Biol. Stud. 2015: 9(3–4); 49–56 • DOI: https://doi.org/10.30970/sbi.0903.452
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC 616.345-008.6-02+615.33.065]:612-092.9

THE DISTURBANCE OF OXIDANT-ANTIOXIDANT BALANCE IN RAT COLONIC MUCOSA AFTER ANTIBIOTIC THERAPY

Y. Holota, O. Tjapko, T. Dovbynchuk, G. Tolstanova

Taras Shevchenko National University of Kyiv
Educational and Scientific Center "Institute of Biology"
60, Volodymyrska St., Kyiv 01033, Ukraine
e-mail: gtolstanova@gmail.com

Antibiotic treatment increases susceptibility to development of inflammatory bowel diseases (IBD) both in children and adults. Oxidative stress plays a prominent role in IBD pathogenesis. The aim of present study was to test an interrelationship between the morphological changes in rat colonic mucosa, the levels of antioxidant enzymes and redox sensitive transcription factors Egr-1 and Sp-1 after treatment with cephalosporin antibiotic ceftriaxone (Cf) with broad spectrum of action. Study was performed on male Wistar rats (180–230 g). Cf (50 mg/kg, i.m.) were injected daily for 5 days. The colonic levels of catalase and superoxide dismutase activity were measured by the colorimetric assays and zymography; levels of Egr-1 and Sp-1 – by Western-blot analysis; histological changes – by morphometric analysis. Body weight and diarrhea were recorded. Systemic administration of the ceftriaxone induced morphological and functional changes in rat colonic mucosa associated with initial stages of the acute inflammation. These changes were accompanied by a decrease of activity of superoxide dismutase and catalase. Levels of redox-sensitive transcription factors Egr-1 and Sp1 were significantly increased, which shows a disturbance of homeostasis of the intestinal barrier.

***Keywords**:* ceftriaxone, colon, antioxidants, transcription factors.

INTRODUCTION

The inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease are characterized by chronic non-specific inflammation and ulcers of the intestinal mucosa of unknown etiology. From 5 to 60 new cases per 100 thousand people are recorded annually and this rate is steadily increased [5, 6].

In 2011, two independent groups reported about positive correlation between long-term use of antibiotics and the risk of IBD in children [2] and adults (average age – 43.4 years) [12]. The mechanisms underlying this phenomenon are hypothetical and require empirical study.

A disruption of the intestinal barrier function is a crucial factor for the IBD pathogenesis [3]. We have found that a disruption of the intestinal barrier integrity at early stages

of the IBD is associated with a development of hypoxia and oxidative stress in the epithelial cells with subsequent activation of the redox sensitive transcription factors [14].

Normal intestinal microbiota plays a crucial role in the formation and maintenance of the intestinal barrier homeostasis. Sterile environment (without bacteria) leads to a disruption of the intestinal barrier and immunity formation in germ-free animals. These animals have immature spleen and thymus; decreased number of lymphoid follicles and mature plasma cells which sensitize IgA; an imbalance at the level of anti- and pro-inflammatory cytokines; hypoplastic changes of Peyer patches [10]. Maintaining stability of microbiota composition is an essential factor for stimulating the expression of the antimicrobial peptides – defensins [1], the tight junction proteins [15] and mucins [11]. Considering a fact that antibiotics disturb the intestinal microbiota composition, we hypothesized that antibiotic treatment may lead to a disruption of the intestinal barrier integrity via a shift in the redox status of the intestinal mucosa cells.

The aim of a present study was to test an interrelationship between the morphological changes in rat colonic mucosa, the levels of antioxidant enzymes and redox sensitive transcription factors Egr-1 and Sp-1 under treatment with cephalosporin antibiotic ceftriaxone.

MATERIALS AND METHODS

Male Wistar rats (180–230 g, n = 14) were bred and housed in a conventional animal facility of the ESC “Institute of Biology” Taras Shevchenko National University of Kyiv (Kyiv, Ukraine) under standard environmental conditions (12 h light/dark cycle at a constant temperature of 22 °C). All animals had unlimited access to animal chow and tap water throughout the study. To normalize gut microbiota, rats from all groups were kept in the same room and maintained by the same personal. Study was approved by the bioethical committee of ESC “Institute of Biology” Taras Shevchenko National University of Kyiv (Protocol No 8 issued by Nov, 2, 2015).

Ceftriaxone (Kyivmedpreparat JSC, Ukraine) was injected 50 mg/kg intramuscularly, daily for 5 days (n = 7). Control rats (n = 7) were treated with sterile water (0.1 ml/rat, intramuscularly).

Animals were weighted before water or ceftriaxone treatment and next day after treatment stop. Diarrhea was estimated daily by a softness of feces. Animals were labeled as positive or negative for diarrhea sign.

Rats were euthanized by CO_2 inhalation followed by cervical dislocation next day after antibiotic withdrawal (6^{th} day of the experiment).

At autopsy, 2 cm of colonic tissue was embedded in 10% buffered formalin following paraffin for further histological analysis. Rest of colon was cut along the anti-mesenteric side and thoroughly rinsed in cold PBS. Colon was gently wiped with paper towel and flat by mucosa side up on ice. Mucosa was gently scraped by using metal spatula from the muscular layer and embedded in a liquid nitrogen for further biochemical assays.

Morphological signs of the colonic mucosa were estimated by the morphometric analysis on 3–5 μm histological sections stained with hematoxylin and eosin.

Catalase activity in colon was measured colorimetrically in a reaction with 0.03% H_2O_2 solution. The reaction was stopped by the molybdate ammonium (Alfarus, Ukraine) and measurement was taken at a wavelength of 410 nm. The activity of superoxide

dismutase (SOD) was determined by a zymography method, which was carried out in polyacrylamide gel with the addition of bromophenol blue to the samples. After the electrophoretic separation, the gel was incubated for 20 min in a solution of the nitroblue tetrazolium, TEMED and riboflavin (Sigma-Aldrich, Germany). Further it was kept under light until the manifestation of the reaction in the form of transparent spots on the light-blue background of the gel. Total concentration of proteins was measured by the Bradford method using a set of "Bio-Rad protein assay" (Bio-Rad, USA).

Separation and detection of proteins (150 μg proteins per sample) was run by the Western-blot analysis in 10% SDS polyacrylamide gel followed by transfer to the Hybond-ECL nitrocellulose membrane (Amersham Biosciences, USA) according to a standard protocol of Bio-Rad Company. Anti-Egr-1 (1:300) and Sp-1 (1:500) antibodies (Santa-Cruz, USA) were used to determine the level of the corresponding proteins in the colonic mucosa, followed by incubation with secondary HRP-conjugated antibodies (1:5,000, Santa-Cruz Biotech, USA). The loading controls were performed by using a mouse monoclonal antibody to β-actin (1:500) (Sigma-Aldrich, Germany).

Data are presented as M±SD. Statistical significance was determined by the Student's t-test. P-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Antibiotic treatment induced diarrhea in 30% rats. Despite of that, we did not detect a significant difference in body loss between control and antibiotic-treated rats. At the end of the experiment, the rats of control and experimental groups lost 5% and 6% of bodyweight, respectively.

The morphometric analysis of histological sections of rat colon revealed that 5 days of ceftriaxone administration increased the thickness of the mucous membrane (from 238.77±60.32 to 371.95±60.32 μm, $P < 0.001$, $n = 5$), the depth of intestinal crypts (from 224.0±32.0 to 310.4 ± 70.1 μm, $P < 0.001$, $n = 5$), and the area of the colonocytes nuclei (from 25.69±8.4 to 35.46±8.63 μm^2, $P < 0.001$, $n = 5$). We observed a swelling and desquamation of the mucosa that indicated an increase of functional activity of colonocytes. It was accompanied by an increase in number of the goblet cells, but a decrease in their size (from 126.23±54.67 to 107.40±49.42 μm^2, $n = 5$). These results indicate the pro-inflammatory changes and homeostasis disturbance of the intestinal barrier.

Lipid peroxidation processes (LPO) are major metabolic reactions, whose intensity is at certain level in the tissue. Disturbances of LPO after damaging effects are early and universal non-specific reactions in pathogenesis of many diseases. One of the pathogenic factors of LPO activation may be a deficiency of antioxidant activity in the colonic mucosa [8]. It is known that SOD catalyzes dismutation of the superoxide into oxygen and hydrogen peroxide. The hydrogen peroxide was formed during dismutation of the superoxide anion radical, is reduced to water, mainly with catalase and glutathione peroxidase.

An injection of ceftriaxone reduced the activity of SOD and catalase in rat colonic mucosa 1.1- and 4.5-folds, respectively (Fig. 1). A reduced activity of SOD and catalase may indicate a disturbance of physiological protection system from an excessive lipid peroxidation after administration of ceftriaxone.

These data are consistent with the results of other study [13]. It was shown that the injection of different doses of cephalosporin antibiotics to rats for 15 days reduced the activity of catalase and SOD as early as at the 1st–2nd days after administration of the antibiotic.

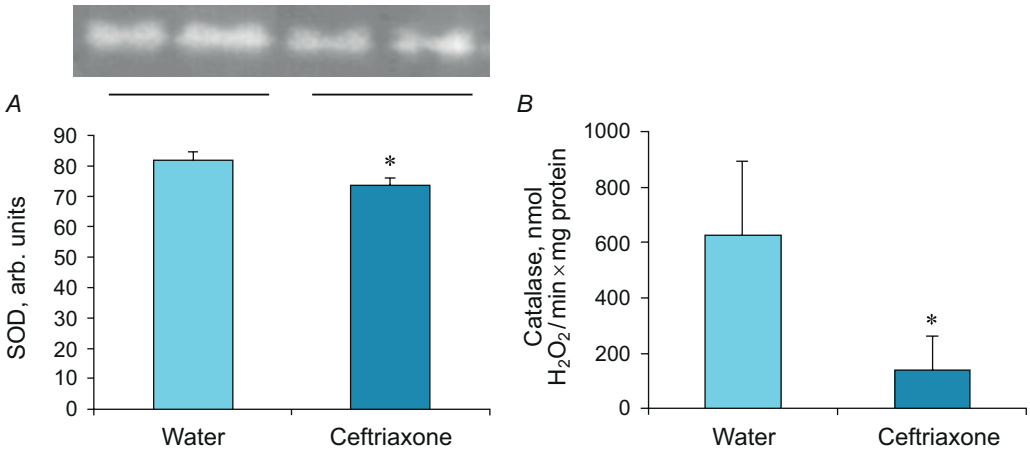

Fig. 1. Activity of antioxidant enzymes – superoxide dismutase (*A*) and catalase (*B*) in rat colonic mucosa after 5 days administration of ceftriaxone (50 mg/kg, i.m.), n = 5; M±SD; * P < 0.05 vs. control group (Water)

Рис. 1. Активність антиоксидантних ензимів супероксиддисмутази (*A*) і каталази (*B*) у слизовій оболонці товстої кишки щурів після 5-денного введення цефтріаксону (50 мг/кг, в/м), n = 5; M±SD; * P < 0,05 щодо показників у контрольній групі (Вода)

Despite a fact that the mechanisms of lipid peroxidation after antibiotic treatment have been reported earlier [8, 13], changes at the level of redox sensitive transcription factors have not been studied. Egr-1 is an early response protein that refers to a group of redox-sensitive transcription factors. Its activity depends on intracellular balance between oxidants and antioxidants. DNA-binding domain of Egr-1 contains cysteine residues. Oxidation the thiol (SH)-groups of cysteine leads to a disruption of the inter/intramolecular disulfide bonds and, conformation changes of protein molecules that affect DNA binding activity of transcription factors. Egr-1 is an inducible factor that does not occur in normal colonic mucosa, but it is activated by an oxidative stress. Egr-1 can activate expression of many angiogenic factors (bFGF, PDGF-A, PDGF-B, VEGF, VEGFR-1, angiopoentin-1, proteases), as well as pro-inflammatory mediators (ICAM-1, VCAM-1, TNF-α, IL-1β, IL-2, monocyte chemotactic protein-1, tissue factor, GM-CSF) by interaction with the proximal promoter region of gene or by protein-protein interaction with other transcription factors [7, 14].

The transcription factor Sp-1 plays an important role in the regulation of cellular processes such as metabolism, growth, differentiation, apoptosis and angiogenesis. It is involved in regulation of expression of the housekeeping genes, genes that direct development of erythroid, lymphoid and monocytic lineages. Sp-1 modulates gene transcription of tissue plasminogen activator which depends on the retinoic acid and cAMP. A protective role of Sp-1 was found during oxidative stress in brain neurons [9, 14].

The regulation of gene expression by Egr-1 and Sp-1 transcription factors showed an interaction between them, since DNA-binding domains of these factors overlap [14, 17]. We [14], and others [4, 16], showed that Egr-1 and Sp-1 play an important role in pathogenesis of the inflammatory bowel diseases.

In present study, we showed an increase in the levels of Egr-1 and Sp-1 in rat colonic mucosa after administration of the ceftriaxone. The levels of Egr-1 were increased by 1.7-fold (Fig. 2, *A*), while Sp1 – 1.6-fold (P < 0.05 *vs.* water-treated group) (Fig. 2, *B*).

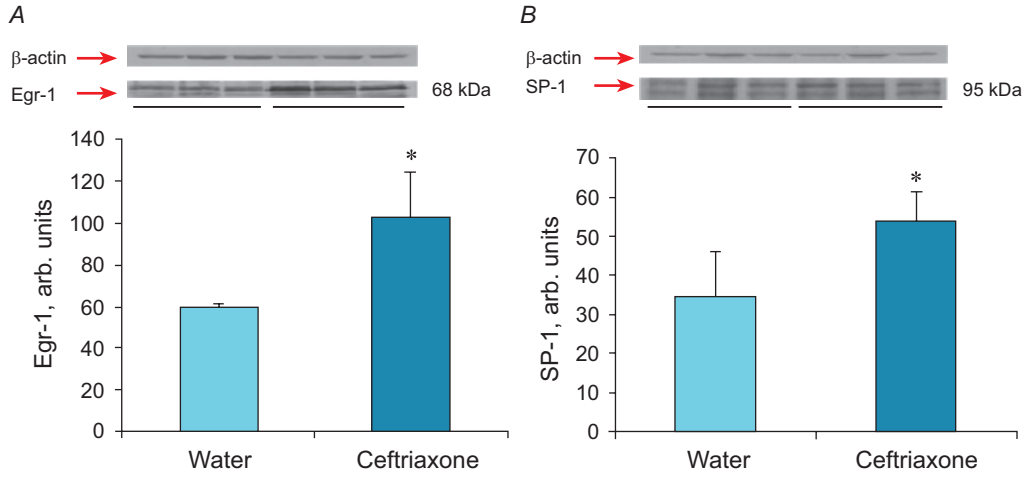

Fig. 2. Expression of redox-sensitive transcription factors Egr-1 (*A*) and Sp-1 (*B*) in rat colonic mucosa after 5 days administration of ceftriaxone (50 mg/kg, i.m.). Western-blot analysis, n = 3; M±SD; * P < 0.05 *vs.* control group (Water)

Рис. 2. Експресія редокс-чутливих транскрипційних факторів Egr-1 (*A*) і Sp-1 (*B*) у слизовій оболонці товстої кишки щурів після 5-денного введення цефтріаксону (50 мг/кг, в/м). Вестерн-блот аналіз, n = 3; M±SD; * P < 0,05 щодо показників у контрольній групі (Вода)

Considering a fact that an increase levels of Egr-1 and Sp-1 can be an early sign of the development of inflammatory bowel disease, our findings may indicate their involvement in the protective mechanisms in a response to side effects of the ceftriaxone.

CONCLUSIONS

Systemic administration of the cephalosporin antibiotic ceftriaxone leads to morphological changes in rat colonic mucosa whose nature indicates the initial stages of the acute inflammation. These changes are accompanied by a decrease in activity of the antioxidant enzymes in the colonic mucosa, and increase in the levels of redox-sensitive transcription factors Egr-1 and Sp1, that shows a disturbance in homeostasis of the intestinal barrier.

1. *Hooper L., Stappenbeck T., Hong C., Gordon J.* Angiogenins: a new class of microbicidal proteins involved in innate immunity. **Nature Immunology**, 2003; 4(3): 269–273.
2. *Hviid A., Svanstrцm H., Frisch M.* Antibiotic use and inflammatory bowel diseases in childhood. **Gut**, 2011; 60: 49–54.
3. *Kaser A., Niederreiter L., Blumberg R.* Genetically determined epithelial dysfunction and its consequences for microflora-host interactions. **Cellular and Molecular Life Sciences**, 2011; 68(22): 3643–3649.
4. *Keates A.C., Keates S., Kwon J.H., Arseneau K.O.* et al. ZBP-89, Sp1, and nuclear factor-kappa B regulate epithelial neutrophil-activating peptide-78 gene expression in Caco-2 human colonic epithelial cells. **The Journal of Biological Chemistry,** 2001; 276(47): 43713–43722.
5. *Lakatos L., Kiss L.S., David G.* et al. Incidence, disease phenotype at diagnosis, and early disease course in inflammatory bowel diseases in Western Hungary, 2002-2006. **Inflammatory Bowel Diseases**, 2011; 17(12): 2558–2565.
6. *Latella G., Fiocchi C., Caprili R.* News from the ”5th International Meeting on Inflammatory Bowel Diseases” CAPRI 2010. **Journal of Crohn’s and Colitis,** 2010; 4(6): 690–702.
7. *Pagel J., Deindl E.* Disease progression mediated by Egr-1 associated signaling in response to oxidative stress. **International Journal of Molecular Sciences**, 2012; 13(10): 13104–13117.
8. *Rush G.F., Heim R.A., Ponsler G.D., Engelhardt J.* Cephaloridine-induced renal pathological and biochemical changes in female rabbits and isolated proximal tubules in suspension. **Toxicologic Pathology**, 1992; 20(2): 156–168.
9. *Ryu H., Lee J., Zaman K.* et al. Sp1 and Sp3 are oxidative stress-inducible, antideath transcription factors in cortical neurons. **The Journal of Neuroscience,** 2003; 23(9): 3597–3606.
10. *Sekirov I., Russell S., Antunes L., Finlay B.* Gut microbiota in health and disease. **Physiological Reviews,** 2010; 90(3): 859–904.
11. *Sharma R., Young C., Neu J.* Molecular modulation of intestinal epithelial barrier: contribution of microbiota. **Journal of Biomedicine and Biotechnology**, 2010; 2010: 1–15.
12. *Shaw S., Blanchard J., Bernstein C.* Association between the use of antibiotics and new diagnoses of Crohn’s disease and ulcerative colitis. **The American Journal of Gastroenterology,** 2011; 106(12): 2133–2142.
13. *Suzuki Y, Sudo J.* Lipid peroxidation and generations of oxygen radicals induced by cephaloridine in renal cortical microsomes of rats. **Japan Journal Pharmacology,** 1990; 52(2): 233–243.
14. *Tolstanova G., Ostaptchenko L.* Interaction of transcription factors Egr-1 and Sp-1 in pathogenesis of inflammatory bowels diseases. **Medical Chemistry**, 2010; 12(6): 23–27. (In Ukrainian).
15. *Wittchen E., Haskins J., Stevenson B.* Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. **The Journal of Biological Chemistry**, 1999; 274(49): 35179–35185.
16. *Yu W., Lin Z., Hegarty J.P., Chen X.* et al. Genes differentially regulated by NKX2-3 in B cells between ulcerative colitis and Crohn’s disease patients and possible involvement of EGR1. **Inflammation**, 2012; 35(3): 889–899.
17. *Zhang X., Liu Y.* Suppression of HGF receptor gene expression by oxidative stress is mediated through the interplay between Sp1 and Egr-1. **American Journal of Physiology. Renal Physiology,** 2003; 284(6): 1216–1225.

ПОРУШЕННЯ ОКСИДАНТНО-АНТИОКСИДАНТНОГО БАЛАНСУ В СЛИЗОВІЙ ОБОЛОНЦІ ТОВСТОЇ КИШКИ ЩУРІВ УНАСЛІДОК АНТИБІОТИКОТЕРАПІЇ

Ю. В. Голота, О. П. Тяпко, Т. В. Довбинчук, Г. М. Толстанова

Київський національний університет імені Тараса Шевченка
Навчально-науковий центр "Інститут біології", вул. Володимирська, 60, Київ 01033, Україна
e-mail: gtolstanova@gmail.com

Антибіотикотерапія підвищує сприйнятливість до розвитку запальних захворювань кишечника (ЗЗК) у дітей і дорослих. Оксидативний стрес відіграє важливу роль у патогенезі ЗЗК. Метою цього дослідження було з'ясувати взаємозв'язок між морфологічними змінами у слизовій оболонці товстої кишки щурів, рівнем ферментів антиоксидантної системи та редокс-чутливих транскрипційних факторів Egr-1 і Sp-1 після введення антибіотика широкого спектра дії, цефтріаксону (Цф). Дослідження проведено на щурах-самцях лінії Вістар (180–230 г). Цф (50 мг/кг, в/м) вводили щоденно упродовж 5 днів. Активність каталази і супероксиддисмутази (СОД) у товстій кишці щурів визначали колориметрично та методом зимографії; рівні Egr-1 і Sp-1 методом Вестерн-блот аналізу; гістологічні зміни оцінювали морфометрично. Проводили моніторинг маси тіла тварин і наявності діареї. Системне введення цефтріаксону призводило до морфологічних і функціональних змін у слизовій оболонці товстої кишки щурів, асоційованих з початковими стадіями гострого запалення. Ці зміни супроводжувалися зниженням активності ензимів антиоксидантної системи супероксиддисмутази і каталази. Рівні редокс-чутливих транскрипційних факторів Egr-1 і Sp1 були значно вищими, що свідчить про порушення гомеостазу кишкового бар'єра.

Ключові слова: цефтріаксон, товста кишка, антиоксиданти, транскрипційні фактори.

НАРУШЕНИЕ ОКСИДАНТНО-АНТИОКСИДАНТНОГО БАЛАНСА В СЛИЗИСТОЙ ОБОЛОЧКЕ ТОЛСТОЙ КИШКИ КРЫС ВСЛЕДСТВИЕ АНТИБИОТИКОТЕРАПИИ

Ю. В. Голота, А. П. Тяпко, Т. В. Довбынчук, А. Н. Толстанова

Киевский национальный университет имени Тараса Шевченко
Учебно-научный центр "Институт биологии", ул. Владимирская, 60, Киев 01033, Украина
e-mail: gtolstanova@gmail.com

Антибиотикотерапия повышает восприимчивость к развитию воспалительных заболеваний кишечника (ВЗК) у детей и взрослых. Оксидативный стресс играет важную роль в патогенезе ВЗК. Целью данного исследования было выяснить взаимосвязь между морфологическими изменениями в слизистой оболочке толстой кишки крыс, уровнем ферментов антиоксидантной системы и редокс-чувствительных транскрипционных факторов Egr-1 и Sp-1 после введения антибиотика широкого спектра действия, цефтриаксона (Цф). Исследование проведено на крысах-самцах линии Вистар (180–230 г). Цф (50 мг/кг, в/м) вводили ежедневно в течение 5 дней. Активность каталазы и супероксиддисмутазы

(СОД) в толстой кишке крыс определяли колориметрически и методом зимографии; уровень Egr-1 и Sp-1 методом Вестерн-блот анализа; гистологические изменения оценивали морфометрически. Проводили мониторинг массы тела животных и наличия диареи. Системное введение цефтриаксона приводило к морфологическим и функциональным изменениям в слизистой оболочке толстой кишки крыс, ассоциированным с начальными стадиями острого воспаления. Эти изменения сопровождались снижением активности энзимов антиоксидантной системы супероксиддисмутазы и каталазы. Уровни редокс-чувствительных транскрипционных факторов Egr-1 и Sp1 были значительно выше, что свидетельствует о нарушении гомеостаза кишечного барьера.

Ключевые слова: цефтриаксон, толстая кишка, антиоксиданты, транскрипционные факторы.

Одержано: 16.11.2015