LOSS OF CAS3 AND INCREASE OF BAX EXPRESSION ASSOCIATED WITH PROGRESSION OF CERVICAL INTRAEPITHELIAL NEOPLASIA

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Apoptosis plays the critical role in the maintenance of tissue homeostasis in human body [1]. In addition, it has been shown that deregulated apoptosis plays the major role in human cancer development, including cervical cancer [1]. Morphologically, apoptosis is characterised with the formation of small, spherical, membrane bound organelles, which are known as apoptotic bodies [2].

Apoptosis represents the tightly regulated process, in which several different families or proteins are involved, including the proteins of Bcl2 and Caspase family [1]. Bcl2 associated X gene, so called BAX gene represents the pro-apoptotic member of Bcl2 family, which is shown to be deregulated during the progression of different cancers [3]. The role of BAX in the progression of cervical intraepithelial neoplasia and cervical cancer less known.

Caspase 3 (Cas3) protein represents, the important member of cysteine-aspartic acid protease family, which plays the major role in the executive phase of apoptosis [4]. It has been suggested that caspase activity is related to the aggressive features of cancer cells, including cervical cancer, which is developed from grade 1 to 3 cervical intraepithelial lesions. It has been shown that Cas3 activity is deregulated during the progression of cervical cancer. Particularly, in 16% of CIN3 cases it is upregulated and in 28% of CIN3 cases it is downregulated, whilst it is gradually decreased in the progression of cervical carcinoma from stage I to stage II-IV [5]. The changes of Cas3 in cervical intraepithelial lesions is not very well studied.

The aim of our study was to evaluate the apoptotic activity the progression of cervical intraepithelial neoplasia, using Cas3 and BAX proteins and to decipher their association with the expression of proliferation marker Ki67 and ER.

Material and methods. Formalin fixed and paraffin embedded tissue material was retrieved from the Research, Diagnostic and Teaching Laboratory of Tbilisi State Medical University, Georgia. Study included altogether 140 tissue samples, divided into two major groups: cases without co-infections (n=54) and cases with co-infections (n=86). Co-infections included *bacterial vaginosis, chlamydia trachomatis and candida albicans.* Cases without co-infections were divided into following subgroups: normal cervix (10 cases), CINI (18 cases), CINII (14 cases), CINIII (7 cases), invasive carcinoma (5 cases); Cases with co-infections were divided into following subgroups: cervix with only infections (15 cases), CINI (29 cases), CINII (19 cases), CINIII (15 cases), invasive carcinoma (8 cases).

4μ FFPE tissue sections were deparaffinized in xylene, rehydrated by using serial dilutions of ethanol (96%, 80%, 70%) and heat mediated antigen retrieval has been performed. Ready to use antibodies against the following antigens were used: Ki67, Cas3, BAX and ER. Staining and visualisation has been performed using Bond polymer refine detection system. The expression of all markers was evaluated as the percentage of marker positive cells.

Comparisons between groups were made using Kruskal-Wallis test. The Kruskal-Wallis test is a nonparametric (distribution free) test, and is used when the assumptions of one-way ANOVA are not met. The Kruskal-Wallis test can be used for both continuous and ordinal-level dependent variables. Correlations were assessed using Spearman's rank correlation. The Spearman's rank correlation is also used when data is non-parametrically distributed. P values <0.05 were considered as significant. All statistical tests were performed using SPSS software V20.00.

Results and discussion. The study of Cas3 in specimens without co-infections indicated that, the average pro-apoptotic index was 56±10.2 in normal cervix, 44±4.9 in CINI, 31±5.6 in CINII, 22±4.4 in CINIII and 9±2.3 in cervical invasive carcinoma. The study of BAX indicated that the average BAX index in normal cervix was 7±2.2, in CINI it was 16±3.9, in CINII it was 19±5.2, in CINIII it was 24±7.6 and in cervical invasive carcinoma it was 55±9.8. The proliferation index measured as Ki67 labelling index was following: 4±1.2 in normal cervix, 13±5.2 in CINI, 15±6.9 in CINII, 20±5.1 in CINIII and 35±10.6 in invasive carcinoma. The average ER distribution was 19±2.2 in normal cervix, 21±5.7 in CINI, 24±5.7 in CINII, 11±3.4 in CINIII and 6±1.1 in invasive carcinoma of the cervix.

Table 1. Distribution apoptotic, proliferation and ER markers in cervical lesions without co-infections. CA, carcinoma

	Without co-infection			
	Cas3	Bax	Ki67	ER
Normal cervix	56	7	4	19
CINI	44	16	13	21
CINII	31	19	15	24
CINIII	22	24	20	11
Invasive CA	9	55	35	6

Table 2. Distribution of apoptotic, proliferation and ER markers in cervical lesions with co-infections. CA, carcinoma

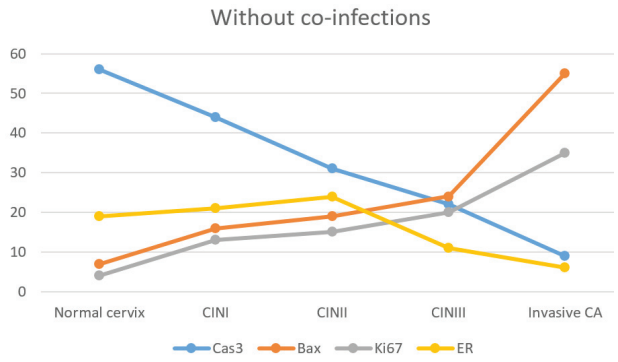
	With Co-infection			
	Cas3	Bax	Ki67	ER
Cervix with Infection	52	9	6	20
CINI	43	21	17	22
CINII	29	25	21	27
CINIII	18	30	26	14
Invasive CA	5	61	42	8

The study of pro-apoptotic marker Cas3 in specimens with co-infections indicated that, the average pro-apoptotic index was 52±3.2 in cervix with infections only, 43±6.9 in CINI, 29±2.2 in CINII, 18±3.6 in CINIII and 5±4.1 in cervical invasive carcinoma. The study of BAX indicated that the average BAX index in normal cervix was 9±3.3, in CINI it was 21±5.8, in CINII it was 25±5.5, in CINIII it was 30±6.7 and in cervical invasive carcinoma it was 61±10.8. The proliferation index measured as Ki67 labelling index was following: 6±1.9 in normal cervix, 17±6.3 in CINI, 21±7.8 in CINII, 26±8.2 in CINIII and 42±10.7 in invasive carcinoma. The average ER distribution was 20±2.4 in normal cervix, 22±3.6 in CINI, 27±4.4 in CINII, 14±2.8 in CINIII and 8±3.6 in invasive carcinoma of the cervix.

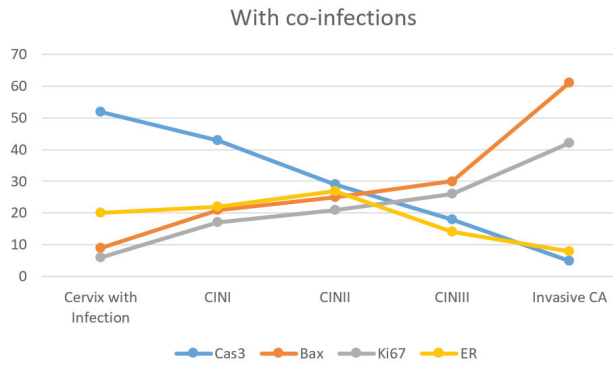
The analysis of the results indicated that the expression of pro-apoptotic protein Cas3 is progressively lost during the progression of cervical intraepithelial neoplasia in the group of without co-infections. Particularly, the expression of Cas3 is almost two-times lower in CINI and CINII compared to normal cervix and it is 3 times lower in CINIII and almost 6 times lower in cervical carcinoma. On the other hand, the expression of BAX is progressively increased during the progression of cervical intraepithelial lesions in the same group. Particularly, the expression of BAX is almost 2-times higher in CINI and CINII compared to normal cervix, 4-times higher in CINIII and almost 6-times higher in cervical CA. The distribution of Ki67 proliferation marker, it is also progressively increased during the progression of CIN. With regards to the expression of ER, the weak expression was seen in normal cervix. The expression of ER was moderate in CINI and CINII lesions, whilst it was again decreased to weak expression in CINIII and almost negativity to cervical carcinoma. The distribution patterns of mentioned markers were similar in the groups with co-infections. However, the loss of Cas3, as well as high expression of Ki67 and the loss of ER was more pronounced in specimens with co-infections. Which might indicate that co-infections further modulate the expression of these markers and therefore playing a role during the CIN progression.

Correlation analysis, in all specimens, indicated that the expression of BAX negatively correlates with the expression of Cas3 ($r=-42.4$, $p<0.05$) and ER ($r=-33.4$, $p<0.05$) and positively correlates with the expression of proliferation marker Ki67 ($r=56.3$, $p<0.05$).

Cervical cancer is developed from cervical pre-cancerous lesions called cervical intraepithelial neoplasia. There are three grades of CIN, including CINI, CINII and CINIII. Interestingly not all CIN lesions progress to cervical cancer and many cases of CIN undergo the spontaneous resolution. Particularly, only about 10% of patients with CINI lesion develop cervical cancer, whilst this number reaches up to 40% in case of CINIII lesions. Therefore, only morphological detection and diagnosis of different grades of CIN is not enough to predict the development of cervical carcinoma from pre-cancerous lesions and there is the need to discover the molecular markers of CIN progression. It has been shown that in the process of cervical carcinogenesis there is the deregulation of apoptosis through various mechanisms, including the accumulation of mutations and/or epigenetic changes in tumor suppression genes. In addition, the expression levels of apoptotic proteins reflect the actual status of cells apoptotic ability and it might be used as a marker for



Graph 1. The distribution of Cas3, BAX, Ki67 and ER in CIN and cervical CA without co-infections



Graph 2. The distribution of Cas3, BAX, Ki67 and ER in CIN and cervical CA with co-infections

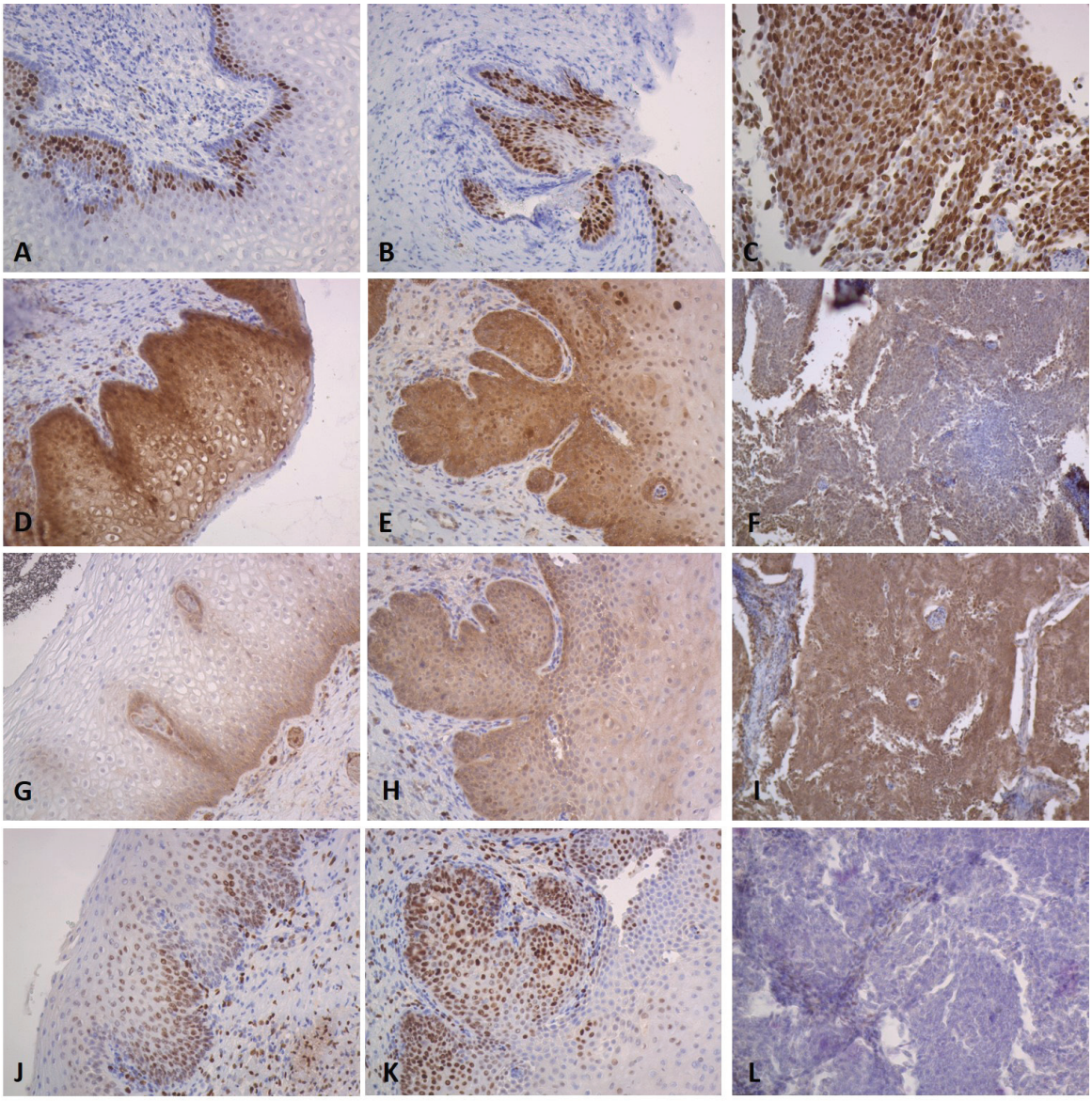


Fig. Expression of Ki67 in A. normal cervix, B. CINIII and C. in cervical CA; expression of Cas3 in D. normal cervix, E. CINIII and F. in cervical CA; expression of BAX in G. normal cervix, H. CINIII and in I. cervical CA; expression of ER in J. normal cervix, K. CINIII and L. cervical carcinoma, IHC, x200

the progression of CIN disease. The measurement of caspase activity is frequently used in experimental studies of apoptosis [6]. Therefore, it has been suggested that the expression of these proteins might also serve as biomarkers on a tissue level [7]. According to the study of Lu et al., the expression of Cas3 was significantly increased in cervical cancer compared to normal tissue [8]. Whilst the others reported that Cas3 expression is decreased in later stages of cervical cancer [5]. Therefore, the existing literature shows the conflicting results. In our study, we have shown that Cas3 is progressively lost during the progression of CIN, showing almost complete loss in cervical cancer. In addition, Cas3 expression is negatively associated with the proliferation marker Ki67 in our study. Hence, we speculate that the expression of Cas3 represents the marker of cells apoptotic potential and therefore the decrease in its expression reflects the decrease of the apoptosis, which is detected as increased proliferation of the cells in CIN and cervical cancer.

To the best of our knowledge, we are first who investigated the expression of BAX in the progression of CIN lesions and in cervical cancer and demonstrated that the expression of BAX is significantly increased at later stages of the disease. Our study is in line with one previous report regarding BAX expression in cytology smears, which showed the increased expression of BAX in abnormal smears, compared to normal smears [9]. In addition, in our study increased BAX expression was correlated to the loss of Cas3. Therefore, we suggest that in cases of Cas3 loss, the increased BAX expression might reflect the over activation of the initiation phase of apoptosis, as a result of decreased ability of cells to execute the final phase of apoptosis.

Conclusions. The results indicate that the increased expression of Bax and Cas3 loss, as well as the increase in proliferation index measured as Ki67 expression is significantly related to the progression of CIN into cervical carcinoma. Therefore, the measuring of mentioned protein expression could be used as the markers of the progression of CIN.

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SUMMARY

LOSS OF CAS3 AND INCREASE OF BAX EXPRESSION ASSOCIATED WITH PROGRESSION OF CERVICAL INTRAEPITHELIAL NEOPLASIA

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Apoptosis plays one of the major roles in the progression of human cancers including cervical carcinoma. The aim of our study was to analyse the expression of Cas3, Bax and their correlation with the proliferation index and ER expression status during the progression of cervical intraepithelial neoplasia (CIN). Study included altogether 140 specimens, divided into two major groups, such as: cervical lesions without co-infections and with co-infections. Standard immunohistochemistry was used to detect antigens: Ki67, Cas3, Bax and ER. The study results showed that the expression of Cas3 is significantly decreased whilst the expression of Bax is significantly increased during the progression of CIN in both groups with and without co-infections. The expression of Bax negatively correlates with the expression of Cas3 ($r=-42.4$, $p<0.05$) and ER ($r=-33.4$, $p<0.05$) and positively correlates with the expression of proliferation marker Ki67 ($r=56.3$, $p<0.05$). The results indicate that the deregulated apoptosis measured as increased expression of Bax and Cas3 loss, as well as the increase in proliferation index measured as Ki67 expression is significantly related to the progression of CIN into cervical carcinoma. Therefore, the measuring of mentioned protein expression could be used as the markers of the CIN progression.

Keywords: CAS3 and BAX proteins; Co-infections; Cervical Lesions.

РЕЗЮМЕ

ПОТЕРЯ CAS3 ДОСТОВЕРНО АССОЦИИРУЕТСЯ С ПРОГРЕССИЕЙ ИНТРАЭПИТЕЛИАЛЬНЫХ НЕОПЛАЗИЙ ШЕЙКИ МАТКИ

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Апоптоз играет значимую роль в прогрессии процесса многих видов карцином, в том числе и карцином шейки матки.

Целью исследования явилось изучение экспрессии проапоптозного белка Cas3 и анти-апоптозного белка Bax в процессах прогрессии интраэпителиальных неоплазий шейки матки и их корреляционных связей с экспрессей маркеров Ki67 и ER. Исследования проведены на 140 образцах тканей, разделенных на 2 основные группы: поражения шейки матки с наличием инфекции (n=86) и без инфекции (n=54). Стандартным иммуногистохимическим методом изучены молекулярные маркеры Ki67, Cas3, Bax и ER.

Результаты исследования показали, что в процессе прогрессии интраэпителиальных неоплазий шейки матки экспрессия про-апоптозного белка Cas3 значительно уменьшается, а экспрессия анти-апоптозного белка Bax значительно увеличивается в обеих группах. Экспрессия Bax находится в негативной корреляционной связи с экспрессией Cas3 ($r=-42.4$, $p<0.05$) и ER ($r=33.4$, $p<0.05$) и в позитивной корреляционной связи - с экспрессией Ki67 ($r=56.3$, $p<0.05$).

Результаты исследования выявили увеличение экспрессии Bax и потерю Cas3, также увеличение пролиферативного индекса Ki67, что достоверно связано с процессом прогрессии интраэпителиальных неоплазий шейки матки в карциному. Следовательно, эксперссию данных белков возможно использовать как маркер прогрессии интраэпителиальных неоплазий шейки матки.

რეზიუმე

Cas3-ის დაკარგვა და BAX-ის მომატებული ექსპრესია სარწმუნოდ ასოცირდება საშვილონოს ყელის ინტრაეპითელური ნეოპლაზიების პროგრესიასთან

გ.ფხაკაძე, ზ.ბოხუა, თ.ასათიანი, თ.მუზაშვილი, გ.ბურკაძე

თბილისის სახელმწიფო სამედიცინო უნივერსტეტი, საქართველო

აპოპტოზი მნიშვნელოვან როლს თამაშობს მრავალი ტიპის კარცინომის, მათ შორის საშვილონოს ყელის კარცინომის პროგრესიის პროცესში.

კვლევის მიზანს წარმოადგენდა პრო-აპოპტოზური ცილა Cas3-ის და ანტი-აპოპტოზური ცილა Bax-ის ექპრესიის შესწავლა საშვილონოს ყელის ინტრაეპითელური ნეოპლაზიების პროგრესიის პროცესში და მათი კორელაციური კავშირის დადგენა პროლიფერაციული მარკერების Ki67-ის და ER-ის ექსპრესიასთან.

კვლევაში გამოყენებული იყო ქსოვილის 140 ნიმუში, რომელიც გაყოფილი იყო 2 ჯგუფად: საშვილონოს

ყელის დაზიანებები ინფექციით (n=86) და ინფექციის გარეშე (n=54). სტანდარტული იმუნოჰისტოქიმიური მეთოდით გამოვლენილია მარკერები: Ki67, Cas3, Bax და ER.

კვლევის შედეგებმა აჩვენა, რომ პრო-აპოპტოზური ცილის Cas3-ის ექსპრესია მნიშვნელოვნად მცირდება, ხოლო ანტი-აპოპტოზური ცილის Bax-ის ექსპრესია იზრდება საშვილონოს ყელის ინტრაეპითელური ნეოპლაზიების პროგრესიის პროცესში ორივე საკვლევ ჯგუფში. Bax-ის ექსპრესია ნეგატიურ კორელაციაშია Cas3-ის (r=-42.4, p<0.05) და ER ექსპრესიებთან (r=33.4, p<0.05) და პოზიტიურ კორელაციაშია პროლიფერაციული მარკერის Ki67-ის ექსპრესიასთან (r=56.3, p<0.05). კვლევის შედეგებმა გამოავლინა, რომ Bax-ის მომატებული ექსპრესია და Cas3-ის დაკარგვა, ისევე როგორც მომატებული Ki67 პროლიფერაციული ინდექსი სარწმუნოდ არის დაკავშირებული საშვილოსნოს ყელის ინტრაეპითელური ნეოპლაზიების კარცინომად პროგრესიასთან. შესაბამისად, აღნიშნული ცილების ექსპრესია შესაძლებელია გამოყენებული იყოს საშვილონოს ყელის ინტრაეპითელური ნეოპლაზიების პროგრესიის მარკერად.

MORPHOLOGICAL CHARACTERISTICS OF SMALL INTESTINE MUCOSA IN DYSBIOSIS AND AFTER ITS CORRECTION BY PROBIOTICS AND ENTEROSORBENTS

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Through the recent years, the world medical community has been demonstrating an increasing interest in studying normal human microbiota. Nowadays the term “normal microbiota” is more commonly replaced by the term “microbiome”, proposed in 2001 by an American geneticist Joshua Lederberg [1]. The human microbiome is known to be involved in some essential biological processes: it protects against harmful germs and compounds; it produces considerable impact on the structural and functional state of the internal organs, on the immune system, as well as plays an important role in regulating some vital functions. It should be emphasized that the advance in studying microbiome and its role in maintaining human overall health is considered as one of the key achievement in modern biology and medicine. The editorial board of one of the most significant and authoritative scientific journals, *Science*, has acknowledged the study of the human microbiome as one of the greatest scientific successes in the first decade of the 21st century [2-5].

Considering the importance of the microbiome for human health, the issue of its physiological functioning is attracting a growing attention from researchers and practitioners. Recent scientific reports have convincingly shown that from 70% to 90% of the world population suffers from dysbiosis of varying degrees that, undoubtedly, corroborates their social and environmental significance [6,7]. At present, dysbiosis is defined as a state of imbalance in the microbial ecosystem, i. e. there is simultaneous impairment of the normal functioning and mechanisms of interacting between its major components: a macroorganism and indigenous microbiota associated with the mucous membranes of the cavities and skin [8].

Among the numerous causes of dysbiotic disorders, the use of chemotherapeutic antimicrobials, often of broad-spectrum action and for per-oral administration, is ranking the top position. Especially dangerous in this regard is the use of antibiotics for prophylactic purposes [9]. However, some other groups of medicines can also contribute to the development of dysbiosis by affecting the kinetics of the mucosal epithelium and, accordingly, the mucin composition. This group may include non-steroidal anti-inflammatory drugs, laxatives, cholagogues, coating agents with adsorbing properties and some others [10]. Irrational, baseless and often uncontrolled use of antibacterial agents in medical practice leads to artificial selection of polyresistant strains of opportunistic microorganisms.

The last decade has been marked by a considerable increase in the interest of healthcare representatives of fields to develop new approaches and to improve existing ones towards the correction of dysbiotic conditions. Among them, the concept of probiotic supplementation is occupying a leading position. According to the WHO definition, probiotics are «microorganisms, which when administered in adequate amounts, confer a health benefit on the host organism” [11]. In recent years, there has been a growing interest in both fundamental and clinical research of probiotics. The mechanisms responsible for various effects produced by probiotics are usually associated with the ability of probiotics to inhibit the development of pathogenic microbes, to demonstrate immunomodulatory properties, to stimulate the proliferation and differentiation of epithelial cells, and to promote the intestinal barrier [12].

At the same time, an imbalance in microbial ecology, as a rule, results from the contamination of the internal body environment with toxic compounds of both exogenous and endogenous nature; therefore, some types of enterosorbents can be attributed to beneficial agents for microflora normalization. The mechanism of their action is largely due to the sanitation of the intestinal lumen resulting in the improved condition for the vital activity of the physiological microbiota. Enterosorption is a non-invasive method of efferent therapy and, when an adequate sorbent selected, can promote effective cleansing the body of allergens, mediators, by-products of allergic or inflammatory processes, metabolites, toxins, viruses and other components. Improvement of biotopes is able to optimize the conditions for normal human microflora functioning [13 - 15]. In the face of increasing resistance to antibacterial agents, the addition of enterosorbents in the integrated therapy of dysbioses is an important and pathogenetic-based approach.