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EXPERIMENTAL STUDY OF COLON PREEPITHELIAL MUCOUS LAYER MICROFLORA IN ALBINO RATS WITH INDUCED DIABETES MELLITUS

Key words: *aloxan-induced diabetes mellitus, colonization resistance, microflora.*

Abstract. The aim of research. *This experimental issue deals with microbiological investigation of quantitative and qualitative composition of microflora of pre-epithelium mucous layer of colon in albino rats with aloxan-induced diabetes mellitus.* ***Methods.*** *Case-control experimental study conducted on white 20 albino rats with weight from 200 to 220 g: 10 intact animals (control group) and 10 rats in basic group with aloxan-stimulated diabetes mellitus. The pieces of colon served as research material for microbiological investigation with obtaining pure cultures of microorganisms. Population level of microflora of pre-epithelium mucous layer of colon was displayed in logarithm of colony-forming units (lg CFU/g). Statistical analysis was performed by Student's t-test.* ***Results.*** *Aloxan-induced diabetes mellitus in experimental animals leads to abnormalities of quantitative composition – the rising of constancy index (CI) of species that related to additional and residual microbiota: E.coli Hly^+ was isolated with CI 30%, Clostridium – 70%, and Staphylococcus, Proteus, Peptococcus – 40%. Qualitative abnormalities include decrease of Bifidobacteria populational level – 5.60±0.29 vs 6.65±0.27 lgCFU/g ($p<0.05$), Bacteroides – 2.21±0.15 vs 6.50±0.18 lgCFU/g ($p<0.001$), E.coli – 2.25±0.16 vs 5.77±0.19 lgCFU/g ($p<0.001$) and Enterococcus 2.08±0.07 vs 6.17±0.22 lgCFU/g ($p<0.01$). Meanwhile Peptostreptococcus populational level had moderate increase to 4.13±0.19 vs 3.22±0.19 lgCFU/g ($p<0.05$).* ***Conclusions.*** *In basic group the pre-epithelial mucous layer of colon was contaminated with opportunistic pathogenic Peptococcus, Clostridium, Proteus, Staphylococcus. Autochtonous obligatory Bifidobacteria, Lactobacilli, Bacte*roides *remain dominant role, only* Eubacterium *was completely eliminated. It probably had great impact on the further weakness of colonization resistance of gut, accompanying diabetes in animal model.*

Background

In any biotope the highest concentration of microorganisms is present on a surface that divides the internal environment of human organism and external world. This testifies an active participation of indigenous microflora in protective reactions and regulation of interaction between host and external environment [5].

Any kinds of microorganisms, as free living so parietal, have immobilized life style. In natural conditions the majority of microorganisms is fixed to receptors of corresponding cells and creates on their surfaces microbial colonies. During formation of colonies, microorganisms produce the exo-polysaccharide glycocalyx, inside of which the growth and replication of bacteria takes place and they realize intercellular connections [3]. Multiple microbial populations of normal microflora are concentrated in special integral structure – pre-epithelial mucus layer (PML). PML of colon is the main reservoir of endogenous microflora in the human organism, which totally has up to 10^6 CFU (colony-forming units) of microorganisms [3, 6]. During the formation of PML separate autochthonous obligate bacterial cells by realizing of complicated mechanism of communicative interactions are specifically consolidated by adhesion and creating microcolonies aimed to serve as colonization resistance of intestinal mucous membrane, which belongs to nonspecific immune anti-infectious protective gut system [1, 8].

Assessment and characterization of gut microbiota become a significant research area in human diseases, including 1 & 2 types of diabetes mellitus, the most prevalent endocrine disease worldwide [2, 4, 9]. During the reviewing of current available scientific literature we didn't find the study aimed to analyze the PML microflora in animal model simulating diabetes mellitus.

Research purpose

To establish qualitative and quantitative composition of colon PML microflora in albino rats with experimental diabetes mellitus.

Material and methods

Experiments have been conducted on white outbreed albino rats with weights from 200 to 220 g. Animals were divided into two groups (basic group includes 10 albino rats with stimulated diabetes mellitus; control group includes 10 intact animals), all underwent quarantine for 10-14 days in vivarium [7]. Before the starting of investigation all rats were examined for presence of any pathology and reaction on visual and acoustic irritants was studied. Water has been given in unlimited quantity. The animals were fed once a day in the morning, with calorific value from 5.6 to 6.2 kJ per kg daily.

Modeling of diabetes mellitus was conducted by the single intraperitoneal administration of aloxan (100 mg/kg), followed by maintenance in stationary vivarium conditions for 20 days. The verification of aloxan-induced diabetes mellitus was performed by the monitoring of glucose level in the peripheral blood of animals. Inclusion criteria: glucose blood level exceeding 10 mmol/l (11,2±0,47 mmol/l) in comparison with intact animals less than 7 mmol/l (6,0±0,78 mmol/l). On the 20th day the animals withdrew from the experiment by decapitation during deep anesthesia, which achieved by the introduction of excessive amounts of sodium thiopental. All intervention and slaughter animals were conducted in compliance with the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1985) and "General Principles of animal experiments" approved by Fourth National Congress on Bioethics (Kyiv, 2010). The Commission on Biomedical Ethics of Bukovinian State Medical University (BSMU) didn't found any breaches of ethical standards during experimental study.

The pieces of colon with a size of 3 cm, which were taken in sterile conditions, served as research experimental material. They were separated from mesenterial layer by sterile scissors. Content was extracted from piece of colon with a forceps. After removing of that segment of colon it was cut along by sterile scissors. Seven times the segment was washed well in tape water for maximal clearance, and in the isotonic solution in Petri dish too. Colon sections were weighed on sterile wax paper; put it into the sterile porcelain mortar; with addition of isotonic solution in the tenfold volume, carefully grinded to getting of homogenous mass in dilution 1:10 (10^{-1}). From the homogenate of colon wall the row of tenfold serial dilution on the base of isotonic solution from 10^{-2} to 10^{-7} were prepared. Every time it was used a new sterile pipette. From each tube row by sterile micropipette were taken 0.1 ml of solution and applied to the corresponding solid nutrient medium optimal for each kind of microbe.

Cultures of facultative anaerobic and aerobic bacteria were cultured in incubator (37° C) for 24-48 hours. Cultures of obligate anaerobic bacteria were cultivated in stationary anaerostat "CO_2-Incubator T-125" (Sweden) for 5-7 days (to appearance of growth), sometimes up to 14 days. Then received single-type colonies were studied for each genus of the microbes. Pure culture were identified by genus (species) by morphological, tinctorial, cultural and biochemical properties.

Because the number of bacteria and yeast-fungi of the genus Candida per unit volume reaches millions and billions, for easier data presentation a quantity where calculated in logarithm of quantitative indicators of microflora (lg CFU/g).

Mathematic, statistical analysis of the results was performed by the method of variation statistics with the definition of average value, average error and probability of possible error by statistical Student's t-test.

Results and their discussion

One of the factors of nonspecific anti-infectious mucous membrane protection of conditionally open cavities is microflora of PML that has extraordinary heterogenic structure. That related to species and strain composition of mucosal microbiota, physiological status of epithelial cells, size and structure of spreading of microcolonies, physical composition and viscosity of matrix, immunoglobulin's content in it, especially secretory immunoglobulin A (sIgA), immunocompetent cells and other components of mucus, its quality, status of biological layer etc.

As initial stage of identification of colonization resistance of mucous membrane of colon in experimental animals with diabetes mellitus, the qualitative composition of PML gut microbiota in rats both (basic and control) groups was studied (table 1).

Colonization resistance of PML in intact (control) albino rats was formed by mucosal microflora that consist of autochthonous obligate anaerobic bacteria of genera *Bifidobacterium, Lactobacillus, Bacteroides* and facultative anaerobic bacteria of genera *Escherichia*, *Enterococcus.* They related to main (dominant) microflora and are constant for this biotope. Rarely *Eubacterium* and *Peptostreptococcus* occur on a gut mucous layer of albino rats.

Development of diabetes mellitus in experimental animals leads to abnormalities of quantitative composition of microflora: arising of CI (constancy index) and FOR (frequency of occurrence rate) of

Table 1

Qualitative composition of microbiota of pre-epithelial mucous layer of colon in albino rats with experimental (aloxan) diabetes mellitus

Microorganisms	Basic group (n=10)			Control group (n=10)		
	Isolated strains	CI	FOR	Isolated strains	CI	FOR
Bifidobacterium spp.	10	100,0	0,14	9	90,0	0,16
Lactobacillus spp.	10	100,0	0,14	10	100,0	0,18
Eubacterium spp.	0	-	-	2	20,0	0,04
Bacteroides spp.	10	100,0	0,14	10	100,0	0,18
Peptostreptococcus spp.	6	60,0	0,08	2	20,0	0,04
Peptococcus niger	4	40,0	0,06	0	-	-
Clostridiums pp.	7	70,0	0,10	0	-	-
E. coli	10	100,0	0,14	10	100,0	0,18
E. coli Hly⁻	3	30,0	0,04	0	-	-
Proteus spp.	4	40,0	0,06	0	-	-
Enterococcus spp.	3	30,0	0,04	7	70,0	0,14
Staphylococcus	4	40,0	0,06	0	-	-

Note. CI – constancy index; FOR – frequency of occurrence rate

Table 2

Population level of microbiota of pre-epithelial mucous layer of colon in albino rats with experimental (aloxan) diabetes mellitus

Microorganisms	Basic group (n=10)			Control group (n=10)		
	lg CFU/g	QDC	SC	lg CFU/g	QDC	SC
Bifidobacterium spp.	5,60±0,29	185,40	0,19	6,65±0,27*	103,40	0,18
Lactobacillus spp.	6,60±0,21	218,54	0,22	6,83±0,14	118,00	0,21
Eubacterium spp.	0	-	-	5,34±0,27	18,40	0,04
Bacteroides spp.	2,21±0,15	73,18	0,07	6,50±0,18***	113,80	0,20
Peptostreptococcus spp.	4,13±0,19	80,45	0,11	3,22±0,19*	11,10	0,02
Peptococcus niger	1,84±0,09	23,90	0,04	0	-	-
Clostridiums pp.	1,85±0,11	42,05	0,06	0	-	-
E. coli	2,25±0,16	73,05	0,10	5,77±0,19***	99,70	0,18
E. coli Hly+	1,66±0,05	16,17	0,02	0	-	-
Proteus spp.	3,75±0,14	48,70	0,07	0	-	-
Enterococcus spp.	2,08±0,07	20,26	0,03	6,17±0,22**	74,60	0,15
Staphylococcus spp.	1,89±0,05	24,55	0,04	0	-	-

Note. * – the statistical degree of evidence corresponds $p<0.05$; ** – the statistical degree of evidence corresponds $p<0.01$; *** – the statistical degree of evidence corresponds $p<0.001$; QDC – quantitative dominancy coefficient; SC – significance coefficient

microbial species that related to additional and residual microbiota of colon PLM. The contamination of this biotope with opportunistic pathogenic bacteria of genera *Peptococcus, Clostridium, Proteus, Staphylococcus* etc. is accompanied with above mentioned process. Simultaneously the quantitative composition of main microflora including mucous membrane remains stable. Thus autochthonous obligate anaerobic bacteria of genera *Bifidobacterium, Lactobacillus, Bacteroides, Peptococcus* and facultative anaerobic bacteria of *Escherichia* genus are related to constant and represent dominant PML of colon microflora. But *Enterococcus* was identified only in 1/3 of animals with diabetes mellitus. Also in PML of colon of albino rats with experimental endocrine disorder the contamination of this biotope with pathogenic (enterotoxigenic *Escherichia*) and opportunistic pathogenic *Enterobacteria, Proteus, Staphylococcus, Peptococcus* and bacteria of genus *Clostridium* was identified.

Next research stage was purposed to make assessment the qualitative composition of PML colon microflora in albino rats with experimental diabetes mellitus; results are represented in table 2.

In intact albino rats according to population level, quantitative dominance coefficient (QDC) and significance coefficient (SC) colon PML microflora are presented by autochthonous obligate anaerobic

bacteria of genera *Lactobacillus, Bifidobacterium, Bacteroides, Peptostreptococcus* and facultative anaerobic bacteria of genera *Escherichia* and *Enterococcus.* Role of other (bacteria of genus *Eubacterium*) is minimal. Also opportunistic pathogenic *Peptococcus, Clostridium, Proteus, Staphylococcus* and enterotoxigenic *Escherichiae* were not obtained from this biotope even in minimal quantity from any animal.

In experimental diabetes mellitus of albino rats the quantity of autochthonous obligate anaerobic bacteria of genus *Lactobacillus* practically was not changed and they become dominant in colon PML. Quantity of others (bacteria of genera *Bifidobacterium, Bacteroides, Escherichiae, Enterococcus*) considerably ($p<0,05$-$0,001$) was decreased and their role in microbiocenosis of colon PML was reduced. Despite of the deep changes of quantitative composition of different bacterial taxonomic groups, autochthonous obligate anaerobic microbes of genera *Lactobacillus, Bifidobacterium* and *Peptostreptococcus* remain dominant.

So, opportunistic pathogenic bacteria of genera *Escherichia, Proteus, Staphylococcus, Peptococcus, Clostridium* were identified in minimal quantity, and by the QDC and SC data these microorganisms do not play important role in microbiocenosis of colon PML in animals with experimental diabetes mellitus. This status in microflora has been created by autochthonous obligate anaerobic bacteria, which displayed significant inhibitive activity against pathogenic and opportunistic pathogenic *Enterobacteria* and other microbes. Previously mentioned proves the stability of the colonization resistance of mucous layer of colon in albino rats with experimental diabetes mellitus on the corresponding level.

Conclusions

1. Formation of experimental diabetes mellitus in albino rats is accompanied with changes of qualitative composition only of additional and residual colon PML microbiota by the contamination of it in some animals (20-70%) with bacteria of genera *Peptococcus, Clostridium, Proteus, Staphylococcus* etc. Dominant microbiota of this biotope saves the stability of its qualitative composition.

2. In experimental diabetes mellitus in PML of colon is registered an expressed deficiency of genera *Bifidobacterium, Bacteroides, Escherichia, Enterococcus.*

3. By quantitative dominance coefficient (QDC), significance coefficient (SC) and constancy index (CI) autochthonous obligate anaerobic bacteria of genera *Bifidobacterium, Lactobacillus , Peptostreptococcus* genera remain dominant in PML. The colonization resistance of mucous layer of colon formed by these microbes supports inhibition of growth and multiplication of pathogenic and opportunistic pathogenic microorganisms of genera *Escherichia, Proteus, Staphylococcus, Peptococcus, Clostridium,* which are identified in this biotope in minimal quantities.

Further investigations prospects

Received results can become a base for the further investigation of microflora of distal part of ileum in animals with experimental diabetes mellitus.

References. 1.Qin J., Li Y., Cai Z., Li S., Zhu J., Zhang F., Liang S., Zhang W., Guan Y., Shen D. *Nature*, 2012, no. 490 (7418), pp. 55-60. 2.Atkinson M.A., Chervonsky A. *Diabetologia*, 2012, no.55(11), pp. 2868-2877. 3.Burcelin R. *Physiology*, 2012, no. 27 (5), pp. 300-307. 4.De Goffau D.C., Luopajdrvi K., Knip M., Ilonen J. *Diabetes*, 2012, no. 28, pp. 356-370. 5.Ibrahim M., Anishetty S. *Biochem. biophys. res. Commun,* 2012, no. 428 (2), pp. 278-284. 6.Ivanov I., Honda K. *Cell host microbe*, 2012, no. 12 (4), pp. 496-508. 7.Lolas F. *Biol. res*., 2008, no. 41 (1), pp. 119-123. 8.Hara N., Alkanani A., Ir D., Robertson C. *Clin. immunol*., 2012, no. 146 (2), pp. 112-119. 9.Sydorchuk L.I., Sydorchuk A.S. *Pivdenno ukrains'kyi medychnyi naukovyi zhurnal*, 2013, no. 1(01), pp. 147-151.

ЭКПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ МИКРОФЛОРЫ ПРИЭПИТЕЛИАЛЬНОЙ СЛИЗИСТОЙ ОБОЛОЧКИ КИШЕЧНИКА У БЕЛЫХ КРЫС С ИНДУЦИРОВАННЫМ САХАРНЫМ ДИАБЕТОМ

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Резюме. Статья посвящена исследованию количественного и качественного состава микрофлоры приэпителиальной слизистой оболочки кишечника. У белых крыс с моделированным сахарным диабетом (алоксан-индуцированным) происходит контаминация кишечника условно патогенными бактериями родов *Peptococcus, Clostridium, Proteus, Staphylococcus*. Одновременно большая часть автохтонных облигатных бифидобактерий, лактобацил, бактероидов остаются доминантными. Микроорганизмы *Eubacterium* элиминируют из числа составляющих колонизационную резистентность тонкой кишки.

Ключевые слова: аллоксан-индуцированный сахарный диабет, колонизационная резистентность, микрофлора.

ЕКПЕРИМЕНТАЛЬНЕ ВИВЧЕННЯ МІКРОФЛОРИ ПРИЕПІТЕЛІАЛЬНОЇ СЛИЗОВОЇ ОБОЛОНКИ КИШЕЧНИКУ БІЛИХ ЩУРІВ З ІНДУКОВАНИМ ЦУКРОВИМ ДІАБЕТОМ

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Резюме. Стаття присвячена дослідженню кількісного та якісного складу мікрофлори приепітеліальної слизової оболонки кишечнику. В білих щурів з модельованим цукровим діабетом (алоксан-індукованим) відбувається контамінація умовно патогенними бактеріями родів *Peptococcus, Clostridium, Proteus, Staphylococcus*. Одночасно більша частина автохтонних облігатних біфідобактерій, лактобацил, бактероідів залишаються домінантними. Мікроорганізми *Eubacterium* елімінують з числа тих, що становлять колонізайну резистентність тонкої кишки.

Ключові слова: алоксан-індукований цукровий діабет, колонізаційна резистентність, мікрофлора.

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