Cytokine and antimicrobial peptide levels in cholelithiasis

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Уровни цитокинов и антимикробных пептидов при желчнокаменной болезни

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Abstract

Objective. To study the cytokines and antimicrobial peptides concentrations in patients with cholelithiasis during 1 year treatment before and after the surgery.

Materials and methods. 38 patients with a cholelithiasis were examined and operated in the Educational–Surgical Clinic of the Azerbaijan Medical University. According to the treatment regimen after the operation the patients were divided into two groups: patients of the 1st group (n = 17) received standard treatment; patients of 2nd group (n = 21), in addition to standard treatment hepatoprotectors, combined enzyme preparations, pre- and probiotics were prescribed. The control group consisted of 14 apparently healthy individuals. The levels of inflammatory cytokines (interleukin-6 and tumor necrosis factor- α) in the blood and antimicrobial peptides (zonulin, calprotectin, and lactoferrin) in feces we studied with enzyme immunoassay method.

Results. In patients with cholelithiasis of the 1st group, the concentration of interleukin-6 was significantly increased by 5 times (p < 0.001), the concentration of tumor necrosis factor- α – by 3.5 times (p < 0.001), of zonulin – by 2.2 times (p = 0.012), of calprotectin – by 5.8 times (p < 0.001, the concentration of lactoferrin – by 60 times (p < 0.001), and in the 2nd group – by 4.8 times (p < 0.001), by 3.1 times (p < 0.001), on 91.7% (p = 0.011), by 5.8 times (p < 0.001) and by 52.8 times (p < 0.001) respectively in comparison with the control.

Conclusions. Standard postoperative treatment supplemented by hepatoprotectors, combined enzyme preparations, pre- and probiotics prescription leads to a positive dynamics of inflammatory mediators concentration and its normalization.

Keywords: cholelithiasis; interleukin-6; tumor necrosis factor- α ; zonulin; calprotectin; lactoferrin.

Реферат

Цель. Изучение концентрации цитокинов и антимикробных пептидов в динамике лечения в течение 1 года до и после операции.

Материалы и методы. В Учебно–хирургической клинике Азербайджанского медицинского университета обследовано и оперировано 38 пациентов с диагнозом желчнокаменная болезнь. После операции согласно схеме лечения пациенты были распределены на две группы: пациенты 1–й группы (n = 17) получали стандартное лечение; пациентам 2–й группы (n = 21) в дополнение к стандартному лечению были назначены гепатопротекторы, комбинированные ферментные препараты, пре- и пробиотики. Контрольную группу составили 14 практически здоровых лиц. С помощью иммуноферментного анализа изучали уровни воспалительных цитокинов (интерлейкина-6 и фактора некроза опухоли- α) в крови и антимикробных пептидов (зонулин, кальпротектин и лактоферрин) в кале.

Результаты. По результатам исследования у больных с желчнокаменной болезнью 1–й группы была достоверно повышена в 5 раз (p < 0,001) концентрация интерлейкина-6, в 3,5 раза (p < 0,001) концентрация фактора некроза опухоли- α , в 2,2 раза (p = 0,012) концентрация зонулина, в 5,8 раза (p < 0,001) концентрация кальпротектина и в 60 раз (p < 0,001) концентрация лактоферрина, во 2–й группе – соответственно в 4,8 раза (p < 0,001), в 3,1 раза (p < 0,001), на 91,7% (p = 0,011), в 5,8 раза (p < 0,001), в 52,8 раза (p < 0,001) по отношению к контролю.

Выводы. Стандартное послеоперационное лечение, дополненное применением гепатопротекторов, комбинированных ферментных препаратов, пре- и пробиотиков, приводит к положительной динамике концентрации медиаторов воспаления и ее нормализации.

Ключевые слова: холелитиаз; интерлейкин-6; фактор некроза опухоли- α ; зонулин; кальпротектин; лактоферрин.

Cholelithiasis (GSD) - is a common pathology among the population of economically developed countries, which is caused by the influence of such risk factors as diabetes, obesity, cholecystitis, stress, and others. Violation of the bile-forming and bile-secretory functions of the liver, along with the risk of stone formation, leads to a violation of the processes of fats emulsification and the micelles formation, a decrease in the digestive enzymes activity and development of malabsorption syndrome (MS). MS is a set of symp-

toms, characterized by impaired digestion, absorption, and transport of one or more nutrients in the small intestine, accompanied by metabolic disorders [1-4].

The molecular mechanisms of MS development in various diseases of the hepatobiliary system, including gallstone disease, are not fully understood, but it is assumed that the nature of inflammatory process and the immune status state play a special role in this. Disorders of metabolic and detoxification processes during gallstones evolving weaken the im-

immune system and antimicrobial defense. The development of inflammatory process in the organism leads to disruption of synthesis and secretion of inflammatory mediators, including cytokines and antimicrobial peptides (AMP) [5-7].

It is known that opportunistic bacteria and products of their reproduction in the intestine with cholelithiasis cause local and systemic inflammatory reactions in the intestinal wall. Contact of pathogenic bacteria with the intestinal wall leads to activation of the innate immune system and acceleration of secretion and expression of genes, followed by inflammatory cyto- and chemokines production. [5,8].

Endogenous AMPs are nonspecific factors of the innate humoral immune system, that provide protection against a wide range of bacteria, viruses, and fungi. Cytokines are involved in inflammatory reactions of the liver, apoptosis and necrosis, cholestasis and fibrosis. Overproduction of cytokines can lead to the development of systemic inflammatory disease as well as septic shock. Fibro- and necrotic damage to hepatic cells is directly related to progression of inflammatory process in the organism tissues with acceleration of the inflammatory cytokines synthesis [5, 8-10]. The study of the cytokines and AMPs levels in cholelithiasis is of great scientific and practical importance for understanding of pathophysiological mechanisms of MS, the development of enzyme replacement therapy, and improving the patients' quality of life. Early and differential diagnosis of MS, that occurs after hepatobiliary surgery, can provide adequate treatment for the underlying disease and contribute to the clinical improvement of patients' condition.

Objective: to study the secretion of cytokines and AMP among patients with cholelithiasis during the treatment before and after surgery.

Materials and methods

Patients, who took part in the study, were selected among those, examined and operated on for gallstones in the Educational-Surgical Clinic of the Azerbaijan Medical University. A total of 38 patients were selected at the age of 30-60 yrs old with diagnosis of gallstone disease. The control group consisted of 14 apparently healthy individuals of the corresponding age. The diagnosis of gallstones was confirmed on the basis of instrumental examination (ultrasound, magnetic resonance imaging and computed tomography).

The concentration of cytokines (interleukin-6 - IL-6 and tumor necrosis factor- α -TNF- α) in the blood of patients and the concentration of AMP (zonulin, calprotectin and lactoferrin) in the feces of patients were studied by method of enzyme immunoassay in the dynamics of treatment: before and after surgery (after 3, 6 months and 1 year). In this case, reagent kits from «Vector Best» (Russian Federation) were used to determine the concentration of cytokines and from «Immun Diagnostic» (Germany) to determine the concentration of AMP.

The samples were examined using a STAT FAX 303 Plus microstrip reader (USA) at a wavelength of 450 nm. After the operation, according to the treatment regimen, the pa-

tients were divided into two groups. The patients of the 1st group (n = 17) received a standard treatment, while to the patients of group 2 (n = 21), in addition to standard treatment, hepatoprotectors, combined enzyme preparations, pre- and probiotics were prescribed [11].

Statistical processing of materials and the research results was carried out, using the "Excel-2013" program of the "Microsoft Office" statistical packages. The statistical significance of differences in the normal distribution was assessed using the Student's t-test, and in the case of an abnormal distribution, Wilcoxon signed-rank test was used.

Results

The concentration of IL-6 in the control group ranged from 0.8 to 4.7 pg / ml and averaged (3.2 ± 0.3) pg/ml. In the 1st group, the concentration of IL-6 was increased by 5 times ($p < 0.001$) compared with that in the control group, averaging (16.0 ± 1.4) pg / ml. In group 2, IL-6 concentration was on average (15.4 ± 1.3) pg/ml, and it was a statistically significant increase in this index in 4.8 times ($p < 0.001$), compared with its value in the control group (see table).

The concentration of TNF- α in the blood serum of practically healthy individuals was ($4.6 - 0.5$ pg / ml) and ranged from 1.9-7.2 pg / ml. The concentration of TNF- α in the 1st group of patients with cholelithiasis have averaged (16.2 ± 1.9) pg / ml, and in the 2nd group - (14.4 ± 1.7) pg / ml and was statistically significantly increased in comparison to indicators in the control group - in 3.5 ($p < 0.001$) and 3.1 ($p < 0.001$) times, respectively.

The concentration of zonulin in the control group varied from 0.4 to 1.7 μ g / g and averaged (0.93 ± 0.12) μ g / g. The concentration of zonulin in the 1st group of patients with cholelithiasis was (2.06 ± 0.31) μ g / g, while in the 2nd group - (1.77 ± 0.28) μ g / g. There was a statistically significant increase in concentration of zonulin among patients with cholelithiasis: in 2.2 times ($p = 0.012$) in group 1 and by 91.7% ($p = 0.011$) - in group 2, compared to that in the control group. The value of calprotectin in the control group was 19.8 - 2.0 mg / g and ranged from 10.0 to 29.6 mg / g. In the 1st group of patients with cholelithiasis, the value of calprotectin was significantly increased - in 5.8 times ($p < 0.001$), compared to the control group and averaged (114.1 ± 13.7) mg / g. In the 2nd group, there was a statistically significant increase in the value of calprotectin - in 5.8 times ($p < 0.001$), compared with the control group - up to (114.1 ± 18.0) mg / g.

In the control group, the lactoferrin value was 0.9-0.1 μ g / g, its fluctuation was noted in the range of 0.2-1.5 μ g / g. The concentration of lactoferrin in the coprofiltrate of patients in the 1st group have averaged (53.5 ± 6.1) μ g / g, in the 2nd group - (47.1 ± 7.6) μ g / g. There was a statistically significant increase in concentration of lactoferrin in coprofiltrate of patients with cholelithiasis: 60 times ($p < 0.001$) in group 1 and 52.8 times ($p < 0.001$) in group 2. During the postoperative period in the 1st group among patients with GSD, the concentration of IL-6 have decreased

Concentration of cytokines and AMP in patients with cholelithiasis ($\bar{x} \pm m$)

Indicators	Control	Groups	Before surgery	After operation		
				3 months	6 months	1 year
IL-6, pg/ml	3,2±0,3 (0,8-4,7)	1st	16,0±1,4* (3,6-28,4)	9,3±1,0*^ (1,0-15,7)	10,8±1,0*^ (2,8-17,0)	11,2±1,5*^ (3,1-22,4)
		2nd	15,4±1,3* (3,4-24,7)	9,7±0,7*^ (6,1-18,0)	4,3±0,4*^ (1,2-6,6)	3,5±0,2^ (1,8-4,9)
TNF-α, pg/ml	4,6±0,5 (1,9-7,2)	1st	16,2±1,9* (6,7-35,5)	11,3±1,2* (1,7-19,9)	10,9±1,4*^ (1,6-22,8)	11,6±1,0* (5,2-18,3)
		2nd	14,4±1,7* (4,2-35,0)	10,4±1,1* (2,9-16,9)	5,9±0,6^ (2,7-13,3)	4,9±0,4^ (2,3-7,8)
Zonulin, μg/g	0,93±0,12 (0,4-1,7)	1st	2,06±0,31* (0,4-3,8)	1,44±0,15* (0,6-2,1)	1,77±0,23* (0,2-2,9)	1,61±0,25* (0,9-3,8)
		2nd	1,77±0,98* (0,8-3,5)	1,43±0,14* (0,8-2,2)	1,21±0,14 (0,5-2,0)	1,16±0,17 (0,4-1,9)
Calprotectin, mg/g	19,8±2,0 (10,0-29,6)	1st	114,1±13,7* (53,8-184,3)	87,4±15,5* (20,8-164,9)	63,1±9,6 (18,9-103,2)	79,1±5,7 (52,2-115,0)
		2nd	114,0±18,0* (45,6-224,7)	80,8±9,9* (35,0-128,4)	34,5±4,1*^ (10,3-51,7)	30,5±5,4* (10,3-63,4)
Lactoferrin, μg/g	0,9±0,1 (0,2-1,5)	1st	53,5±6,1 (20,6-78,0)	18,5±2,4 (7,3-29,6)	17,3±2,5*^ (1,1-30,4)	14,3±1,7^ (6,7-25,8)
		2nd	47,1±7,6 (16,2-93,8)	15,7±1,9 (3,2-24,8)	5,0±0,6 (2,2-7,9)	2,1±0,2 (1,1-3,8)

Note. * - in comparison with the control group; ^ - in comparison with the indicators before the operation.

in comparison with the preoperative values -by 41.8% ($p = 0.003$) - after 3 months, by 32.3% ($p = 0.006$) after 6 months and by 30.3% ($p = 0.006$) after 1 year. At the same time, it remained higher than in the control group: 2.9 times ($p < 0.001$) - after 3 months, 3.4 times ($p < 0.001$) - after 6 months and 3.5 times ($p = 0.002$) - after 1 year after postoperatively. In the group 2 patients, who received hepatoprotective therapy in addition to standard treatment, the concentration of IL-6 have decreased, compared to values before treatment: by 37.1% ($p = 0.003$) - after 3 months, in 3.6 times ($p < 0.001$) - after 6 months and in 4.4 times ($p < 0.001$) - after 1 year after surgery. At the same time, the concentration of IL-6 have exceeded the control values: in 3.0 times ($p < 0.001$) - after 3 months, and by 34.7% ($p < 0.001$) - after 6 months after surgery. One year after the surgery, the concentration of this cytokine was very insignificantly different from its values before it. In the group 1 in the patients with cholelithiasis a decrease in concentration of TNF-α was observed: 3 months after surgery - by 30.3% ($p = 0.068$), after 6 months - by 33.1% ($p = 0.028$), after 1 year - by 28.5% ($p = 0.098$), compared with preoperative values. In this group, concentration of this cytokine have exceeded the control values in 2.5 times ($p < 0.001$) - after 3 months, 2.4 times ($p = 0.001$) - after 6 months and 2.5 times ($p < 0.001$) - after 1 year postoperatively.

In the 2 group of patients with cholelithiasis concentration of TNF-α was by 27.6% lower ($p = 0.073$) in 3 months after surgery, and in 2.4 times ($p < 0.001$) - after 6 months, compared with preoperative values. After 1 year, a 2.9-fold decrease in the concentration of TNF-α was observed ($p < 0.001$). According to the results of comparative analysis,

concentration of this cytokine was in 2.3 times higher ($p = 0.001$) after 3 months, and in 2.4 times ($p = 0.006$) - after 6 months, compared with the control, but after 1 year the differences were less noticeable.

The concentration of zonulin in the group 1 of patients have decreased by 30.2% ($p = 0.062$) after 3 months, by 14% - after 6 months ($p = 0.477$), and by 22.1% ($p = 0.248$) - 1 year after surgery, compared to the values before the operation. The value of this AMP in the dynamics of treatment have remained high, compared to the control, and the difference was 55.4% ($p = 0.023$) after 3 months, 91.5% ($p = 0.007$) - after 6 months and 73.6% ($p = 0.010$) - after 1 year.

During treatment, the decrease in concentration of zonulin in the 2nd group of patients with GSD was 19.6% ($p = 0.790$) 3 months after surgery, 31.4% ($p = 0.583$) - after 6 months and 34.7% ($p = 0.092$) - after 1 year, compared to preoperative values. The level of zonulin during the treatment was higher than in the control group by 54.1% ($p = 0.011$) after 3 months, and by 31.3% ($p = 0.021$) - after 6 months. However, it have approached the norm 1 year after surgery.

In the 1st group of patients with cholelithiasis the concentration of calprotectin have decreased in comparison with preoperative values by 23.5% ($p = 0.328$) 3 months after surgery, by 44.7% ($p = 0.016$) - after 6 months and by 30.7% ($p = 0.075$) - after 1 year. It was found, that concentration of calprotectin in the dynamics of treatment have remained 4.4 times higher than the control values ($p < 0.001$) after 3 months; 3.2 times ($p = 0.001$) - after 6 months, 4.0 times ($p < 0.001$) - 1 year after surgery. Concentration of calprotectin in the 2nd group of patients with cholelithiasis have decreased in comparison to preop-

erative values 3 months after surgery by 29.1% ($p = 0.347$), after 6 months – in 3.3 times ($p = 0.002$) and after 1 year – in 3.7 times ($p = 0.005$). At the same time, the concentration of calprotectin in the dynamics of treatment have remained higher than in the control, after 3 months - in 4.1 times ($p < 0.001$), after 6 months - by 74.3% ($p = 0.009$), after 1 year - by 54.2% ($p = 0.166$).

In the 1st group of patients with cholelithiasis there was a decrease in concentration of lactoferrin, compared with the preoperative values in 2.9 times ($p = 0.003$) 3 months after surgery, 3.1 times ($p = 0.004$) - after 6 months and 3.7 times ($p = 0.003$) - after 1 year. During the treatment the concentration of this AMP have remained 20.7 times higher than the control ($p < 0.001$) after 3 months, 19.4 times ($p < 0.001$) - after 6 months, and 16.1 times ($p < 0.001$) - 1 year after surgery.

In the 2nd group of patients with cholelithiasis, the concentration of lactoferrin have decreased in 3.0 times ($p = 0.005$) after 3 months, 9.5 times ($p = 0.002$) - after 6 months, and 22.0 times ($p = 0.002$) - after 1 year, compared to preoperative values. At the same time, the lactoferrin level have exceeded the control values in 17.6 times ($p < 0.001$) after 3 months, 5.6 times ($p < 0.001$) - after 6 months, and 2.4 times ($p < 0.001$) - after 1 year after surgery.

Discussion

It was found that the course and intensity of the inflammatory response in the intestine is regulated by action of IL-6 on inflammation. IL-6 is one of the most important mediators of acute phase of inflammation, triggering the inflammatory response by activation of monocytes and macrophages [12].

TNF- α is a main inflammatory cytokine. It plays an important role in formation of the cytokine cascade in the organism and is mainly synthesized in macrophages and T-killer cells by viruses, endotoxins, and other cytokines. TNF- α induces expression of inflammatory cytokines, such as IL-1, IL-6, IL-12, and IL-18. It also participates in several biochemical reactions, inducing apoptosis due to activation of T-lymphocytes and neutrophils at the site of inflammation [13].

As was mentioned above, during the intestine inflammatory processes, the study of AMP is of particular importance. Among AMPs in the pathogenesis of gallstone disease, zonulin, lactoferrin, and calprotectin are of greater interest.

Zonulin plays an important role in development of MS. It is excreted through the intestinal mucosa, provides a close connection between the cells of the small intestine mucosa, and participates in regulation of intestinal permeability. Overproduction of zonulin causes an increase in intestinal permeability. This leads to the expansion of intercellular connections, which can be considered an indicator of diarrhea - one of the main symptoms of MS of any genesis. In this regard, zonulin can be considered as the main laboratory biomarker of intestinal permeability. As a result of increased intestinal permeability, numerous foreign bod-

ies, antigens, food additives, toxic metals are released from the intestinal walls, which leads to activation of cells of the immune system. From this point of view, zonulin levels can not only determine the degree of intestinal permeability, but the control of the anti-inflammatory therapy effectiveness well [14, 15].

Calprotectin is a calcium-binding protein of active phase. He is involved in an active inflammatory process, releasing the activation and destruction of neutrophils and tissue macrophages. In the inflammatory process in the intestine, as a result of the immune response, a large amount of calprotectin is released, so its concentration in the feces may be 6 times higher than in the blood. Determination of the concentration of calprotectin in coprofiltrates reflects the degree of amplification of the inflammatory process in the gut and can be of high interest to monitor during the course of treatment and MS [16 - 18].

Lactoferrin is a multifunctional protein, which belongs to the transferrin family. It is found in various secretory fluids and constitutes a glycoprotein with a molecular weight of 80 kDa. In addition to the functions of the iron binding and transport, lactoferrin has antibacterial, antiviral, antifungal, antiparasitic and antioxidant activity, and provides a microbalance in the alimentary channel. Thus, lactoferrin constitutes a component of the organism immune system, which participates in nonspecific humoral immune responses, and affects the activity of immunocompetent cells. In inflammatory and neoplastic diseases of the digestive system the concentration of this protein in feces may increase in 10-100 times, therefore it is considered a sensitive and specific marker for the diagnosis of these diseases [19, 20].

Thus, the revealed increased concentrations of cytokines and AMPs among patients with cholelithiasis indicates activation of the inflammatory process in the organs. The standard treatment, carried out in postoperative period, is turned to be less effective in reducing the concentration of inflammatory cytokines and AMPs, while the use of hepatoprotectors, combined enzyme preparations, pre- and probiotics, in addition to standard treatment, led to a positive dynamics in the concentration of these inflammatory mediators.

Conclusions

1. Among patients with cholelithiasis the concentration of IL-6, TNF- α , zonulin, calprotectin and lactoferrin significantly increases.

2. Standard postoperative treatment, supplemented by application of hepatoprotectors, combined enzyme preparations, pre- and probiotics, leads to positive dynamics of the inflammatory mediators concentrations and their normalization.

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