carbon 42, 3153–3158. doi: 10.1016/j.carbon.2004.07.029

Kenny, B., DeVimney, R., Stein, M., Reinscheid, D. J., Frey, E. A., and Finlay, B. B. (1997). Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91, 511–520. doi: 10.1016/S0092-8674(00)80437-7

Kiyokawa, N., Taguchi, T., Mori, T., Uchida, H., Sato, N., Takeda, T., et al. (1998). Induction of apoptosis in normal human renal tubular epithelial...
cells by *Escherichia coli* Shiga toxins 1 and 2. *J. Infect. Dis.* 178, 178–184. doi: 10.1086/315592

Kodama, T., Akeda, Y., Kono, G., Takahashi, A., Imura, K., Iida, T., et al. (2002). The EspB protein of enterohemorrhagic *Escherichia coli* interacts directly with alpha-catenin. *Cell. Microbiol.* 4, 213–222. doi: 10.1046/j.1462-5822.2002.00176.x

Konowalchuk, J., Speirs, I. J. L. and Stavric, S. (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* 18, 775–779. doi: 10.1128/IAI.18.11.775-779.1977

Le Nours, J., Paton, A. W., Byres, E., Troy, S., Herdman, B. P., Johnson, M. D., et al. (2013). Structural basis of subtype cytotoxicity SubAβ assembly. *J. Biol. Chem.* 288, 27505–27516. doi: 10.1074/jbc.M113.462622

Lingwood, C. A., Law, H., Richardson, S., Petric, M., Brunton, J. L., De Grandis, S., et al. (1987). Glycolipid binding of purified and recombiant *Escherichia coli* produced verotoxin in vitro. *J. Biol. Chem.* 262, 8834–8839. doi: 10.1016/S0021-9258(17)47986-4

Majowicz, S. E., Scallan, E., Jones-Bitton, A., Sargeant, J. M., Stapleton, J., Angulo, F. J., et al. (2014). Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathog. Dis.* 11, 447–455. doi: 10.1089/fpd.2013.1704

Mazurenko, I., Clement, R., Byrne-Kodjabachian, D., de Poulpiquet, A., Tsujimura, S., and Akatsuka, W. (2014). Exceptionally high glucose current on a hierarchically structured porous carbon electrode with "wired" Flavin adenine dinucleotide-dependent glucose dehydrogenase. *J. Am. Chem. Soc.* 136, 14324–14337. doi: 10.1021/jacs.0405376

Wilson, R. K., Shaw, R. K., Daniell, S., Knutton, S., and Frankel, G. (2001). Role of EcoS, a putative needle complex protein, in the type III protein translocation system of enteropathogenic *Escherichia coli*. *Cell. Microbiol.* 3, 753–762. doi: 10.1111/j.1462-5822.2001.00159.x

Wong, C. S., Jalacic, S., Habeel, R. L., Watkins, S. L., and Tarr, P. I. (2008). The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N. Engl. J. Med.* 342, 1930–1936. doi: 10.1056/NEJM200803062240261

Yamagami, S., Motoki, M., Kimura, T., Izumi, H., Takeda, T., Katsuraya, Y., and et al. (2001). Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. *J. Infect. Dis.* 184, 738–742. doi: 10.1086/323082

Conflict of Interest: MU, belongs to a commercial company, Kureha corp. This author contributed to the study design and data interpretation, but did not directly participate in data collection.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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