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ORIGINAL PAPERS

Neomycin and bacitracin reduce the intestinal permeability in mice and increase the expression of some tight-junction proteins

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ABSTRACT

Background: Tight-junction (TJ) proteins regulate paracellular permeability. Gut permeability can be modulated by commensal microbiota. Manipulation of the gut microbiota with antibiotics like bacitracin and neomycin turned out to be useful for the treatment of diarrhoea induced by *Clostridium difficile* or chemotherapy drugs.

Aim: To evaluate the effects of the microbiota depletion evoked by the oral administration of neomycin and bacitracin on the intestinal permeability and expression of TJ proteins in mice.

Methods: Mice received neomycin and bacitracin orally for 7 days. Intestinal permeability was measured by the fluorescein-isothiocyanate-dextran (FITC-dextran) method. The gene expression of TJ proteins in the intestine was determined by real time-PCR.

Results: FITC-dextran levels in serum were reduced by half in antibiotic-treated mice, indicating a reduction of intestinal permeability. Antibiotics increased the expression of zonula occludens 1 (ZO-1), junctional adhesion molecule A (JAM-A, and occludin in the ileum and ZO-1, claudin-3, and claudin-4 in the colon.

Conclusion: The combination of neomycin and bacitracin reduce intestinal permeability and increase the gene expression of ZO-1, junctional adhesion molecule A (JAM-A), and occludin in the ileum and ZO-1, claudin-3, and claudin-4 in the colon.

Key words: Antibiotics. Microbiota. Tight-junction proteins. Intestinal permeability.

INTRODUCTION

The intestinal epithelium provides a selective, permeable barrier achieved by the presence of intercellular tight-junction (TJ) structures, which regulate paracellular permeability. The TJ protein complex consists of transmembrane and intracellular scaffold proteins. Four transmembrane proteins, occludin, claudins (24 members), junctional adhesion molecule (JAM), and tricellulin, have been identified. These transmembrane proteins interact with cytosolic scaffold proteins such as zonula occludens (ZO) proteins (1).

Intestinal epithelial cells are continuously interacting with an extensive intestinal microbiota, which modulate the intestinal permeability directly through the release of toxins, cellular structural components, or metabolites, or indirectly through its effects on host immune cells (2).

Antibiotic intake for the treatment of various diseases of bacterial origin obviously produces an alteration of the intestinal microbiota. This alteration of the intestinal microbiota can have beneficial effects and contribute to the restoration of intestinal homeostasis. Bacitracin has been used orally for the treatment of *Clostridium difficile*-associated diarrhoea and colitis (3,4). Oral neomycin is indicated for suppression of intestinal bacterial flora in patients undergoing colorectal surgery (5,6) and as a means of decreasing colonic bacteria and the production of ammonia in hepatic encephalopathy (7). Recently, it has been reported that the oral administration of neomycin plus bacitracin prevents diarrhoea induced by chemotherapy drugs used in the treatment of advanced colorectal cancer (8). However, prolonged use of antibiotics has also been described as a factor that, by altering the intestinal microbiota, can contribute to the development and/or maintenance of chronic intestinal diseases such as inflammatory bowel disease or irritable bowel syndrome, pathologies with altered intestinal permeability (9).

The aim of the present study is to evaluate the effects of the microbiota depletion induced by the oral administration of neomycin and bacitracin on intestinal permeability and the expression of certain TJ proteins in mice.

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METHODS

Animals and treatment with antibiotics

All procedures were approved by the Ethics Committee for Animal Experiments from the University of Zaragoza, Spain (Project Licence PI36/12).

Female C57BL/10 mice (5 to 7 weeks old) received a combination of non-absorbable antibiotics (neomycin 20 mg and bacitracin 20 mg per mouse, AppliChem, Barcelona, Spain) by oral gavage (0.2 mL) for 7 consecutive days. Bacitracin and neomycin were diluted in sterile, deionized water, and the pH of the solution was adjusted to 4.0 to prevent inactivation of bacitracin. Pimaricin (5 µg per mouse) was added to the antibiotic solution to prevent yeast overgrowth. Control mice received sterile, deionized water (0.2 mL). This combination of antibiotics has been used previously by our group to induce significant depletion in commensal microbiota (10).

We used 2 groups of 8 mice (control and treated) to assess intestinal permeability in vivo and 2 groups of 8 mice (control and treated) to study the gene expression of the TJ proteins.

Intestinal permeability *in vivo*

After a 14 h fast, mice were gavaged with 60 mg per 100 g body weight of fluorescein-isothiocyanate-dextran (FITC-dextran, FD4, 3.000-5.000 kD, Sigma-Aldrich, Madrid, Spain) in a volume of 0.2 mL. Blood samples were obtained by cardiac puncture at 4 h after administration of FITC-dextran, as it has been described that dextran levels in serum are at maximum at this point (11). The blood was centrifuged at 3,000 rpm for 5 min at room temperature to obtain the serum. Fluorescence intensity of each serum sample (DTX 880 Multimode Detector, Beckman Coulter, CA, USA) was measured, and the concentrations of FITC-dextran were determined from standard curves generated by the serial dilution of FITC-dextran. Results were expressed as ng FITC-dextran mL^{-1} serum per 100 g body weight (bw).

Study of mRNA expression of TJ proteins

The expression of zonula occludens 1 (ZO-1), junctional adhesion molecule A (JAM-A), occludin, and claudin-3, -4, and -7 mRNA was quantified by real time-PCR. Ileum (i.e. the last 6 cm of the small intestine) and proximal colon samples from control and antibiotic-treated mice were extracted and preserved in RNAlater solution (Ambion, Life Technologies, Madrid, Spain) at 4 °C overnight. The samples were kept at -80 °C until the RNA extraction with the RNeasy mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized by reverse transcription using the AffinityScript Multiple Temperature cDNA synthesis Kit (Stratagene, La Jolla, CA, USA), and it was used to determine the expression levels of mRNA of the TJ proteins. Reactions were run using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsband, California, USA). The reaction mixture (10 µL) comprised 4.5 µL FastStart Universal SYBR Green Master (Roche, Mannheim, Germany), 0.5 µL of each primer 30 µM (Table I), 2.5 µL of sterile distilled water, and 2 µL of DNA template (100 ng μL^{-1}). Each sample was run in triplicate, and the mean Ct was determined from the 3 runs. Relative TJ proteins' mRNA expression under each experimental condition (control or treatment) was expressed as ΔCt = Ct TJ protein – Ct calibrator. GAPDH housekeeping gene expression was used as a calibrator after verification of its stability under our experimental conditions. Then, relative TJ proteins' mRNA expression was calculated as $\Delta\Delta Ct = \Delta Ct$ treatment – ΔCt control. Finally, the relative gene expression levels were converted and expressed as fold change respective to the control ($=2^{-\Delta\Delta Ct}$).

Data analysis and statistics

Results were expressed as the mean ± S.E.M. with *n* denoting the number of animals used. The Mann-Whitney U test was used to compare the data, and differences with P-values < 0.05 were considered to be statistically significant.

Table I. Primers used for quantification of TJ proteins by RT-PCR

Gene	*Reference*	*GenBank accession number*	*Sense and antisense primers*
ZO-1	(22)	NM_001163574.1	ACTCCCACTTCCCCAAAAAC CCACAGCTGAAGGACTCACA
Ocludina	(22)	NM_008756.2	ACTGGGTCAGGGAATATCCA TCAGCAGCAGCCATGTACTC
JAM-A	(22)	NM_172647.2	CTGATCTTTGACCCCGTGAC ACCAGACGCCAAAAATCAAG
Claudina-3	(17)	NM_009902.4	AAGCCGAATGGACAAAGAA CTGGCAAGTAGCTGCAGTG
Claudina-4	(17)	NM_009903.2	CGCTACTCTTGCCATTACG ACTCAGCACACCATGACTTG
Claudina-7	(17)	NM_016887.6	AGGGTCTGCTCTGGTCCTT GTACGCAGCTTTGCTTTCA
Beta-actina	Primerbank	NM_007393.3	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT

RESULTS

Effect of neomycin and bacitracin on intestinal permeability

As shown in figure 1, water-drinking mice showed FITC-dextran levels in serum of 2009 ± 473.5 ng mL^{-1} serum per 100 g of body weight. Mice treated with antibiotics showed a reduction of approximately half in FITC-dextran levels (973.4 ± 172.8 ng mL^{-1} serum per 100 g of body weight) respective to controls.

Effect of neomycin and bacitracin on mRNA expression of TJ proteins

Figure 2 shows the mRNA expression levels of ZO-1, JAM-A, occludin, and claudins-3, -4, and -7 found in the ileum (Fig. 2A) and colon (Fig. 2B) of mice treated with antibiotics respective to controls. Antibiotic treatment increased the expression of ZO-1, JAM-A, and occludin in the ileum and ZO-1, claudin-3, and claudin-4 in the colon.

DISCUSSION

Previous results by our group have shown that oral administration of neomycin and bacitracin induces a large depletion of the intestinal microbiota, a slight intestinal inflammation, a reduction of faecal output, a slowing of intestinal transit, and a reduction of the contractility *in vitro* in the ileum and colon in mice (10). In this work, we have evaluated in these same mice treated with antibiotics the permeability of the small intestine and colon to FITC-dextran after 4 h of administration. Other authors have shown that the FITC-dextran can reach the colon at 3 h (12), and the maximum concentrations of FITC-dextran are found in the serum at 4 h after its oral administration (11). Our results indicate that mice treated with neomycin and bacitracin show reduced intestinal permeability to FITC-dextran. However, others have shown that bacitracin increases paracellular uptake of FITC-dextran (4,000 kD) in epithelial cells of human colon carcinoma (Caco-2) (13). Moreover, neomycin increases the absorption of sugars and has direct effects on the morphology of rat intestinal epithelium. However, neomycin does not produce these effects on hamsters, indicating that there are specific differences between species in terms of the susceptibility of the epithelium to neomycin (14). The fact that intestinal transit is slowed down in mice treated with neomycin and bacitracin might explain the lower arrival of FITC-dextran to the intestinal epithelial cells of the ileum and colon and therefore, less paracellular absorption.

On the other hand, there are several examples showing that antibiotic treatment is effective in restoring normal intestinal permeability. Thus, ciprofloxacin and metronidazole improve the impaired mucosal barrier function in mice with cystic fibrosis (15). Oral rifaximin prevents

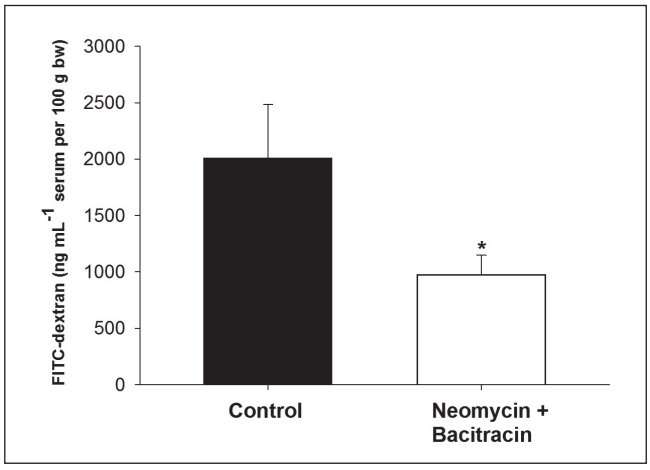

Fig. 1. FITC-dextran levels in serum from control and bacitracin + neomycin-treated mice as a measure of intestinal permeability. Results are expressed in mean values (ng mL^{-1} serum per 100 g body weight, bw) and the vertical bars indicate the S.E.M. of 8 mice per group. *p < 0.05 respective to the control.

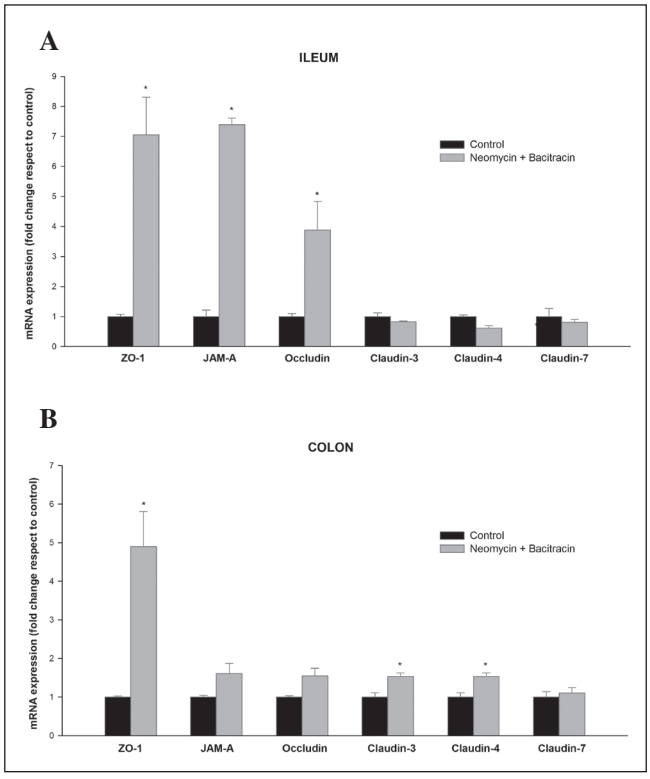

Fig. 2. Levels of mRNA expression of the TJ proteins in A ileum and B colon from control and bacitracin + neomycin-treated mice. Results are expressed as mean values (fold change respect to control), and the vertical bars indicate S.E.M. of 8 mice per group. *p < 0.05 respective to the control.

the impairment of intestinal barrier function induced by water-avoidance stress (16).

Among the mechanisms that may modify the intestinal permeability are alterations in TJ proteins' expression, localization, or function (2). In our study, we evaluate the direct effect of the combination of neomycin and bacitracin on gene expression of some TJ proteins. All segments of the small and large intestine of mice show high levels of expression of ZO-1, JAM-A, occludin, and claudins-3, -4, and -7 (17). In our study, the depletion of the microbiota by the administration of neomycin and bacitracin induced an increase in gene expression of ZO-1, JAM-A, and occludin in the ileum and ZO-1, claudin-3, and claudin-4 in the colon. These results indicate that the intestinal microbiota might modulate the transcription of these proteins and, in turn, regulate intestinal permeability. However, it should be noted that changes in gene expression do not always imply a change in functional protein levels and, therefore, a change in the functionality of the TJs.

Our study shows that the microbiota might regulate gene expression of TJ proteins differently in the ileum and colon. Thus, increased expression of ZO-1, JAM-A, and occludin proteins may contribute to the reduced permeability of FITC-dextran observed in the ileum. It has been described that ZO proteins play an important role in regulating the assembly of TJs (1). *In vivo* and *in vitro* studies demonstrate that JAM-A participates in the regulation and maintenance of the TJ barrier (1). The JAM-A knockout mice exhibit higher permeability to dextran in the colon compared to wild-type mice (18). Studies in Caco-2 cells and mouse intestines have shown that occludin knockdown induces an increase in paracellular permeability to macromolecules (19).

Conversely, increased expression of ZO-1, claudin-3, and claudin-4 might contribute to reduced permeability to FITC-dextran observed in the colon. Thus, claudin-3 and claudin-4 are involved in barrier formation and decrease the paracellular permeability (1). However, the level of claudin-7 is not altered in the ileum or colon, and this protein is involved in pore formation and increased paracellular permeability (1), which supports our results.

Finally, other antibiotics have been shown to modify the expression of TJ proteins. Oligomycin alleviated the IFN-γ and TNF-α caused morphological redistributions of TJ proteins ZO-1 and occludin (20). Minocycline exerted intestinal anti-inflammatory effects and attenuated the reactivation of colitis by increasing the expression of ZO-1 (21).

In conclusion, the depletion of the microbiota induced by neomycin and bacitracin reduces intestinal permeability and increases expressions of ZO-1, JAM-A, and occludin in the ileum and ZO-1, claudin-3, and claudin-4 in the colon.

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