Effects of Erythropoiesis-stimulating Agents on Intestinal Flora in Peritoneal Fibrosis

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Introduction. This study aimed to investigate the effects of erythropoiesis-stimulating agents (ESAs) on intestinal flora in peritoneal fibrosis.
Methods and Methods. Twenty-four Wistar albino rats were divided into 3 groups as the control group, which received 0.9% saline (3 mL/d) intraperitoneally; the chlorhexidine gluconate (CH) group, which received 3 mL/d injections of 0.1% CH intraperitoneally, and the ESA group, which received 3 mL/d injections of 0.1% CH intraperitoneally and epoetin beta (3 doses of 20 IU/kg/wk) subcutaneously. On the 21st day, the rats were sacrificed and the visceral peritoneum samples were obtained from left liver bowel. Blood samples were obtained from abdominal aorta and intestinal flora samples were obtained from transverse colon.
Results. Histopathologically, the CH, ESA, and control groups had peritoneal thickness of 135.4 ± 22.2 µm, 48.6 ± 12.8 µm, and 6.0 ± 2.3 µm, respectively. *Escherichia coli* was the predominant bacterium in the intestinal flora in the control group. Significant changes in microbial composition of intestinal flora towards *Proteus* species and *Enterobacter* species was seen among the groups ($P < .001$). There was no significant difference between the ESA and CH groups regarding the isolates from blood cultures. However, the bacterial isolates from cultures of intestinal flora among these groups were significantly different ($P < .05$).
Conclusions. Erythropoiesis-stimulating agents change intestinal flora by a clinically significant amount in experimental peritoneal fibrosis. We consider that ESAs achieve this via regulating intestinal peristaltism.

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INTRODUCTION

The most common problem for patients with end-stage renal disease (ESRD) carrying out peritoneal dialysis (PD) is peritonitis, accounting for considerable mortality and hospitalization. Getting prompt treatment for peritonitis is very important; otherwise, the infection can get worse. It is usually caused by gram positive organisms and less commonly gram negative organisms or other microorganisms.[1,2] The route of transmission for pathogens in PD-associated peritonitis could be direct contamination of the connection device or bacterial translocation through the gastrointestinal tract.[3,4]

Intestinal organisms such as *Enterococcus*, *Campylobacter*, *Salmonella*, and *Shigella* account for a small proportion *of* PD-associated peritonitis. These enteric pathogens may also gain access to the peritoneal cavity by touch contamination during periods of fecal shedding or by transmural

migration through inflamated intestine. However, method of spread of these organisms from the gastrointestinal tract to the peritoneal cavity remains speculative.[1,5,6]

In addition, exposure to high-glucose PD fluids results in structural and functional changes over time in the peritoneal membrane among ESRD patients on PD.[7,8] Peritonitis is still the leading cause of technique failure among long-term PD patients. Peritonitis attacks can eventually cause progressive peritoneal fibrosis, loss of peritoneal surface area, decreased permeability, and ultrafiltration failure, making PD less effective.[9-11]

Erythropoiesis-stimulating agents (ESAs) are used in millions of patients to treat anemia. It has been shown that ESAs have other effects besides treating anemia. Administration of human recombinant erythropoietin for acute ischemic kidney injury model formed by 2-sided ischemic-reperfusion inhibits apoptosis, enhances tubular epithelial regeneration, and promotes kidney functional recovery.[12] Furthermore, ESAs have cellular protective effects.[12-14]

Because of direct trasmission of bacteria from intestinal flora to peritoneum or the hematogenous route may play an important role in the development of peritonitis.[15] We aimed to investigate the effects of ESA on istestinal flora in chemically induced peritoneal fibrosis in rats. To the best of our knowledge, this is the first study about the effects of ESAs on intestinal flora.

METHODS AND METHODS

Animals

This study was undertaken after approval by the Institutional Ethics Committee for Experimental Animal Investigations. For homogenisation of study groups, a total of 24 female Wistar albino rats, 6 to 8 months old, weighing between 200 g and 230 g were used. The rats were housed in multidisciplinary laboratory for 21 days in standard hutchs (4 rats per hutch) with a standard supply of food and water. Development of infections and systemic reactions other than peritoneal sclerosis or any other reason for morbidity were the exclusion criteria.

Study Design

The rats included in the study were weighed before the study and divided into 3 groups (8 rats per group). Duration of study was 21 days. To induce peritoneal fibrosis, we used the model described by Ishii and colleagues comprising chlorhexidine gluconate (CH; Dogsan Drugs, Balgat, Ankara, Turkey), made up as a 0.1% CH solution in 15% ethyl alcohol dissolved in 0.9% physiological saline in aseptic conditions.[16] Epoetin beta (Neorecormon, F Hoffmann-La Roche Ltd, Basel, Sweden) was used as 1000 IU per 0.3 mL (3 doses of 20 IU/kg/wk). In the control group, the rats were given intraperitoneal injection of 0.9% saline (3 mL per 200 g) through a 21-gauge needle for 20 days. In the CH group, the rats were given daily intraperitoneal injections of 200 g (3 mL) of CH through a 21-gauge needle for 20 days. In the ESA group, the rats were given the same protocol as the CH group plus epoetin beta, subcutaneously through a 21-gauge needle.

On the 21st day of the study, the rats were sacrificed by using ether at a toxic dose. A midline thoracoabdominal incision was used for exploring the abdominal cavity (Figure 1). Samples from the visceral peritoneum were obtained from the left liver and immediately placed in tubes containing 10% formaldehyde for pathological evaluation. For each rat, a blood sample of 2 mL was obtained from abdominal aorta and was placed in blood culture tubes (Hemoline, Biomerieux, France). The bottles were incubated for 7 days. Gram stains were performed on the samples taken from the tubes, in which there was bacterial incubation. For determining the intestinal flora, samples were obtained from transverse colon and inoculated immediately on Glombia agar with 5% sheep

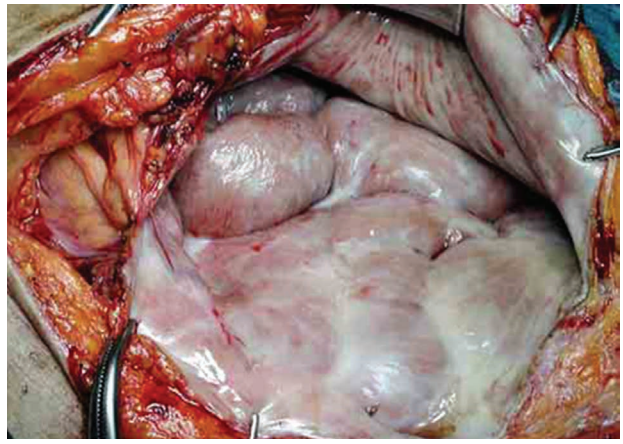

Figure 1. Exploring of the abdominal cavity with midline thoracoabdominal incision.

blood, and eosin methylene blue agar (Biomerieux, Marcy I'Etoile, France), and incubated at 36°C for 24 hours. The incubating bacteria were identified with standard conventional and automatic methods (API system, Biomerieux, France). Susceptibility testing was performed using the disk diffusion method according to the National Committee for Clinical Laboratory Standards criteria.[17]

Susceptibility was tested on Mueller-Hington agar at 37°C by using the disk diffusion test against amikacin, amoxicillin, amoxicillin-clavulanic acid, ampicillin, cefepime, cefuroxime, cefazolin sodium, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, meropenem, levofloxacin, trimethoprim/sulfamethoxazole, gentamicin, piperasillin/tazobactam, and vancomycin.

Histopathologic Analysis

From formaldehyde-fixed visceral peritoneum samples, vertical sections of 3-mm thickness were prepared, embedded in paraffin, and stained with hematoxylin-eosin. The thickness of the visceral peritoneum was examined by a light microscope.

Light Microscopic Examination

Microscopic images of the hematoxylin-epsin-stained sections were transferred to a computer through a camera (Olympus BX50, Olympus Optical Co, Tokyo, Japan) to measure peritoneal thickness. The thickness of the visceral peritoneum was measured from the inner surface of the muscle to the mesothelium in 10 different areas by the same researcher using image analysis software. The mean value of 10 different areas was taken for analysis.[18]

Statistical Analysis

Statistical analyses were performed with the SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA). Distribution of data was determined by the Shapiro-Wilk test. Continuous variables were expressed as mean ± standard deviation, and categorical variables, as frequency and percentage. The chi-square test was used to determine the difference between the three groups for categorical variables. Continuous variables were compared with the Mann-Whitney U test for 2 group. The Kruskal-Wallis test was used to determine the differences between the three groups. The Bonferroni-corrected Mann-Whitney U test was used for post-hoc test after the Kruskal-Wallis test. A P value less than .05 was considered significant for all tests.

RESULTS

All 24 rats completed the 21 days of the study. We observed macroscopically visceral peritoneal thickness and adhesions on the liver and abdominal wall in the rats of the CH and ESA groups, but not in the rats of the control group. The visceral peritoneal thicknesses of the CH, ESA, and control groups were 135.4 ± 22.24 µm, 48.56 ± 12.8 µm, and 6.04 ± 2.32 µm, respectively. Visceral peritoneal thickness was significantly less in the ESA group compared to the CH group ($P < .001$). Increases in the thickness of visceral peritoneum in both of the ESA and CH groups were significantly higher than those in the control group ($P < .001$; Figure 2).

Escherechia coli was isolated from all cultures of intestinal samples of all of the rats in the control group, whereas blood cultures were sterile in

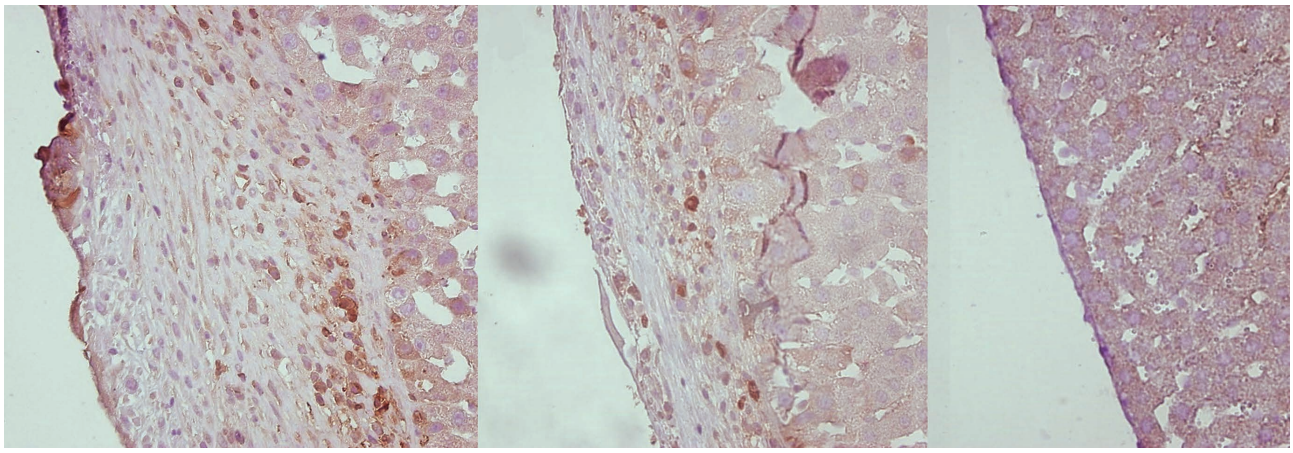

Figure 2. Histopathologic images of visceral peritoneal thickness are shown. Left, the chlorhexidine gluconate group; erythropoiesis-stimulating agent group; Right, the control group.

this group. In the CH group, *Proteus* species was isolated from 6 (75%) cultures and *Enterobacter* species from 2 (25%) cultures. One rat with *Proteus* species in the intestinal flora had positive blood culture for the same bacteria.

In the ESA group, *Escherichia coli* was isolated from the intestine cultures in 4 (50%) rats, but blood cultures of these rats were sterile. *Proteus* species was isolated from 4 (50%) of the remaining intestinal culture and 1 (12.5%) rat with *Proteus* species in the intestinal flora had positive blood culture for the same bacteria.

The bacterial isolates from cultures of intestinal flora among these groups were significantly different ($P < .001$). There was no significant difference between the ESA and CH groups regarding the isolates from blood cultures. Antibiotic susceptibility testing showed that the bacteria which were isolated from blood and intestinal cultures were susceptible to all antibiotics. The intestinal flora in the CH group changed compared to the ESA and control groups ($P = .04$ and $P < .001$, respectively), whereas there was no significance difference between the ESA group and the control group ($P = .08$). It was observed that intestinal flora changed as the peritoneal thickness increased among the groups ($P < .001$).

DISCUSSION

The results of our study indicated that ESA was effective on the gut flora in the treatment of chlorhexidine induced peritoneal fibrosis. The thickness of the peritoneum increases progressively during peritoneal dialysis, and it disrupts intestinal motility.[19,20] Erythropoiesis-stimulating agents reduce the thickness of the peritoneum significantly and improves intestinal motility and consequently the intestinal flora.

The ESA used in this study was not pure molecule. Epoetin beta is used as adjunct to some ingredients such as urea, sodium chloride, polysorbate, sodium dihydrogen phosphate, monohydrogen sodium phosphate, calcium chloride, glycine, leucine, izoleusine, treonin, glutamic acid, and phenylalanine. In conclusion, since ESA caused a decrease in peritoneal fibrosis we suggest that neither low dose ESA nor other adjunctive materials affected development of peritoneal fibrosis. If ever either ESA or any adjunctives cause peritoneal fibrosis, this also might be prevented. Low-dose ESA is introduced as a compound and used clinically subcutaneously; thus, we could not use pure ESA, and the effect of adjunctive substances in the compound on peritoneal fibrosis could not be estimated.

Peritonitis remains a common complication of peritoneal dialysis. The incidence of PD-related peritonitis has progressively declined since the 1980s due to developing technology and innovations. Microorganisms which cause peritonitis reach the peritoneal cavity through catheter lumen. Therefore, the dialysis pouch-transfer set and transfer set-catheter connections can be contaminated. Also, the splits and fissures can form the entry. Periluminal space around the catheter can be the pathway. Bacteria can reach the peritoneum via transmural, hematogenic, or transvaginal routes. Intestinal flora plays an important role in peritonitis occurring after bacteremia, which is caused by the gut flora or the transmural transaction of the intestinal bacteria during peritoneal dialysis.[15,21]

As *Escherichia coli* is the predominant aerobic gram-negative species in normal intestinal flora, much more attention has been paid to the possible role of its subtypes. Besides commensal strains, certain clones possess virulent properties and cause disease in humans; the diarrheagenic subtypes of *Escherichia coli* belong to this latter group, showing properties such as adherence to the gut mucosa, production of enterotoxins and cytotoxins, and tissue invasion.[22] In this study, *Escherichia coli* was the predominant bacterium in the intestinal flora among control group. A complete change in microbial composition of intestinal flora towards *Proteus* species and *Enterobacter* species (75% and 25%, respectively), has been seen in *the CH* group compared to the controls. In ESA group, the predominant bacteria were *Escherichia coli* (50%), and *Proteus* (50%), respectively. Compared to the control group, the reproduction differences in the CH group was found to be significant. Although blood cultures of the ESA and CH groups were found to be insignificant, different reproductions in the intestinal flora were significant. This study showed that while the intestinal flora at the beginning was *Escherichia coli*, as the peritoneal thickness increases the intestinal flora transforms to *Proteus* and *Enterobacter* The bacteria reproduced both in intestinal flora and blood are susceptible to all antibiotics on the antibiogram. We thought

that as the laboratory animals had no exposure to antibiotics before, they are all susceptible.

Currently, it is demonstrated that ESAs have other effects than just for therapy of chronic kidney failure-related anemia. They inhibited apoptosis in experimental acute kidney failure model using bilateral ischemic reperfusion model, increased tubular epithelial regeneration and accelerated gain of kidney function.[12] Also, they decreased interstitial fibrosis and tubular injury. Eritropoietin, which was used at 5000 U/kg in an experimental peritoneal fibrosis study, is impossible to apply clinically.[23] We used ESA in decreased doses. We chose small doses because we tried to evaluate the effects of ESA other than for anemia treatment and if necessary make it possible to use clinically.

As the peritoneal thickness increases, it is observed that the intestinal flora changes. Compared to the control group flora in the CH group totally changed. However, there was no significant difference between the ESA group and the control group.

We thought that the intestinal flora change was due to intestinal motility deterioration, which causeed thickening of visceral peritoneum. Also, ESA decreased the thickness of peritoneum and had a more physiological effect on the intestinal flora, which caused protection of the normal flora.

CONCLUSIONS

The normal intestinal flora is *Escherichia coli*, but in experimental peritoneal fibrosis, when the peritoneal thickness increases, the flora changes to *Proteus* and *Enterobacter* species. Erythropoesis-stimulating agent both decreases the thickness of visceral peritoneum and protects the intestinal flora according to changes in the physiological range. Further studies conducted in animal models of peritoneal dialysis will contribute to clarify the effects of ESA on the intestinal flora.

CONCLUSIONS

None declared.

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