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Original Article

The effect of broad-spectrum antibiotic ceftriaxone on net colonic water and ion transport *in vivo*

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ABSTRACT

Introduction: The molecular mechanism of idiopathic antibiotic-associated diarrhea is not clear. Ceftriaxone, a third-generation cephalosporin, is a broad-spectrum antibiotic and diarrhea is the main side-effect of ceftriaxone treatment. The present study tested the hypothesis that ceftriaxone-induced diarrhea is associated with a shift in microbiota composition followed by the alteration in colonic water/ion transport, the expression pattern of transporters and epithelial barrier function.

Methods: Male Wistar rats were treated daily with ceftriaxone (50 mg/kg, i.m.) for 5 or 14 days. Epithelial net water and ion transport (Na^+, K^+, Cl^-) were evaluated on the 6th or 15th day respectively by isolated colonic loop perfusion technique *in vivo*. Gene expression by RT-PCR, glycoproteins levels by PAS-staining, and microbiota by culture method on the elective medium were evaluated.

Results: Decreases in Na^+ and water absorption, surface mucus layer, and *Scnn1b* and *Aqp8* gene expression were associated with more severe diarrhea after 5 days-antibiotic treatment. After 14-days of antibiotic treatment, fewer animals with diarrhea were observed. At the same time, there was a decrease of Cl^- and an increase in Na^+ absorption, along with increased mucus secretion and upregulation of *Cftr*, *Scnn1b*, *Slc9a3*, *Muc2*, *Ocln*, and *Tjp1* gene expression. These changes were accompanied by an increase in the number of culturable conditionally pathogenic microbiota after 5 days of treatment which almost returned to the control value after 14 days of treatment.

Conclusion: We concluded that the observed transitory antibiotic-associated diarrhea was a well-orchestrated physiological defense response at the molecular level driven by the shift in normal microbiota composition.

Keywords:
Antibiotic-associated diarrhea
Ceftriaxone
Colon
Ion transport
Epithelial barrier

Introduction

Diarrhea is the most common side effect of broad-spectrum antibiotic treatment, which occurs in 5 to 62% of cases and is called antibiotic-associated diarrhea (AAD). AAD may develop at any time point from the initiation of therapy up to two months after the end of the treatment. Most cases of AAD can be classified into two categories: 1) *Clostridium difficile* (*C. difficile)* - in-

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duced diarrhea; 2) idiopathic diarrhea whereby no infection agent is recognized. *C. difficile* colonic overgrowth and its subsequent toxin production are responsible for only 2-25% of AAD cases, the rest of the cases have idiopathic etiology (Benjamin et al, 2018, Nelson et al, 2011). Hove et al. (1996) found that only 2 out of 13 patients with AAD were culture-positive for *C. difficile* during antibiotic therapy. After cessation of antibiotic therapy, none of the patients had diarrhea even though 6 patients were *C. difficile* positive.

The mammalian colonic epithelium is a typical electrolyte-transporting system, that can efficiently transport significant amounts of salt and water from a mucosal toward the serosal side or *vice versa*. Colonic epithelium transport is characterized by a net absorption of NaCl, short-chain fatty acids (SCFA), and water, allowing the extrusion of feces with minimal water and salt content (K^+, Cl^-, HCO_3^-) (Kunzelmann, 2002, Kiela et al, 2009). The absorptive capacity of the colonic mucosa is dependent on the activity and several transporters, such as water channel (mostly *Aqp*8), electroneutral Na^+/H^+-exchanger (*Slc9a3*), electrogenic Na^+ channel (*Scnn1b*), electrogenic Cl^- channel cystic fibrosis transmembrane conductance regulator (*Cftr*), Cl^- channel down-regulated in adenoma (DRA), Na^+/HCO_3^-, HCO_3^-/SCFA exchanger and others (Kunzelmann,2002).

Scanty data on the effects of antibiotics on intestinal epithelium water and ion transport (Giannella et al, 1981, Roberts et al, 1991, Goldhill et al, 1996) support the idea of their involvement in AAD pathogenesis. All previous studies have examined changes in water and ion transport following direct exposure to antibiotics such as intraluminal perfusion (Giannella et al, 1981), cell culture incubation (Goldhill, 1996) or bath perfusion (Roberts et al, 1991)). They did not take into account the possible effect of altered microbiota composition and its metabolic activity on colonic net absorption as a mechanism of AAD, despite the fact that non-pathogenic bacteria can directly affect the secretory and absorptive function of the gut epithelium (Lomasney et al, 2013). The molecular mechanism underlying the disturbed function of the colonic epithelium during AAD is also unclear.

The third-generation cephalosporin ceftriaxone is a broad-spectrum antibiotic used as the first-line medication for the treatment of lower airway infections, intra-hospital and post-surgical perturbations (Durham et al, 2017). Hypersensitivity reactions and gastrointestinal disorders such as diarrhea are the main side effects associated with ceftriaxone treatment (Holota et al, 2019, Neuman et al, 2014, Thomson et al, 2017). The present study was designed to test the hypothesis that ceftriaxone-induced diarrhea is associated with a shift in the microbiota composition which subsequently affects colonic water/ion transport, the expression pattern of transporters (*Cftr*, *Aqp*8, *Scnn1b,* and *Slc9a3*), and epithelial barrier function. The study was conducted *in vivo* on Wistar rats, treated with ceftriaxone for either 5 or 14 days to reproduce the clinical treatment protocol.

Material and Methods

Experimental animals

Male Wistar rats (180-230 g) were bred and housed in the conventional animal facility of the ESC "Institute of Biology and Medicine" at Taras Shevchenko National University of Kyiv (Kyiv, Ukraine) under standard environmental conditions (12-h light/dark cycle at a constant temperature of 22°C). Throughout the study, all animals had unlimited access to animal chow and tap water. To ensure standardized gut microbiota, rats from all groups were housed in the same room and cared for by the same personnel.

The experiments were conducted under the ethical principles described in "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011). The study underwent review and received approval from the animal committee of the ESC "Institute of Biology and Medicine" at Taras Shevchenko National University of Kyiv (Protocol # 8, on November 2, 2015).

Antibiotic treatment protocol and experiment design

Rats were randomly divided into control and ceftriaxone-treated groups. Ceftriaxone 1g powder for solution for injection (Arterium, Ukraine) was dissolved in sterile water immediately before injection and administered intramuscularly once a day at a dose of 50 mg/kg for 5 (n=14) or 14 (n=12) consecutive days. Control rats recieved daily treatment with 100 µl of sterile water for either 5 days (n=19) or 14 days (n=12).

Animals were weighed immediately before the water or ceftriaxone treatment and the following day after treatment cessation. Diarrhea was assessed daily based on the consistency of feces, and Animals were classified as either positive or negative for diarrhea signs.

Effects of ceftriaxone treatment were evaluated on the day after treatment cessation. a subset of treated rats (7 rats per group) was assigned for measurements of colonic net water and electrolyte transport using the isolated colonic loop perfusion technique. All other animals were euthanized by CO_2 inhalation followed by cervical dislocation according to the guidelines of the Institutional Animal Care and Use Committee. During autopsy, stool samples were collected in sterile microtubes for detection of *C. difficile* toxins A and B as well as in sterile saline for bacteriological examination. The entire colon was removed, cut along the anti-mesenteric side, rinsed thoroughly in cold PBS, and gently wiped with a paper towel. A 1 cm section of the distal colon was cut and used for the mucus secretion assay. The remaining colon was flattened with the mucosa facing upward on ice. The mucosa was gently scraped from the muscular layer using a metal spatula and immediately frozen in liquid nitrogen for gene expression.

Processing of fecal specimens for microbiological analysis

During the autopsy fecal materials (1 g) were sampled from at least 5 rats per group and immediately dispersed in 9 ml of sterile saline solution (0.5% NaCl). Serial dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-8}) were prepared for each sample. to detect aerobic microbiota (*Escherichia coli, Staphylococcus spp,* conditionally pathogenic *Enterobacteriaceae: Citrobacter spp, Klebsiella spp, Proteus spp*), 100 µl aliquots from each dilution were plated onto selective solid media (HiMedia Laboratories Pvt. Ltd) and incubated for 12-24 hours at 37°C. For anaerobic bacteria for the detection of *Bifidobacteria spp., Lactobacillus spp., Propionibacteria spp., Clostridium spp*, 1 ml aliquots from each dilution were inoculated in tubes containing 9 ml of selective anaerobically sterilized media (HiMedia Laboratories Pvt. Ltd) and incubated for 24-72 h at 37°C under anaerobic conditions in a 5% CO_2 incubator.

Bifidobacterium was determined on *Bifidobacterium* Agar (HiMedia, India); creamy white colonies obtained after 48 h of anaerobic incubation at 37°C were considered. Randomly picked-off colonies were Gram-stained. All Gram-positive pleomorphic non-performing rods were tentatively identified as *Bifidobacterium*. To isolate representatives of the genus *Lactobacillus*, 1 ml of 10-fold dilutions of feces was inoculated into MRS Agar (HiMedia, India) with the addition of chalk, and cultivated under anaerobic conditions for 48 hours. This was followed by microscopy of lenticular, triangular, cotton-like colonies that formed a chalk-dissolving zone around the colony. *Lactobacillus* included Gram-positive, non-spore-forming, catalase-negative bacilli. Iron Sulphite Agar (HiMedia, India) was applied for *Clostridia*. Black colonies obtained after 48 h of anaerobic incubation at 55°C were considered *Clostridium.*

Staphylococcus was cultured on Mannitol Salt agar (HiMedia, India). Both mannitol-positive (subsequently called *Staphylococcus aureus)* and negative (subsequently called *Staphylococcus spp.)* colonies observed after 2 days of incubation at 37°C in ambient air were counted. Simmons Citrate Agar (HiMedia, India) was used to differentiate members of the *Enterobacteriaceae* based on their utilization of citrate as the sole carbon source. Colonies that changed the color of the pH indicator in the media from green to blue were considered and subsequently called conditionally pathogenic enterobacteria. A presumptive test for members of the coliform group was performed on Endo Agar (HiMedia, India). After incubation at 37C for 24 hours of cultivation, dark crimson colonies (lactose-fermenting E. coli strains) and light pink (lactose-non-fermenting E. coli strains) were considered. The second test medium was Triple Sugar Iron Agar (TSI, HiMedia, India). Both lactose-positive and lactose-negative colonies from the Endo Agar were transferred on the TSI. Lactose-positive, gas-forming, H_2S-negative colonies were considered *Escherichia coli* with normal enzymatic properties (subsequently called *E. coli* lactose "+"). Lactose-negative, gas-forming, H_2S-negative colonies were considered *Escherichia coli* with altered enzymatic properties (subsequently called *E. coli* lactose "-"). Simultaneously, the pink colonies without metallic sheen were subcultured onto Simons medium. Colonies that grew on this medium and changed the color of the medium from green to blue were attributed to opportunistic *Enterobacteria*. If no growth was observed during reseeding, it was classified as lactose-negative *E. coli*. Hemolytic bacteria counts were obtained after incubation at 37°C on Blood Agar Base (HiMedia, India) supplemented with 5% w/v sterile defibrinated sheep blood (Hemostat Laboratories, USA).

Following incubation, different colony types were counted, isolated in pure culture, and identified at the

genus level. All isolates were analyzed based on Gram-stain appearance and colony morphology followed by routine biochemical tests. Colony forming units (CFU) were counted and log CFU/g of feces was calculated.

Immunochromatographic assay of Clostridium difficile toxins A and B in feces

C. difficile toxins A and B were detected in rats' feces by Duo toxin A+B check-1 (VEDALAB, France) according to the manufacturer's protocol. The Duo Toxin A+B-Check-1 is a lateral flow immunochromatographic rapid test designed for the qualitative detection of *C. difficile* Toxin A and Toxin B.

Perfusion techniques

The rates of the net fluid and electrolyte movement were measured by perfusion an isolated colonic loop *in vivo* (Sladen, 1972, Schedl, 1966). After an overnight fasting, the animals were anesthetized by urethane (110 mg/100 g, i.p.; Sigma, Aldrich). A tracheotomy was performed immediately after anesthetization to prevent respiratory insufficiency caused by glottic edema. The colonic loop was prepared and perfused using a modified version of previously described methods (Sladen, 1972, Schedl, 1966).

The abdomen was opened along the midline. A 15-cm segment of the colon was gently pulled out from the abdominal cavity and cannulated with a polyethylene tube about 0.5 cm below the cecum where it was secured with a nylon ligature. The distal colon (2 cm from the anus) was ligated before the colonic loop was filled with prewarmed saline at 37°C. The distal colon was then incised, and another polyethylene tube was inserted and sutured in place to collect the aspirated perfuse solution. The resulting closed colonic loop (perfused length 8-7 cm) was perfused with prewarmed modified Krebs-Henseleit solution (mM per L: NaCl – 117; KCl – 5.9; $NaHCO_3$ – 24.8; NaH_2PO_4 – 1.2; $MgCl_2$ – 1.2; $CaCl_2$ – 2.5; glucose – 5.5; T 37°C; pH 7.3-7.4) containing unabsorbed marker phenol red (20 mg/l). The perfusion was carried out using a perfusion pump (Gilson ® Minipuls 3, France) at a rate of 0.2 ml/min.

After a 60 min stabilization period, the perfusate was collected for each subsequent 20 min period of perfusion, with a total perfusion time of 180 min. The data obtained for each 20-min period were then averaged and expressed as the Mean/min. The rectal temperature was kept at 37˚C by appropriately positioned table lamps. At the end of the experiment, the animal was euthanaized by an overdose of anesthetic and the colon segment was immediately excised, opened longitudinally, and gently wiped with filter paper. After that, it was dried out for 20 h at a tempreture of 60°C to obtain its dry weight.

Measurement of water and electrolyte net transport rates

The rate of the net water transport was estimated by measuring the level of the unabsorbed marker phenol red in the fluid effluent by spectrophotometry (Schedl, 1966). CPRF/CPRE (CPRF represents the phenol red concentration in the perfusion solution; while CPRE represents the phenol red concentration in the effluent solution) was calculated based on the phenol red absorbencies measured at 520, 560, and 600 nm using the following formula: corrected absorbency.

$$= 560 - \frac{1}{2}(520 + 600)$$

This corrects the linear background absorbencies in this region of the spectrum. Concentrations of Na^+ and K^+ were determined by flaming spectrophotometry and the concentration of Cl^- was measured using a chloridometer. These indices were then calculated by formulas (Sladen, 1972, Schedl, 1966).

Water net transport (Jnet water) was calculated as µL/g×min:

Jnet water = [F×(1 – CPRF/CPRE)]/W,

where F – flow velocity in ml/min; CPRF – phenol red concentration in the perfusion solution; CPRE – phenol red concentration in the effluent solution, and W – the dry weight of the intestinal segment in grams. Positive results indicate net absorption, and negative results indicate net secretion into the lumen.

Ions net transport (Jnet ion) was calculated as µM/g×min:

Jnet ion=F×(Cion F – CionE)×(CPRF/CPRE) / W ,

where F – flow velocity in ml/min; CionF – ion concentration in perfusion solution; CionA – the ion concentration in the effluent solution; CPRF – the phenol red concentration in the perfusion solution; CPRE – the phenol red concentration in the effluent solution, and W – the dry weight of the intestinal segment under consideration. Positive results indicate net absorption and negative results indicate net secretion into the lumen.

TABLE 1: The primers sequences and protocol of PCR amplification for detected genes

Gene	Primers sequence (5′–3′)	Denaturation (Initial denaturation - 95 °C, 3 min)	Primers annealing	Extension (Final extension step was performed upon 72° C for 5 min)	Cycles	Product length, b.p.
Cftr	(f) ACTCTTCTTCAGCTGGACCACACCA (r) AAAGCATCGCCGGAGGGCGT	95 °C, 1 min	53ºC, 45 s	72 °C, 1 min 15 s	35	190
Aqp8	(f) CAGATATGTCTGGGGAGCAGACGC (r) CTGCCAGCAGTTCTTCACCTCGAC	95 °C, 1 min	54ºC, 1 min	72 °C, 1 min 30 s	35	110
Scnn1b	(f) ATTCCAGCCCCTTCCAGTAC (r) GGGTACTGGTGGTGTTGGT	95 °C, 1 min	54ºC, 40 s	72 °C, 1 min	30	123
Slc9a3	(f) CCTGATGGGCGAACTGAAGA (r) GCAGTGACTCCCCAAAAACA	95 °C, 1 min	52ºC, 45 s	72 °C, 1 min 15 s	35	149
Muc2	(f) CAGAGTGCATCAGTGGCT (r) CCCGTCGAAGGTGATGTA	95 °C, 1 min	50ºC, 45 s	72 °C, 1 min 15 s	35	265
Ocln	(f)TCTTTGTATAAGTCACCGCCT (r)GTTTCATAGTGT-GTCTGGGTCTG	95 °C, 1 min	50ºC, 45 s	72 °C, 1 min 15 s	35	185
Tjp1	(f)CCATCTTTGGACCGATTGCTG (r)TAATGCT-CCGAGCTCCGATG	95 °C, 1 min	49ºC, 45 s	72 °C, 1 min 15 s	35	123
Actb	(f) TGGGACGATATGGAGAAGAT (r) ATTGCCGATAGTGATGACCT	95 °C, 1 min	49º C, 40 s	72 °C, 1 min	28	521

Semi-quantitative RT-PCR analysis

The total RNA was isolated from frozen colonic tissue by the phenolic method described by Chomczynski and Sacchi (Chomczynski et al, 2006). For cDNA synthesis, a 20 µl reaction mix was prepared containing 2 µg of RNA, 1 mM dNTP, 200 U of reverse transcriptase "Thermo Scientific RevertAid Reverse Transcriptase", the corresponding buffer, 20 U of ribonuclease inhibitor "Thermo Scientific RiboLock RNase Inhibitor" ("Thermo Scientific", Lithuania), and 20 pmol (1,0 µM) of the reverse primer. The synthesis was carried out under the following conditions: 65° C – 5 min, 45° C – 1 hour. Polymerase chain reaction was performed in a 30 µl of reaction mix consisting of 20 ng of cDNA, PCR buffer, 200 µM of each dNTP, 30 pmol (1,0 µM) of each primer, 2,5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase ("Taq DNA Polymerase (recombinant)", "Thermo Scientific", Lithuania). The primer sequences for the detected genes and the specific PCR amplification protocol are summarized in Table 1.

The reproducibility of the amplification results was evaluated in parallel experiments by repeating the PCR reactions with all animals and each primer at least three times. Separation of PCR products was performed electrophoretically on a 1.6% agarose gel with 0.5x TBE buffer, following the protocol by Sambrook et al. (Sambrook et al, 2006). For semi-quantitative analysis of amplicon expression based on densitometry, ImageJ 1.45s program was used. Indices of gene expression were calculated for each sample following the method by Konturek et al. (Konturek et al, 1998).

Measurement of colonic mucus secretion by detection of mucus glycoprotein

Since adherent mucus gel represents a substantial part of secreted mucin, to separate adherent mucus gel, 1 cm of the distal colon (3 cm from the anus edge) was incubated with 2 ml 6N N-acetyl-l-cysteine with constant shaking for 3 min. After that, the contents were centrifuged for 5 min at 3000 rpm (T=4°C) and 1 ml of the supernatant was discarded. The remaining contents were re-suspended, and 500 µl of the solution was transferred

into new tubes and frozen at -20°C. Mucus glycoprotein levels were measured using periodic acid solution (PAS) staining of polyvinylidene difluoride (PVDF) membranes, as described by Akiba et al. (Akiba et al, 2000). Ten microliters of the resuspended sample solution were blotted on PVDF membrane (Hybond-P, Amersham International, Little Chalfont, UK) and placed on wet filter paper for 10-15 min until the sample was fully absorbed. The membrane was briefly rinsed in distilled water for 2 min following incubation in 0.5% KOH in 70% ethanol for 10 min and rinsed again in dH_2O. Subsequently, the membrane was exposed to 0.5% PAS for 10 min, rinsed with distilled water, and exposed to Schiff's reagent (basic Fuchsin 1 g, 20 ml 1N HCl, 1 g NaS_2O_5, dH_2O 200 ml) for 15-30 min with constant shaking. This was followed by two 3-min exposures to 0.6% sodium metabisulfite and rinsing in distilled water for 3 min. After the final distilled water rinse, the membrane was dried in open air (for about 10 min) and its density was digitized using a scanner (Samsung SCX-4x21). Image analysis was performed by measuring of dot-blot density with the image-processing software Sigma Plot.

Data analysis and statistics

Data are presented as mean±standard deviation (SD). The homogeneity of variance was assessed using the Levene test. Statistical analysis of the data was performed using Kruskal-Wallis test or one-way analysis of variance (ANOVA) with the Tukey post hoc test. The difference was considered statistically significant at $P<0.05$. GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, United States) was used for creating graphs.

The Student's t-test has been used to detect reliable differences between two samples and assess their normal distribution. In the case of non-normal distribution, we used the Mann-Whitney Test.

Results

Signs of diarrhea in ceftriaxone-treated rats

Ceftriaxone treatment increased the number of animals with diarrhea from 6% on the 2nd day to 12% on the 6th day of ceftriaxone administration. Over the course of 10 days, we observed a decreased number of animals with diarrhea, ranging from 13% to 6-7% of cases (Fig.

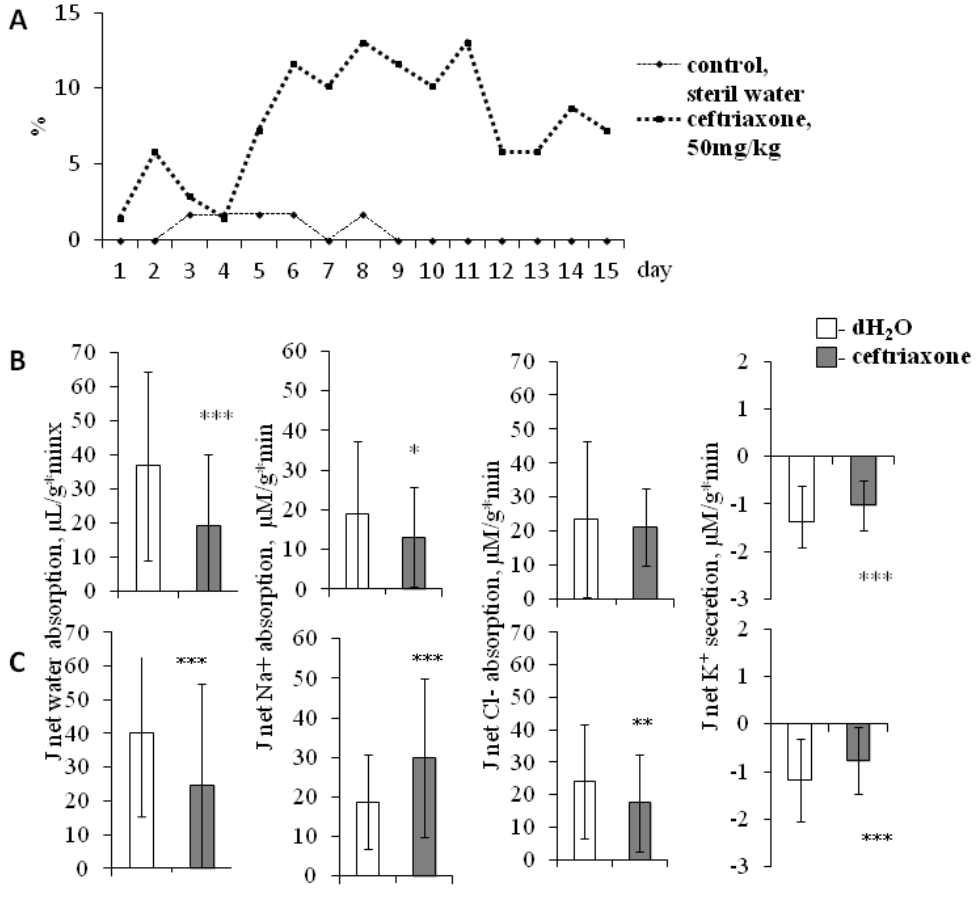

FIGURE 1. Ceftriaxone treatment (50 mg/kg, i.m.) for 5 or 14 days induced changes in water and ion transport in rat's colon. The percentage of rats with diarrhea during the ceftriaxone treatment (n=26) and in the control group (n=31) (A). The levels of colonic net water, Na^+, K^+, and Cl^- transport after 5 days (B) and 14 days (C) of ceftriaxone treatment in rats (n=7 per group). Rates of net fluid and electrolytes' movement were measured by perfusion of an isolated colonic loop in vivo. M±SD; * - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$ vs control values.

TABLE 2: The counts (lg CFU/ g feces) of fecal microorganisms after 5 or 14 days of daily ceftriaxone treatment (50 mg/kg, *i.m.*) in rats

Group of microorganisms	lg CFU/g feces			
	5 days		14 days	
	Control	Ceftriaxone	Control	Ceftriaxone
Bifidobacterium	8,7 ± 0,3	7,7 ± 0,8	9,1+0,3	8,1+0,1
Lactobacillus	6,3 ± 0,4	5,0 ± 1,5	7,2+0,4	6,0+0,9
Propionibacterium spp	8,2 ± 0,9	7,1 ± 0,3	9,2+0,1	7,3+1
Escherichia coli lactose-fermenting	4,5 ± 0,5	**2,1 ± 1,6***	4,5 ± 0,5	**2,1 ± 1,6***
Escherichia coli lactose non-fermenting	4,7 ± 0,3	5,5 ± 0,4	3,4_0,3	2,1+1,5
Citrate-fermenting conditionally pathogenic *Enterobacteria*	2,0 ± 0,2	**4,0 ± 1,0***	3,2+0,3	**0,2+0,1***
Staphylococcus aureus	3,5 ± 1,2	**5,0 ± 0,8***	3,5+0,8	**1,8+0,7***
Staphylococcus spp.	4,5 ± 0,8	3,1 ± 1,2	2,5+0,8	**0***
Clostridium spp.	1,8 ± 0,6	**4,1 ± 1,0***	3,1+0,5	4,1+0,5

* - $P<0.05$ *vs* control.

1A).

Quantitative changes in fecal culturable aerobic and anaerobic microbiota following 5 and 14 days of ceftriaxone treatment

As shown in Table 2, ceftriaxone treatment for 5 days was associated with a decreased number of anaerobic *Lactobacillus, Bifidobacterium, and Propionibacterium* by ca. 1 log CFU/g of feces. The same level of changes was observed after 14 days of ceftriaxone treatment. These alterations were within the range of normal variations and not statistically significant. Counts of *Clostridium spp.* increased by *ca.* 2 log CFU/g of feces ($P<0.05$). *C. difficile* toxins A and B were not detected in rats' feces after 5 days of treatment. After 14 days, the number of *Clostridium spp*. was still increased and fecal samples from 25% of rats (1 out of 4) were *C. difficile* toxins A and B positive.

Counts of aerobic bacteria were different after 5 and 14 days of ceftriaxone treatment. The mean numbers of conditionally pathogenic *Enterobacteriaceae* (*Citrobacter spp, Klebsiella spp., Proteus spp*.) increased by ca. 2 log CFU/g of feces ($P<0.05$) after 5 days of ceftriaxone administration and decreased by *ca.* 2 log CFU/g of faeces ($P<0.05$) after 14 days of ceftriaxone treatment. The same pattern was observed for *Staphylococcus aureus*. Mean counts of *Staphylococcus aureus* decreased after both 5 and 14 days of ceftriaxone treatment, but these alterations were within the range of normal variations and not statistically significant. After both 5 and 14 days of ceftriaxone treatment, the total number of *E.coli* was decreased by ca. 1.5 and 3.5 log CFU/g of feces, respectively ($P<0.05$). This reduction was associated with reduced mean counts of lactose (+) *E.coli* by *ca.* 2.5 log CFU/g of feces ($P<0.05$).

Colonic net water and electrolytes (Na+, K+, Cl-) transport after 5 and 14 days of ceftriaxone treatment

Ceftriaxone treatment for 5 days decreased net water absorption by 47.5 % ($P<0.001$), Na^+ absorption by 28.8% ($P<0.01$), and net K^+ secretion by 25.4% ($P<0.001$). Net Cl^- absorption did not change (Fig. 1B).

Increasing the duration of ceftriaxone treatment for up to 14 days has led to a decrease in net water absorption by 38.8% ($P<0.001$) and K+ secretion by 34.7% ($P<0.001$) (Fig. 1C). In contrast to the 5-day treatment, ceftriaxone treatment for 14 days induced up-regulation of net Na^+ absorption by 27.5% ($P<0.01$) but down-regulation of net Cl^- absorption (27.5%; $P<0.01$) (Fig. 1C).

Levels of Aqp8, Slc9a3, Scnn1b, and Cftr genes expression in rat colon after 5 and 14 days of ceftriaxone treatment

Expression of water channel *Aqp*8 gene was decreased by 1.3-fold ($P<0.05$) after 5 days of ceftriaxone treatment and returned to the baseline level after 14-days of treatment (Fig. 2A). Expression of *Cftr* chloride channel gene was increased by 1.4-fold ($P<0.001$) and 1.9-fold

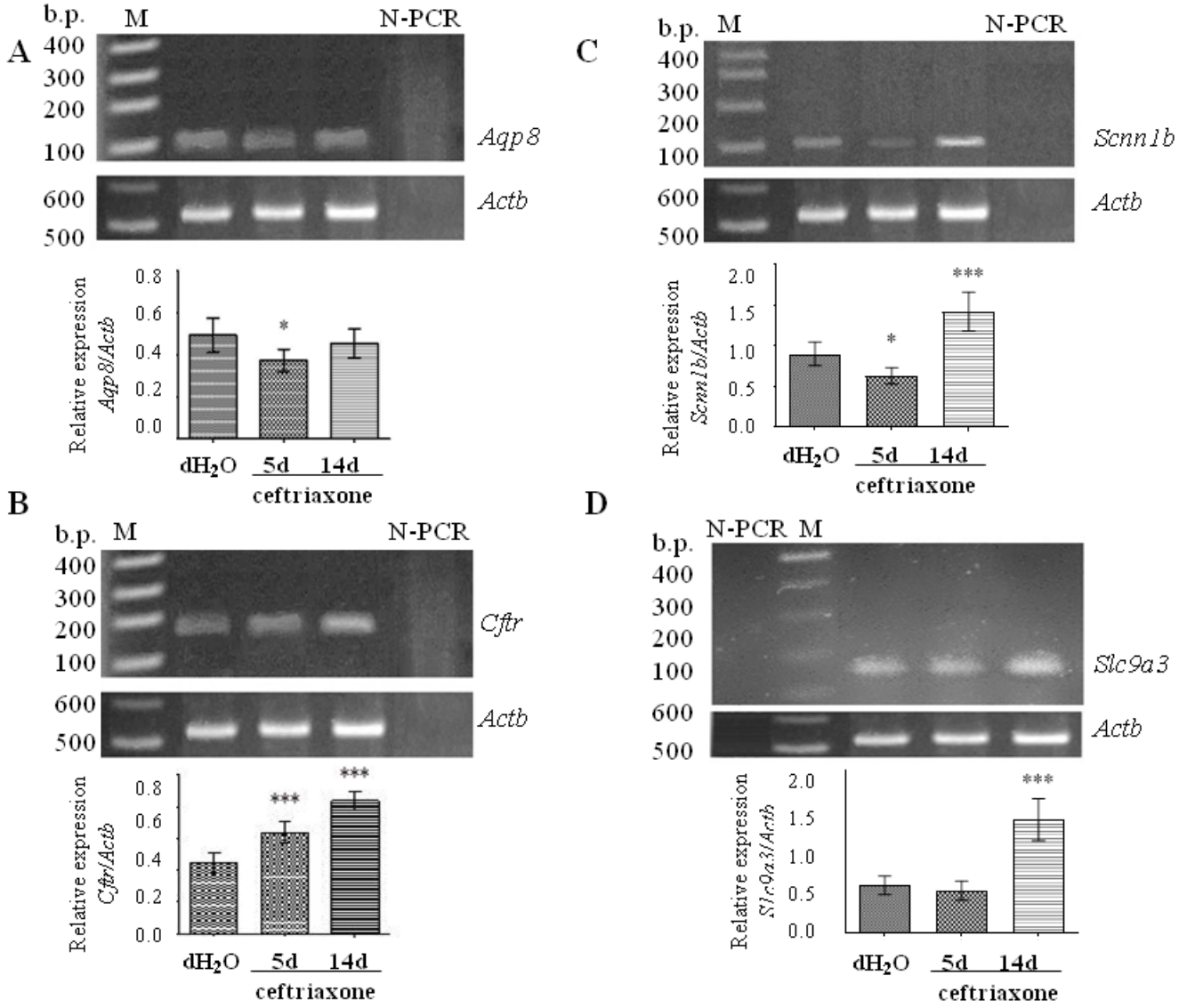

FIGURE 2. Ceftriaxone administration (50 mg/kg, *i.m.*) for 5 or 14 days altered the gene expression of water and electrolyte transporters in the rat colon. Representative electropherogram and relative gene expression histogram (M±SD) of water channel *Aqp8* gene (A), the electrogenic Cl^- channel cystic fibrosis transmembrane conductance regulator (*Cftr*) gene (B), the voltage-dependent Na^+ channels (*Scnn1b*) gene (C) and the electroneutral Na^+/H^+-exchanger 3 (*Slc9a3*) gene (D) in rat colonic epithelium. The gene expression was examined by semi-quantitative RT-PCR analysis (n=5 per group). M – molecular mass marker – "GeneRuler™ 100bp DNA Ladder" ("Fermentas", Lithuania); N-PCR – negative PCR control; * - $P<0.05$; *** - $P<0.001$ vs control values.

($P<0.001$) after 5 and 14 days of ceftriaxone treatment, respectively (Fig. 2B). Expression of the voltage-dependent sodium channel gene (*Scnn1b*) was decreased by 1.4-fold ($P<0.05$) after 5 days of ceftriaxone treatment, but increased 1.6-fold ($P<0.001$) after 14 days of ceftriaxone treatment, respectively (Fig. 2C). Expression of sodium–hydrogen exchanger 3 gene (*Slc9a3*) was not changed after 5 days of ceftriaxone treatment and increased by 2.4-fold ($P<0.001$) after 14 days of treatment compared to water treated rats (Fig. 2D).

Epithelial barrier function after 5 and 14 days of ceftriaxone treatment

Ceftriaxone treatment for 5 days was associated with a 3.1-fold ($P<0.01$) decrease in surface colonic mucus level. In contrast, after 14 days of ceftriaxone treatment, we observed an opposite 3-fold increase in surface colonic mucus level ($P<0.05$ *vs.* water treated-rats) (Fig. 3A). The gene level of *Muc2* mucin was slightly increased by 1.3-fold ($P<0.05$) after 14 days of ceftriaxone treatment compared to water treated-rats (Fig. 3B). The expression levels of tight junction genes *Tjp1* and *Ocln* were increased by 3.6- and 2.5-fold ($P<0.05$ *vs.* water treated-rats), respectively, after 14 days of ceftri-

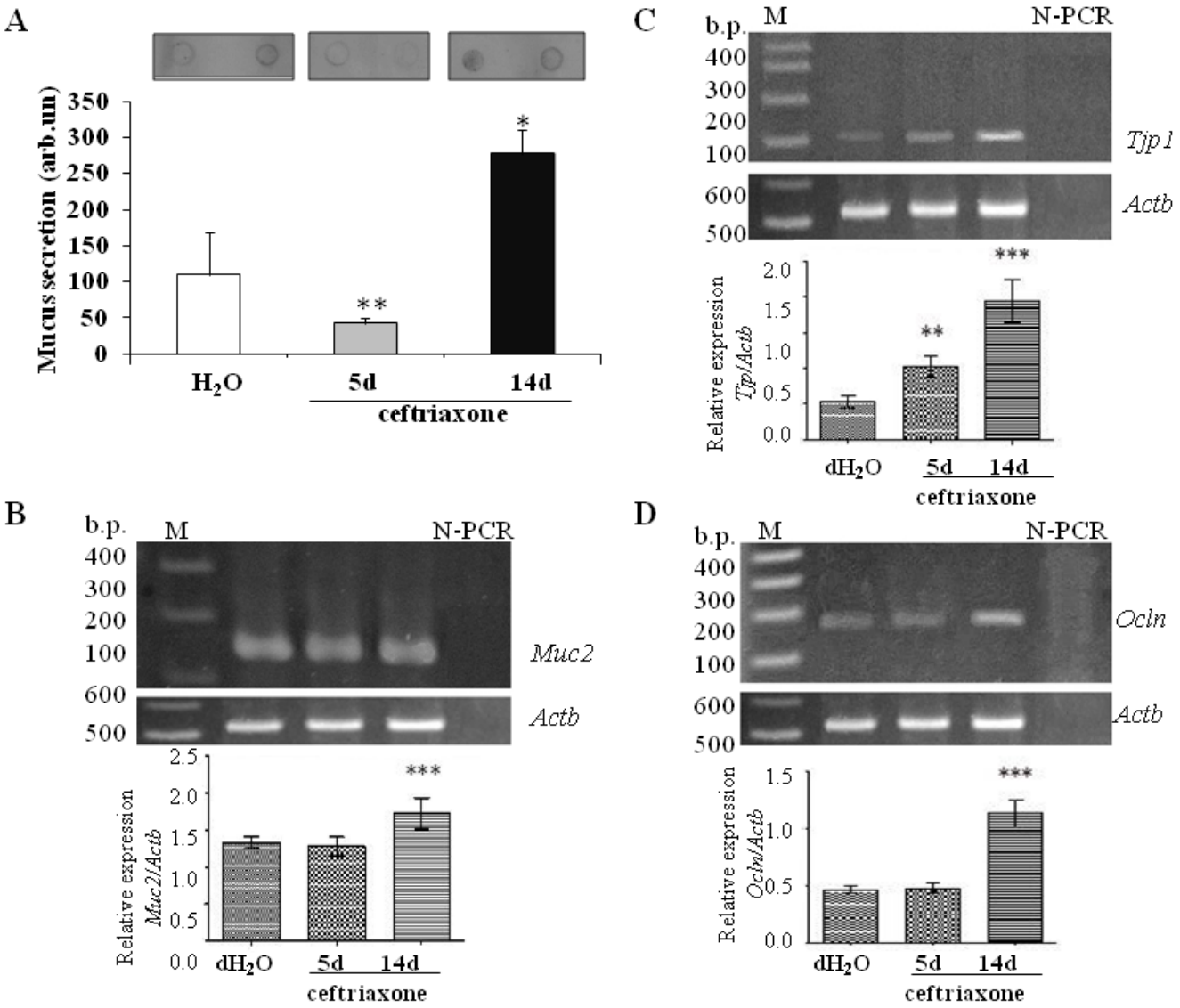

FIGURE 3. Ceftriaxone treatment (50 mg/kg, *i.m.*) for 5 or 14 days compromised epithelial barrier function in rat's colon. The glycoprotein content in the rat colon surface mucus after 5 (n=5) and 14 (n=5) days of ceftriaxone treatment vs the control group (n=5). Schiff reaction method on PVDF-membrane (A). Electropherogram and relative gene expression histogram (M±SD) of *Muc2* mucin gene (B), tight junction protein 1 (*Tjp1*) gene (C), and *Ocln* occludin gene (D) expression in rat colonic epithelium. The gene expression was examined by semi-quantitative RT-PCR analysis. M – molecular mass marker – "GeneRuler™ 100bp DNA Ladder" ("Fermentas", Lithuania); N-PCR – negative PCR control; ** - $P<0.01$; *** - $P<0.001$ vs control values.

axone treatment (Fig. 3C, D).

Discussion

AAD is a common side effect of cephalosporin antibiotics therapy. The mechanism of cephalosporin-induced diarrhea may have idiopathic as well as *C. difficile* etiology (Durham e al, 2017). We showed that after 5 or 14 days of ceftriaxone administration diarrhea developed in 13% or 6% of animals respectively. While there were no *C. difficile* toxins A and B positive rats after 5 days of ceftriaxone administration, 25% of animals were positive after 14 days. In a clinical study on patients who received oral bowel biota suppression therapy for 12 days (cefuroxime, bramycin, nystatin three times daily) diarrhea developed in 87% (13 of 15) of the patients. Only 2 patients were culture-positive for *C. difficile* during therapy, whereas 6 out of 15 patients (45%) became *C. difficile* cytotoxin-positive after cessation of suppression therapy at a time when none had diarrhea. Moreover, diarrhea was self-limiting despite continued antibiotic treatment (Hove et al, 1996). These findings, along with our experimental data, support the idea that cytotoxic effects of *C. difficile* overgrowth are a less common etiologic factor of AAD development.

Despite the fact that increased intraluminal osmolality, active secretion, exudation, and altered motility can all drive diarrhea, disturbance in colonic ion transport is a more common factor.–(Hoque et al 2012). Water absorption occurs in the colon passively through the intercellular pathways driven by ion concentration gradients and facilitated diffusion through specialized cellular channels called aquaporins (e.g. *Aqp*8). There are two pathways for electrolyte absorption through the colonic epithelium: electroneutral (mainly through Na^+/H^+ exchangers for Na^+ and Cl^-/HCO^-_3 for Cl^-) and electrogenic (through the *Scnn1b*). Electrogenic Cl- secretion via the *Cftr* channel is one of the main driving forces of secretory diarrhea (Kunzelmann et al, 2002).

Early studies reported on the direct effect of antibiotics on fluid transport through the intestinal epithelium (Giannella et al, 1981, Roberts et al, 1991, Goldhill et al, 1996). In our study, we used the parenteral route of antibiotic administration which eliminates any direct effect of antibiotics on epithelial cells. Despite this, we observed significant changes in water and ion (Na^+, Cl^-, and K^+) transport via rat colonic epithelium *in vivo*. There were pro-secretory changes in the colon after 5 days of ceftriaxone treatment, which was associated with the down-regulation of *Scnn1b* expression and increased *Cftr* expression. Moreover, the expression of *Aqp*8 was also decreased. Decreased *Aqp*8 has been described in the pathogenesis of diarrhea-predominant irritable bowel syndrome (Camilleri et al, 2019) and inflammation-associated diarrhea (Sakai et al, 2014). Pro-inflammatory changes in the intestinal mucosa with subsequent recruitment of neutrophils and pro-oxidant responses are key points in protecting against intestinal infection and preventing fatal dehydration (Ghosh et al 2011). In our previous study, we found degranulation of colonic mast cells after 5 days of ceftriaxone treatment (Dovbynchuk et al, 2015b). Ghosh et al. (Ghosh et al, 2011) showed that the interaction of mast cells with *E. coli*, an opportunistic pathogen and a constituent of the normal endogenous microbiota of the gut, induced mast cell bactericidal activity, degranulation, and release of inflammatory mediators (Piliponsky et al 2018).

After 14 days of ceftriaxone treatment, we still observed pro-secretory changes in the colonic epithelium but they were less pronounced than after 5 days of treatment. This response was mainly driven by Cl^- secretion, and the expression of *Cftr* channels was also increased. Surprisingly, along with the net Cl^- secretion, net Na^+ absorption was increased as well as expression of the electroneutral Na^+/H^+ exchanger (*Slc9a3*) and the electrogenic *Scnn1b*. The concomitant increase in sodium absorption along with the observed Cl^- secretion might be explained by a complex defense reaction to shift intraluminal colonic homeostasis. On one hand, there is compensation for fluid loss due to an increase in Na^+ absorption. On the other hand, there is protection of the epithelial layer integrity against colonization by pathogenic microbiota and its direct contact with the epithelial cells, achieved by driving mucus secretion and flushing out of invading bacteria due to an increase in Cl^- secretion.

Pathogenic, as well as commensal bacteria, can directly affect colonic fluid transporters by regulating ion transporters. For example, infection with *C. rodentium* resulted in the upregulation of *Cftr*, *DRA,* and *Slc9a3* transporters (Spehlmann et al, 2009). Oral gavage with *Salmonella enterica serovar Typhimurium* reduced the expression of DRA, *Scnn1b* and caused internalization of *Cftr* in mice colonic epithelial cells (Marchelletta et al, 2013). *B. breve* downregulated carbachol-induced Cl^- secretion and, to a lesser extent, Fsk-induced Cl^- secretion in HT29-19A epithelial monolayer (Liu et al, 2020). *L. acidophilus* significantly increased Cl^-/HCO^-_3 exchange activity via an increase in the surface levels of the apical anion exchanger DRA in both *in vitro* and *in vivo* studies (Shubha et al, 2015). Previously we found that the combined administration of multispecies probiotics with antibiotics prevented antibiotic-induced diarrhea in rats by increasing water absorption via the colonic epithelium (Dovbynchuk et al, 2015b).

In the present study, we observed the downregulation of anaerobic *Lactobacillus, Bifidobacterium, and Propionibacterium spp.* in rats' feces after 5- and 14-day ceftriaxone treatment. However, these changes were within the normal range. Counts of aerobic conditionally pathogenic *Enterobacteriaceae* (*Citrobacter spp, Klebsiella spp, Proteus spp*) and *S. aureus* were significantly increased after 5 days of ceftriaxone treatment but returned to baseline after 14 days of treatment. The decreased total number of *E.coli* after both 5 and 14 days of ceftriaxone treatment was mainly due to a reduction in the mean counts of lactose-positive *E.coli*. These dynamic changes in culturable colonic microbiota composition were accompanied by a significant depletion of

the colon surface mucus layer after 5 days of ceftriaxone treatment, but it improved after 14 days of ceftriaxone. That probably was the result of mucus release from Goblet cells as well as secretion *de-novo*, since the gene level of the major constituent of mucus, *Muc*2 mucin, was also slightly increased.

Dynamic changes in the thickness and composition of the mucus layer on the surface of epithelial cells were reported as a part of host defense mechanisms protecting the host from pathogenic invasion during *C. rodentium* infection (Gustafsson et al, 2013, Dovbynchuk et al, 2015b) as well as after transferring *E. coli* from the intestinal contents of conventional suckling rats into germ-free adult rats (Tomas et al, 2015). In all these studies (Gustafsson et al, 2013, Dovbynchuk et al, 2015b, Tomas et al, 2015), alterations in the mucus layer were accompanied by changes in ion secretion, ion transporters, and water channel expression. Generally, fluid secretion functions as an attempt to flush out potential pathogens or noxious agents such as toxins (Gareau et al, 2013). The changes in secretion allow the reformation of the mucus layer, displacing the pathogen to the outer mucus layer, where it is then outcompeted by the returning commensal flora (Gustafsson et al, 2013, Dovbynchuk et al, 2015a).

As further confirmation of the epithelial defense reaction during antibiotic therapy, we detected a significant increase in mRNA levels of major tight junction integral membrane protein occludin (*Ocln*) and its cytoskeletal linker proteins Zonula occludin-1 (*Tjp1*) after 14 days of ceftriaxone treatment in the colon mucosa. Zonula occludins link the cytoplasmic peripheral membrane proteins, e.g. occludin, with the membrane cytoskeleton to form tight junction complexes. These complexes, together with intracellular signaling proteins, are the rate-limiting factor for paracellular permeability; they are programmed to rapidly open and seal the barrier in the event of injury and other signals (Chelakkot et al, 2018).

Conclusion

The present study demonstrated, for the first time, that parenteral treatment with the cephalosporin antibiotic ceftriaxone induced diarrhea, which was associated with changes in colonic net water, ion transport, and mucus secretion driven by a shift in the number of culturable conditionally pathogenic microbiota. The molecular mechanisms of these changes differ after short (5 days) and long (14 days) periods of antibiotic therapy and might explain the observed transitory pattern of idiopathic AAD. However, the current study is limited by its focus on one group of antibiotics and caution must be exercised when translating these findings to the mechanisms of antibiotic-associated diarrhea for other antibiotics groups.

Conflict of interest

The authors declare that there is no conflict of interest.

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Declarations

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Ethics approval

The study was reviewed and approved by the animal committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Protocol # 8)

Consent to participate

All authors have agreed to this submission.

Consent for publication

This publication has been approved by all co-authors and by the responsible authorities at the ESC "Institute of Biology and Medicine" and Institute of High Technologies of Taras Shevchenko National University of Kyiv where the work has been carried out.

Authors' contributions

TD, LZ, TS and AH conducted experiments, TD, LZ and TS analyzed data, LZ and GT conceived and de-

signed research, TC original draft preparation, LO funding acquisition, GT and TS reviewed and edited. All authors read and approved the manuscript. The authors declare that all data were generated in-house and no paper mill was used.

References

Akiba Y, Guth PH, Engel E, Nastaskin I, Kaunitz JD. Dynamic regulation of mucus gel thickness in rat duodenum. Am J Physiol Gastrointest Liver Physiol 2000; 279(2): G437-47. https://doi.org/10.1152/ajpgi.2000.279.2.G437

Benjamin HM, Horace RTW. *Clostridium difficile* infection and antibiotic-associated diarrhea. Clin Med (Lond) 2018; 18(3): 237–41. https://doi.org/10.7861/clinmedicine.18-3-237

Camilleri M, Carlson P, Chedid V, Vijayvargiya P, Burton D, Busciglio I. Aquaporin Expression in Colonic Mucosal Biopsies From Irritable Bowel Syndrome With Diarrhea. Clin Transl Gastroenterol 2019; 10(4), e00019. https://doi.org/10.14309/ctg.0000000000000019

Chelakkot C, Ghim J. Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med 2018; 50, 103. https://doi.org/10.1038/s12276-018-0126-x

Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nature Protocols 2006; 1(2), 581–5. https://doi.org/10.1038/nprot.2006.83.

Dovbynchuk T, Zakordonets L, Baban V, Chervinska T, Tolstanova G. The effect of multi-spicies probiotic on antibiotic-induced changes in colonic ion and water transport. Res J Pharma, Biol Chem Sci 2015a; 6(6): 896-901. https://doi.org/10.1111/j.1572-0241.2007.01547.x

Dovbynchuk T, Zakordonets L, Putnikov A, Vareniuk I, Tiapko O, Roslova N, et al. Net water transport via rat colon epitelium under the experimental dysbiosis. Fiziol Zh 2015b; 61(6):76-85. https://doi.org/10.15407/fz61.06.076

Durham SH, Wingler MJ, Eiland LS. Appropriate Use of Ceftriaxone in the Emergency Department of a Veteran's Health Care System. J Pharm Technol 2017; 33(6):215-8. https://doi.org/10.1177/8755122517720293

Gareau MG, Barrett KE. Fluid and electrolyte secretion in the inflamed gut: novel targets for treatment of inflammation-induced diarrhea. Curr Opin Pharmacol 2013; 13(6): 895–9. https://doi.org/10.1016/j.coph.2013.08.014

Ghosh S, Dai C, Brown K, Rajendiran E, Makarenko S, Baker J, Gibson DL. Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression Am J of Physiol. Gastrointest and Liver Physiol 2011; 301(1): G39-49. https://doi.org/10.1152/ajpgi.00509.2010

Giannella RA, Serumag J, Walls D, Drake KW. Effect of clindamycin on intestinal water and glucose transport in the rat. Gastroenterology 1981; 80(5 pt 1): 907-13. https://doi.org/10.1016/0016-5085(81)90058-5.

Goldhill JM, Rose K, Percy WH. Effects of antibiotics on epithelial ion transport in the rabbit distal colon in-vitro. J Pharm Pharmacol 1996; 48(6):651-6. https://doi.org/10.1111/j.2042-7158.1996.tb05990.x

Gustafsson JK, Navabi N, Rodriguez-Piñeiro AM, Alomran AHA, Premaratne P, Fernandez HR, et al. Dynamic changes in mucus thickness and ion secretion during Citrobacter rodentium infection and clearance. PloS one 2013; 8(12): e84430. https://doi.org/10.1371/journal.pone.0084430

Holota Y, Dovbynchuk T, Kaji I, Vareniuk I, Dzyubenko N, Chervinska T, et al. The long-term consequences of antibiotic therapy: Role of colonic short-chain fatty acids (SCFA) system and https://intestinal barrier integrity. PLoS One 2019; 14(8): e0220642. https://doi.org/10.1371/journal.pone.0220642

Hoque KM, Chakraborty S, Sheikh IA. Woodward OM. New advances in the pathophysiology of intestinal ion transport and barrier function in diarrhea and the impact on therapy. Expert Rev Anti Infect Ther 2012; 10(6): 687-99. https://doi.org/10.1586/eri.12.47

Hove H, Tvede M, Mortensen PB. Antibiotic-associated diarrhoea, Clostridium difficile, and short-chain fatty acids. Scand J Gastroenterol 1996; 31(7): 688-93. https://doi.org/10.3109/00365529609009151

Kiela PR, Ghishan FK. Ion transport in the intestine. Curr Opin Gastroenterol 2009; 25(2): 87-91. https://doi.org/10.1097/MOG.0b013e3283260900

Konturek PC, Brzozowski T, Pierzchalski P, Kwiecien S, Pajdo R, Hahn EG, et al. Activation of genes for spasmolytic peptide, transforming growth factor alpha and for cyclooxygenase (COX)-1 and COX-2 during gastric adaptation to aspirin damage in rats. Aliment Pharmacol Ther 1998; 12(8): 767-77. https://doi.org/10.1046/j.1365-2036.1998.00371.x

Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol Rev 2002; 82(1): 245-89. https://doi.org/10.1152/physrev.00026.2001

Liu Q, Yu Z, Tian F. et al. Surface components and metabo-

lites of probiotics for regulation of intestinal epithelial barrier. Microb Cell Fact 2020; 19, 23. https://doi.org/10.1186/s12934-020-1289-4

Liu Q, Yu Z, Tian F, Zhao J, Zhang H, Zhai Q, et al. Surface components and metabolites of probiotics for regulation of intestinal epithelial barrier. Microb Cell Fact 2020; 19, 23. https://doi.org/10.1186/s12934-020-1289-4

Lomasney KW, Hyland NP. The application of Ussing chambers for determining the impact of microbes and probiotics on intestinal ion transport. Can J Physiol Pharmacol 2013; 91(9): 663-70. https://doi.org/10.1139/cjpp-2013-0027

Marchelletta RR, Gareau MG, McCole DF, Okamoto S, Roel E, Klinkenberg R, et al. Altered expression and localization of ion transporters contribute to diarrhea in mice with Salmonella-induced enteritis. Gastroenterology 2013; 145(6): 1358–68.e1–4. https://doi.org/10.1053/j.gastro.2013.08.054

National Research Council. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press. 2011. https://doi.org/10.17226/12910

Nelson Rl, Kelsey P, Leeman H, Meardon N, Patel H, et al. Antibiotic treatment for Clostridium difficile -associated diarrhea in adults (Review). Cochrane Database Syst Rev 2011; (9):CD004610. https://doi.org/10.1002/14651858.CD004610.pub4

Neuman G, Boodhan S, Wurman I, Koren G, Bitnun A, et al. Ceftriaxone-induced immune hemolytic anemia. Ann Pharmacother 2014; 48(12): 1594-1604. https://doi.org/10.1177/1060028014548310.

Piliponsky AM, Romani L. The contribution of mast cells to bacterial and fungal infection immunity. Immunol Rev 2018; 282(1):188-97. https://doi.org/10.1111/imr.12623

Roberts M, Hladky SB, Pickles RJ, Cuthbert AW. Stimulation of sodium transport by duramycin in cultured human colonic epithelia. J Pharmacol Exp Ther 1991; 259(3): 1050–8.

Sakai H, Sagara A, Matsumoto K, Jo A, Hirosak A, Takase K, Narita M. Neutrophil recruitment is critical for 5-fluorouracil-induced diarrhea and the decrease in aquaporins in the colon. Pharmacol Res 2014; 87: 71–9. https://doi.org/10.1016/j.phrs.2014.05.012

Sambrook J, Russell DW. Separation of RNA according to size: electrophoresis of glyoxylated RNA through agarose gels. CSH Protoc 2006; 1: 23-8. https://doi.org/10.1101/pdb.prot4057

Schedl HP. Use of polyethylene glycol and phenol red as unabsorbed indicators for intestinal absorption studies in man. Gut 1966; 7(2): 159-63. https://doi.org/10.1136/gut.7.2.159

Shubha P, Arivarasu NA, Tarunmeet G, Alip B, Seema S, Ravinder KG, et al. All-*trans*-retinoic Acid Increases *SLC26A3* DRA (Down-regulated in Adenoma) Expression in Intestinal Epithelial Cells via *HNF-1*β J Biol Chem 2015; 290, 15066-77. https://doi.org/10.1074/jbc.M114.566356

Sladen GE, Harries JT. Studies on the effects of unconjugated dihydroxy bile salts on rat small intestinal function in vivo. Biochim Biophys Acta. 1972; 288(2): 443-56. https://doi.org/10.1016/0005-2736 (72)90265-9

Spehlmann ME, Dann SM, Hruz P, Hanson E, McCole DF, Eckmann L. CXCR2-dependent mucosal neutrophil influx protects against colitis-associated diarrhea caused by an attaching/effacing lesion-forming bacterial pathogen. J Immun 2009; 183(5): 3332-43. https://doi.org/10.4049/jimmunol.0900600

Thomson SR, Ommurugan B, Patil N. Ceftriaxone induced hypersensitivity reactions following intradermal skin test: Case Series. J Clin Diagn Res 2017; 11(10) 3,672. https://doi.org/10.7860/JCDR/2017/29088.10758

Tomas J, Reygner J, Mayeur C, Ducroc R, Bouet S, Bridonneau C, et al. Early colonizing Escherichia coli elicits remodeling of rat colonic epithelium shifting toward a new homeostatic state. ISME J2015; 9(1): 46-58. https://doi.org/10.1038/ismej.2014.111