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COMPARISON OF LONG-TERM EFFECT OF TWO DYSBIOSIS MODELS IN WISTAR RATS

Aim. *To compare the changes of intestinal microbial composition of two models of dysbiosis induced by ceftriaxone (Cf) or the mix of ampicillin (Amp) and metronidazole (Met).* ***Methods.*** *The object of the study was the changes of fecal and mucosa-associated microbiota of colon and small intestine of Wistar male rats (m=170–200g, n=19). Cf was injected once a day for 14 days at a dose of 300 mg/kg i.m. The mix of Amp (75 mg/kg) and Met (50 mg/kg) was injected once a day for 3 days per os. Animals were removed from the experiment in 1 day and 56th days after treatment withdrawal. The microbiological analysis of the fecal (CFU/g) and mucosa-associated (CFU/cm^2) biotopes of the rats were carried out bacteriologically by sowing the dilutions into the selective diagnostic media for Bifidobacterium, Lactobacillus, Clostridium, E. coli, opportunistic enterobacteria, Staphylococcus and hemolytic bacteria.* ***Results.*** *It was shown that the changes of fecal microbiota after Cf withdrawal progressed with time and continued until the 56th day of observation. Cf administration induced 2 folds decrease the number of colon mucosa-associated anaerobic bacteria Bifidobacterium and Lactobacillus. Moreover, there was over growth of bacteria in the small intestine in 56 days. The mix of Amp/Met induced dysbiosis on the 1st day after the mix withdrawal. The normalization of the colon microbial composition was observed in 56 days.. The mix of Amp/Met increased only quantity of opportunistic enterobacteria and lactose-positive E.coli in mucosa-associated microbiota of small intestine in 56 days after the mix withdrawal.* ***Conclusions.*** *Injecting of the mix of Amp/Met to rats is more adequate model for modelling acute dysbiosis. Cf use induced long-term profound changes in microbiota composition and might be suitable to model chronic dysbiosis.*

Key words: microbiota, antibiotics, long-term effect, dysbiosis.

There is till to 3–30% of the patients with adverse effects during or after antibiotics treatment. The most common are allergic reactions and the digestive disorders. Dysfunctions of the gastrointestinal tract are especially often observed in children and elderly [2].

The antibiotic-associated dysbiosis was found in 5–30% of the patients, during or immediately after antibiotic therapy [8]. The classic example of the consequence of dysbiosis is antibiotic-associated diarrhea occurs in approximately 9–43% of the patients, treated with cephalosporins of the II and III generations,

20–30% – clindamycin, 23–71% – amoxicillin/clavulanate, 11% of those, treated with other broad spectrum penicillium and 8–16% – macrolides [9].

Antibiotic treatment often reduced metabolic activity of human microbiome and changed the spectrum of microorganisms. These changes cannot even revert to the initial state in several weeks [10] or even in years after antibiotic therapy [7]. Intravenous use of antibiotic ciprofloxacin increased the numbers of intestinal Gram-positive aerobic bacteria and orally use clindamycin induced the shift in microbial population at the genus level up to 12 months of the experiment. Vancomycin administration for 2 weeks orally altered the fecal microbiota structure observed even in 22 weeks [13].

It is important to note that the method and frequency of antibiotic use do not affect the risk of developing of antibiotic-associated complications. But there are no comprehensive comparative data on the long-term changes in the intestinal microbiota after different treatment regime and different antibacterial agents.

Thus, the aim of the study was to compare the changes of intestinal microbial composition of two models induced by ceftriaxone or the mix of ampicillin and metronidazole.

In present study the dysbiosis was induced by 14 days treatment with beta-lactam cephalosporin antibiotic ceftriaxone. Ceftriaxone is one of the most widely used antibiotic that inhibits the synthesis of bacterial cell wall and effects aerobic bacteria *Streptococcus spp., Enterobacter spp., Escherichia coli* and anaerobic – *Bacteroides spp., Clostridium spp.* [15]. This antibiotic is the first on the list according to Pharmacovigilance of Ukraine for 2011 extend of side effects on gastrointestinal tract [5].

Another used-model of dysbiosis is commonly accepted. Simultaneous injection of ampicillin with metronidazole for 3 days *per os* [4]. Antibiotic ampicillin is active against many aerobic Gram-positive (α- and β-hemolytic *Streptococcus, Staphylococcus spp.,*) and Gram-negative bacteria *(Salmonella spp., Shigella spp., Escherichia coli, Neisseria meningitidis).* Metronidazole is one of the main drugs for the treatment of anaerobic infections caused by *Bacteroides spp., Fusobacterium spp., Clostridium spp.* In medical practice this composition is used in obstetric practice in purulent inflammatory diseases where the antibiotics effected aerobic and anaerobic bacteria, obtained bactericidal effect and have a narrow spectrum of action to minimize the effect on the normobiota of intestine are required [6].

Materials and methods

The experimental studies were carried out on Wistar male rats (170–200 g, n=19). All manipulation were carried out in accordance with the rules of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986). All animals were kept under at standard conditions: temperature (22 ºC), lighting (a cycle of 12h of light and 12h of darkness), humidity (30–35%) and diet (complete feed for laboratory animals K-12-4) and water *ad libitum.*

The object of the study was the changes of fecal and mucosa-associated microbiota of the colon and small intestine of rats.

The animals were divided into groups: I group (n=5) – control group (Cf)

was injected once a day for 14 days with 0.1 ml of saline intramuscularly (i.m.); II group (n=5) – the antibiotic Cf (PJSC "Kievmedpreparat", Ukraine) was injected once a day for 14 days, at a dose of 300 mg/kg *(i.m.)*; III group (n=4) – control group (Amp/Met) was gavaged once a day for 3 days with 1 ml of saline *per os;* IV group (n=5) – the mix of Amp (Kyivmedpreparat, Ukraine) at a dose of 75 mg/kg and Met (Ph.C. "Zdorovya", Ukraine) at a dose of 50 mg/kg was gavaged once a day for 3 days [3]. The 1st day of treatment was the 1st day of experiment. The feces for the bacteriological analysis were collected in one and 56 days after antibiotic agents withdrawal. The animals were removed from the experiment in 56 days after treatment withdrawal by CO_2 inhalation followed by cervical dislocation. All the experiments were repeated twice.

During autopsy 1 cm^2 of colon (2 cm from the anus) and 1 cm^2 of small intestine (2 cm from the ileocecal valve) were collected for further bacteriological analysis of the mucosa-associated microbiota as it was described previously [12].

The quantitative and qualitative changes of the intestinal microbiota composition were identified on selective diagnostic media: Bifidobacterium Agar; MRS agar; Endo; Iron Sulphite Agar; Mannitol Salt Agar, Simmons Citrate Agar; Blood Agar Base (HiMedia Laboratories Pvt. Ltd., India) which were cooled to 45–50 °C and aseptically added 5% sterile sheep's blood. Inoculated media were incubated thermostatically at 37 °C for 24–48 h. Bergey's Manual of determinative Bacteriology was used for bacteria identification. Identification was carried out according to morphological and tinctorial characteristics (reaction to plasma coagulation, DNA activity, lysozyme and phosphatase production, sensitivity to novobicin – to differ *S.aureus, S. epidermidis* from *S. saprophyticus;* oxidase test, carbohydrate fermentation tests, Voges-Proskauer's reaction, mobility test, formation of hydrogen sulphide – to differ lactose-negative *E. coli* from opportunistic enterobacteria). The results are presented as M±m lg CFU/g and M±m lg CFU/cm^2.

The statistical analysis of the results were performed using *Excel* and *STATISTICA* 8. We evaluated the differences between control and experimental groups using the Student t-test for independent samples. The P-value < 0.05 was considered statistically significant.

Results and discussion

Treatment with ceftriaxone or the mix of Amp/Met did not significantly effect anaerobic saccharolytic bacteria *(Bifidobacterium* and *Lactobacillus)* isolated from the fecal biopsy (Table 1 and 2).

There were recorded the significant increase of the number of *Clostridium* genus representatives in 56 days after ceftriaxone withdrawal (table 1). Casey M. Theriot et al [15] have shown that treatment with antibiotic cefperazone changed the structure and functions of the intestinal microbiota in mice. These changes were accompanied by elevated levels of primary bile acids and carbohydrates that possibly contributed to the growth of *C. difficile in vitro* and *ex vivo* via 6-weeks after the antibiotic withdrawal. Today it is considered that antibiotics decrease resistance to *C. difficile* colonization that causes 10–30% antibiotic-associated dysbiosis [4].

Table 1

Quantitative and qualitative (M±m lg CFU/g) changes of fecal microbiota after ceftriaxone withdrawal

Group of microorganisms	Control n=5	The first day after Cf withdrawal n=5	The 56th day after Cf withdrawal n=5
Bifidobacterium spp.	8.86 ± 0.24	8.77 ± 0.24	8.80 ± 0.15
Lactobacillus spp.	8.79 ± 0.16	8.18 ± 0.48	9.00 ± 0.21
Clostridium spp.	1.94 ± 0.06	1.74 ± 0.19	3.18 ± 0.48*
Escherichia coli lactose-positive	5.21 ± 0.27	0	7.04 ± 0.51*
Escherichia coli lactose-negative	1.72 ± 0.85	2.22 ± 0.92	4.12 ± 0.78*
Opportunistic enterobacteria	1.08 ± 0.44	4.10 ± 0.13*	6.02 ± 0.68*
Staphylococcus aureus	6.41 ± 0.07	3.95 ± 0.67*	6.57 ± 0.48
Staphylococcus spp.	1.02 ± 0.08	4.68 ± 0.58*	6.69 ± 0.26*
Hemolytic bacteria	4.23 ± 0.23	7.63 ± 0.55*	6.34 ± 0.48*

*P<0.05 compared to the control group

In our study, lactose-positive *E.coli* disappeared from fecal microbiota on the first day after ceftriaxone withdrawal and increased from 5.21±0.21 to 7.04±0.51 lg CFU/g in comparison with control value in 56 days. Lactose-negative *E. coli* and opportunistic enterobacteria increased gradually both straight after discontinuation of the antibiotic and in 56 days.

On the 1st day after the mix of ampicillin and metronidazole withdrawal there were observed the increasing of lactose-positive *E. coli* in fecal microbiota. In 56 days their growth rates were within the control values. Besides, on the first day after the mix withdrawal, there were observed increasing of the lactose-negative *E. coli* (from zero to 4.05±3.07 lg CFU/g) and opportunistic enterobacteria from 0.75±0.33 to 4.00±0.00 lg CFU/g. However, these changes were decreased twice *vs.* the first day after antibiotic agents withdrawal (table 2).

Usually the increase in the population level of *Escherichia* is associated with the decrease of anaerobic saccharolytic bacteria and insufficiency of immune protection. But in our study *Bifidobacterium spp.* and *Lactobacillus spp.* were not changed that represents the first level of dysbiosis according to the classification of the Ministry of Health of Ukraine from 01.05.1996 No.4.

There were observed the increased growth of *Staphylococcus* genus and hemolytic bacteria in 8 weeks after ceftriaxone withdrawal.

Thus, the changes of fecal microbiota after ceftriaxone withdrawal progressed over time, but the mix of ampicillin and metronidazole caused changes on the first day after its withdrawal, they disappeared on their own in 8 weeks.

Zaharova et al [16] suppose that antibiotic-associated dysbiosis that developes in the first days after antibiotic using, more likely associated with the direct effect

of antibiotic on the intestinal microbiota with impaired synthesis of short-chain fatty acids (SCFA) and decreasing in fluid absorption in the lumen of the intestine and increasing its motility. The long-lasted changes of microbiota in the patients after antibiotic using is associated with greater likelihood of infection factors.

Table 2

Quantitative and qualitative (M±m lg CFU/g) changes of fecal microbiota after Amp/Met withdrawal

Group of microorganisms	Control n = 4	The first day after Amp/Met withdrawal n = 5	The 56th day after Amp/Met withdrawal n=5
Bifidobacterium spp.	7.79±0.71	8.99±0.31	8.65±0.62
Lactobacillus spp.	7.75±0.43	8.75±0.06	7.92±0.44
Clostridium spp.	2.42±0.43	3.17±0.23	2.95±0.00
Escherichia coli lactose-positive	6.20±1.79	8.45±0.43*	5.99±0.72
Escherichia coli lactose-negative	0	4.05±3.07*	0
Opportunistic enterobacteria	0.75±0.33	4.00±0.00*	2.16±0.23
Staphylococcus aureus	4.21±0.32	4.28±2.76	4.77±0.33
Staphylococcus spp.	2.3±1.74	4.39±2.91	4.26±0.20
Hemolytic bacteria	0	3.85±2.91*	2.77±1.96

*P<0.05 compared to the control group

The mucosa-associated microbiota has better sustainability and represents overall homeostasis of bacteria. In our study the colon mucosa-associated microbiota showed the significant decreasing in the number of *Bifidobacterium spp.* and *Lactobacillus spp.* in 56 days after ceftriaxone withdrawal (table 3). The quantitative changes of anaerobic saccharolytic bacteria were not noted after the discontinuation of the mix of ampicillin and metronidazole use (table 4).

There was a significant increasing of *Clostridiums spp.* in the mucosa-associated microbiota of colon in 56 days after ceftriaxone withdrawal. *Clostridium* were not found in the colon mucosa-associated microbiota after the mix of ampicillin and metronidazole.

Lactose-positive and lactose-negative *E. coli* disappeared in the colon mucosa-associated microbiota after ceftriaxone withdrawal, but the number of opportunistic enterobacteria increased to 10^6 CFU/cm^2. It indicates the dumping of epithelial cells with bacteria in the cavity of the colon and an increasing number of these bacteria in fecal microbiota (table 1). After treatment with the mix of ampicillin and metronidazole, these bacteria were not found in the mucosa-associated intestinal microbiota (table 4).

The quantity of *S.aureus* in the colon mucosa-associated microbiota was also

Table 3

Quantitative and qualitative (M±m lg CFU/cm^2) changes of intestine mucosa-associated microbiota after ceftriaxone withdrawal

Group of microorganisms	Microbiota of colon		Microbiota of small intestine	
	Control n=5	56th day n=5	Control n=5	56th day n=5
Bifidobacterium spp.	6.40±0.20	4.80±0.20*	5.00±0.50	7.20±0.20
Lactobacillus spp.	4.90±0.10	2.90±0.20*	5.00±0.20	6.00±0.10
Clostridium spp.	0.50±0.20	3.10±0.10*	0	0
Escherichia coli lactose-positive	2.00±0.10	0*	0.80±0.20	0*
Escherichia coli lactose-negative	1.60±0.20	0*	0.50±0.30	0.30±0.30
Opportunistic enterobacteria	0	5.00±0.00*	0	2.10±0.10*
Staphylococcus aureus	0.50±0.20	3.10±0.10*	0	2.00±0.10*
Staphylococcus spp.	0.50±0.20	2.10±0.10	0	2.50±0.50*

*P<0.05 compared to the control group

Table 4

Quantitative and qualitative (M±m lg CFU/cm^2) changes of intestine mucosa-associated microbiota after Amp/Met withdrawa

Group of microorganisms	Microbiota of colon		Microbiota of small intestine	
	Control n=4	56th day n=5	Control n=4	56th day n=5
Bifidobacterium spp.	4.23±0.75	4.05±0.25	5.30±0.00	4.93±0.34
Lactobacillus spp.	3.87±0.42	3.90±0.14	5.17±0.78	5.42±0.08
Clostridium spp.	0	0	0	0
Escherichia coli lactose-positive	2.00±1.83	0*	1.00±0.41	3.21±2.32*
Escherichia coli lactose-negative	1.10±0.56	0*	0	0
Opportunistic enterobacteria	0	0	0	1.11±0.56*
Staphylococcus aureus	1.16±0.64	2.57±1.94	3.86±0.32	2.73±1.99
Staphylococcus spp.	2.33±1.70	2.83±2.01	3.95±0.62	3.83±0.62

*P<0.05 compared to the control group

high after ceftriaxone withdrawal.

Thus, the changes after ceftriaxone were more pronounced in the colon mucosa-associated microbiota than in the fecal microbiota and may lead to serious damage of the macroorganism's homeostasis.

The study of small intestine mucosa-associated microbiota showed a significant increase in anaerobic saccharolytic bacteria in 56 days of ceftriaxone

withdrawal (table 3), at a time when there is a decrease in these microorganisms in the colon. It can be characterized as a syndrome of small intestinal bacterial overgrowth and the associated process of bile acids premature deconjugation [1].

The quantity of *Clostridium, E.coli* lactose-positive and lactose-negative of the small intestine mucosa-associated microbiota were within reference values. Only opportunistic enterobacteria and *Staphylococcus* genus increased to lg 2.10±0.10 CFU/cm^2.

There were observed the significant changes only in quantity of *E.coli* lactose-positive and opportunistic enterobacteria in the small intestine mucosa-associated microbiota after the mix of ampicillin and metronidazole withdrawal.

There was no particular difference in the changes of bacterial species of fecal microbiota after the antibiotics agents withdrawal. But there was the difference in the mucosa-associated microbiota, especially over growth in the small intestine after ceftriaxone using in 56 days.

The microbiota changes of the gastrointestinal tract after ceftriaxone using was stable and affects almost all studied groups of microorganisms. The microbiota changes after the mix of ampicillin and metronidazole withdrawal normalized up to 8 weeks of the experiment. Thus, for the modeling of acute dysbiosis that develops immediately after antibiotic abolition, the model with ampicillin and metronidazole is more adequate. Otherwise, ceftriaxone might be suitable to model chronic dysbiosis.

Today, all data concerning the long-term effects of antibiotics were determined by fecal microbial profile by molecular methods (sequencing, qPCR) and no results concerning the long-term effect in the mucosa-associated microbiota [11]. And for the first time there were showed the changes in the different groups of bacteria in the fecal and mucosa-associated microbiota of the colon and small intestine by cultural methods in one day and 8 weeks after antibiotic agents withdrawal. And there were showed that the mucosa-associated microbiota after ceftriaxone treatment has bigger changes than after the mix ampicillin with metronidazole.

Furthermore, earlier it has been shown that the changes occurring after parenteral use of antibiotics are deeper than their orally use [10]. That we confirmed in our study too.

The question of the long-term consequences of antibiotic therapy, in particular their mechanisms and the role of the intestinal microbiota, is a topical issue of modern medicine and, accordingly biomedical research. The basis for elucidation of the mechanisms of long-term consequences is the answer to the question of which antibiotic drugs or their combination in a clinically relevant dose may cause long-term changes. And perhaps it will help to explaine experimental justification for the prescription of antibiotics.

Conflict of interest

The authors have no conflict of interest.

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ПОРІВНЯННЯ ДОВГОТРИВАЛОГО ЕФЕКТУ ДВОХ МОДЕЛЕЙ ДИСБІОЗУ У ЩУРІВ ЛІНІЇ WISTAR

Реферат

Мета. *Порівняти зміни кишкової мікробіоти двох моделей дисбіозу, які були викликані цефтриаксоном (Цф) або сумішшю ампіциліну (Амп) та метронідазолу (Мет).* ***Методи.*** *Об'єктом дослідження була зміна фекальної та пристінкової мікробіоти товстої та тонкої кишок щурів-самців лінії Wistar (m=170-200 г, n=19). Цф вводили раз на добу впродовж 14 днів у дозі 300 мг/кг в.м. Суміш Амп (75 мг/кг) з Мет (50 мг/кг) вводили per os раз на добу, впродовж 3 діб. Виведення тварин з експерименту здійснювали через 1 та 56 діб після відміни введення речовин. Мікробіологічний аналіз фекального (КУО/г) та пристінкового (КУО/см²) біотопу щурів здійснювали бактеріологічним шляхом при висіві розведень на елективно-діагностичні середовища для Bifidobacterium, Lactobacillus, Clostridium, E. coli, умовно-патогенних ентеробактерій, Staphylococcus та гемолітичних бактерій.* ***Результати.*** *Було показано, що зміни в фекальній мікробіоті після виведення Цф прогресували з часом і тривали до 56 доби спостереження. Введення Цф викликало зміни в кількості анаеробних бактерій Bifidobacterium і Lactobacillus вдвічі у просвітній мікробіоті товстої кишки. І спостерігався надмірний ріст бактерій в тонкому кишечнику через 56 діб. Суміш Амп/Мет індукувала дисбіоз одразу після відміни введення речовин. Нормалізація мікробіоти спостерігалася через 56 діб. Суміш Амп/Мет збільшувала лише кількість умовно-патогенних ентеробактерій і лактозо-позитивних E. coli в тонкому кишечнику через 56 діб після відміни введення речовин.* ***Висновки.*** *Введення суміші Амп/Мет щурам є більш адекватною моделлю для моделювання гострого дисбіозу. Введення Цф викликало глибокі довготривалі зміни і ця модель може бути придатною для моделювання хронічного дисбіозу.*

Ключові слова: мікробіота, антибіотики, віддалені наслідки, дисбіоз.

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СРАВНЕНИЕ ДОЛГОСРОЧНОГО ЭФФЕКТА ДВУХ МОДЕЛЕЙ ДИСБИОЗА У КРЫС ЛИНИИ WISTAR

Реферат

Цель. *Сравнить изменения кишечной микробиоты двух моделей дисбиоза, что были вызваны цефтриаксоном (Цф) или смесью ампициллина (Амп) с метронидазолом (Мет).* ***Методы.*** *Объектом исследования были изменения фекальной и пристеночной микробиоты толстой и тонкой кишок крыс-самцов линии Wistar (m=170-200 г, n=22). Цф вводили раз в сутки в течение 14 дней в дозе 300 мг/кг в.м. Коктейль Амп (75 мг/кг) с Мет (50 мг/кг) вводили per os раз в сутки, в течение 3 дней. Выводили животных из эксперимента через 1 и 56 дней после отмены введения веществ. Микробиологический анализ фекального (КОЕ/г) и пристеночного (КОЕ/см^2) биотопа крыс осуществляли бактериологическим путем при посеве разведений на элективно-диагностические среды к Bifidobacterium, Lactobacillus, Clostridium, E.coli, условно-патогенных энтеробактерий, Staphylococcus и гемолитических бактерий.* ***Результаты.*** *Показано, что изменения в фекальной микробиоте после вывода Цф прогрессировали со временем и продолжались до 56 дня наблюдения. Введение Цф вызвало изменения в количестве анаэробных бактерий Bifidobacterium и Lactobacillus вдвое в просветной микробиоте толстой кишки. Наблюдался чрезмерный рост бактерий в тонком кишечнике через 56 дней. Смесь Амп/Мет индуцировала дисбиоз сразу же после отмены введения веществ. Нормализация микробиоты наблюдалась через 56 дней. Смесь Амп/Мет увеличивала только количество условно-патогенных энтеробактерий и лактозо-положительных E. coli в тонком кишечнике через 56 дней после отмены веществ.* ***Выводы.*** *Введение смеси Амп/Мет крысам является более адекватной моделью для моделирования острого дисбиоза. Введение Цф вызвали глубокие долговременные изменения и эта модель может быть пригодной для моделирования хронического дисбиоза.*

Ключевые слова: микробиота, антибиотики, отдаленные последствия, дисбиоз.

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