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THE EFFECT OF DOXORUBICIN-INDUCED OXIDATIVE STRESS ON RESISTANCE OF INTESTINAL MUCOSA

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The paper presents the findings of our own studies on the evaluation of the effect of anthracycline-induced oxidative stress on the level of N-acetylneuraminic acid and the state of the processes of free radical oxidation and antioxidant protection in the mucous membrane of rat small and large intestines depending on the presence of nonalcoholic steatohepatitis. 30 mature outbred albino rats were involved into studies. The cumulative dose of doxorubicin was 15 mg/kg. In the mucous membrane of the small intestine of rats with nonalcoholic steatohepatitis, administration of doxorubicin resulted in less pronounced oxidative damage and 1.2-fold decrease in N-acetylneuraminic acid concentration compared to rats without nonalcoholic steatohepatitis. It has been proved that administration of doxorubicin led to the development of doxorubicin-induced oxidative stress in the intestinal mucosa of rats with more severe lesions of the small intestine, regardless of the presence of nonalcoholic steatohepatitis.

Keywords: doxorubicin, oxidative stress, lipid peroxidation, antioxidant system, mucosal resistance, intestine.

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ВПЛИВ ДОКСОРУБІЦИН-ІНДУКОВАНОГО ОКСИДАТИВНОГО СТРЕСУ НА РЕЗИСТЕНТНІСТЬ СЛИЗОВОЇ ОБОЛОНКИ КИШКІВНИКА

У статті наведені результати власних досліджень по оцінці впливу антрациклін-індукованого оксидативного стресу на рівень N-ацетилнейрамінової кислоти та стан процесів вільно-радикального окиснення та антиоксидантного захисту у слизовій оболонці тонкої і товстої кишок щурів залежно від наявності неалкогольного стеатогепатиту. Дослідження проведені на 30 білих нелінійних статевозрілих щурах. Кумулятивна доза доксорубіцину склала 15 мг/кг. У слизовій тонкої кишки щурів із неалкогольним стеатогепатитом введення доксорубіцину приводило до менш вираженого оксидативного ураження та зменшення концентрації N-ацетилнейрамінової кислоти у 1,2 раза порівняно з щурами без неалкогольного стеатогепатиту. Доведено, що введення доксорубіцину призводило до розвитку доксорубіцин-індукованого оксидативного стресу в слизовій кишківника щурів з більш тяжким ураженням тонкої кишки незалежно від наявності неалкогольного стеатогепатиту.

Ключові слова: доксорубіцин, оксидативний стрес, перекисне окиснення ліпідів, антиоксидантна система, резистентність слизової оболонки, кишківник.

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Currently, the evidence has been given that a large number of chemotherapeutic drugs can lead to the development of inflammation and dysfunction of the entire gastrointestinal tract (GIT). Thus, chemotherapy-induced mucositis (CHIM) is one of the most common complications of cytostatic therapy. Clinical manifestations of CHIM can be painful ulcerations in the oral cavity, nausea, vomiting, abdominal pain, diarrhea, inflammation and origination of erosions and ulcers of the GIT mucous membrane (MM) [10]. It has been reported that the toxic effects of doxorubicin on GIT led to vomiting and development of mucositis during the first 5-10 days of chemotherapy (CHT). In most patients, GIT MM was restored within the next 10 days of treatment, though, in patients with severe reactions to administration of doxorubicin, ulceration and intestinal necrosis was observed with subsequent development of infectious complications with possible fatal consequences [15]. Furthermore, clinically significant consequences of CHIM may be a reduction or complete cessation of patients' intake of food and oral medications, resulting in the development of anorexic-induced atrophy of GIT MM. The need for intake of oral chemotherapeutic drugs increases the risk for systemic infections. The side effects related to GIT can lead to reduction in the maximum tolerated dose for many chemotherapeutic agents, resulting in lowering of the effectiveness of treatment. Evaluation of the degree of MM damage and GIT dysfunction caused by chemotherapeutic drugs is also challenging. Thus, generally, examinations of patients are limited to the evaluation of the oral cavity, general health state and the use of indirect indices of the functioning of the GIT, namely, hydrogen breath tests, biochemical blood test and microbiological studies [10]. The state of GIT MM resistance and the degree of its damage under the effect of cytostatic drugs can be assessed by the level of N-acetylneuraminic acid (NANA) [3].

Doxorubicin is anthracycline antibiotic that is one of the most effective cytotoxic drugs in the treatment of both solid and hematological malignancies, including Hodgkin's lymphoma and non-Hodgkin's lymphomas, acute and chronic leukemia. Currently, two main antitumor mechanisms of action of doxorubicin have been established. One of them is related to the property of doxorubicin to cause degradation of topoisomerase II, disrupting the process of DNA repair and causing its degradation [8]. The second mechanism of action of the drug is associated with the induction of oxidative stress, which affects both the cell membrane, cell DNA and numerous proteins, namely, NADH dehydrogenase, nitric oxide synthase, xanthine oxidase, glutathione peroxidase, catalase and superoxide dismutase [15]. Thus,

NADPH-cytochrome P-450 converts doxorubicin to a semihinone radical, which in turn initiates the production of superoxide anion and hydroxyl radicals, which cause lipid peroxidation [12].

The probability of developing CHT complications is influenced by the presence of patient's comorbidities. From this point of view, the study of the role of nonalcoholic steatohepatitis (NASH) in the development of cytostatic-induced lesions of the GIT is of particular importance. Oxidative stress is given an important role in the pathophysiological mechanisms of NASH development. Thus, according to numerous clinical observations and experimental studies in animal models, a strong relationship between the degree of oxidative damage and the severity of NASH has been shown [6, 8]. In view of the above, the study the course of oxidative lesions of the intestine with the use of doxorubicin in the presence of NASH is rational.

The purpose of the study was to establish the effect of anthracycline-induced oxidative stress on the level of N-acetylneuraminic acid, the state of the processes of free radical oxidation (FRO) and antioxidant protection in mucous membrane of the small and large intestines of rats depending on the presence of nonalcoholic steatohepatitis.

Materials and methods. Mature outbred albino rats (n=30; male rats=15 (50 %); female rats=15 (50 %)) weighted 160-220 g were involved into the study. The experiment was performed in two stages. At the first stage a group of animals (n=10; 5 males and 5 females) were exposed to simulated NASH during 63 days, induced by a fast-food diet. The ration per one animal for NASH modeling included combination fodder-concentrate granulated (0.04 kg), 72.5 % dairy butter (0.01 kg), refined sunflower oil (0.01 kg), palm oil (0.01 kg) and 4 % aqueous fructose solution as the sole source of liquid. From day 1 to day 63 of the stage I of the experiment, another rats (n=20; 10 males and 10 females) received a regular rations of vivarium, containing combination fodder-concentrate granulated (0.04 kg), low-fat cheese (0.006 kg), carrots (0.02 kg), cabbage (0.015 kg) per one animal.

At the second stage of the experiment the modeling of doxorubicin-induced liver damage was carried out for three days (from day 64 to day 66), according to which the rats were assigned into groups:

Group I (n=10; 5 males and 5 females) rats received a regular rations of vivarium from day1 to day 63. Subsequently, from day 64 to day 66 they were administered with 5mg/kg/day doxorubicin intraperitoneally (the cumulative doze of 15 mg/kg).

Group II (n=10; 5 males and 5 females) rats with overweight were exposed to modeled NASH from day1 to day 63. Subsequently, from day 64 to day 66 they were administered with doxorubicin similar to Group I.

Group III (n=10; 5 males and 5 females) rats received a regular rations of vivarium from day1 to day 63. Subsequently, from day 64 to day 66 they were administered with 0.9 % sodium chloride solution intraperitoneally at a dose of 1 ml.

The rats were sacrificed under thiopental anesthesia on day 67 of the observation.

Dissection of the animals' anterior abdominal wall was performed. The small and large intestines were taken out and cut lengthwise, subsequently washed with 0.9% sodium chloride solution and MM was separated with a scalpel. Then the MM of the small and large intestines was homogenized in 0.9% sodium chloride solution in a ratio of 1: 5 at a speed of 3000 rpm in a homogenizer.

In the homogenate of MM of the small and large intestines, the state of free radical oxidation (FRO) processes was determined by the concentration of substances that form a trimethine complex (TBA-reactants) with 2-thiobarbituric acid [4] and the antioxidant system (AOS) by catalase activity [2]. Resistance of the mucous barrier of the small and large intestines was determined by the concentration of NANA in the mucosal homogenate [1].

Statistical processing of the resulting data was performed using the GraphPad Prism version 5.00 statistical program (GraphPad Software, Inc., San Diego, CA, USA) to perform parametric and nonparametric statistical analysis. With the normal distribution of data, the results were presented in the form of arithmetic means (M) and their error (m). Significance of the differences was calculated using the Student's t-test. Paired nonparametric methods of Wilcoxon and Mann-Whitney rank tests were used in the distribution that differs from the normal one. The relationship between the studied indices was evaluated using Spearman's correlation analysis. Differences at $p<0.05$ were considered statistically significant. The research was carried out in compliance with the Declaration of Helsinki principles.

Results of the study and their discussion. In rats of Group I, administration of doxorubicin in a cumulative dose of 15 mg/kg led to activation of the FRO processes, which was characterized by 2.1 times ($p=0.002$) increased amount of TBA reactants in the small intestine homogenate and a decrease in catalase activity by 3.4 times compared to the control group ($p=0.002$) (table 1). Consequently, administration of doxorubicin in rats leads to imbalance between the FRO production and the activity of AOS enzymes in the MM of the small intestine, resulting in increased levels of free radicals, which is a prerequisite for oxidative damage to enterocytes.

Thus, oxidative stress in rats of Group I caused an increase in the level of NANA in the MM of the small intestine by 1.6 times compared to the control group ($p=0.002$) (table 1), which indicates

doxorubicin-induced activation of free radical damage in its MM. It can be assumed that doxorubicin leads to the development of oxidative stress, which is accompanied by both increased production of free radicals and antioxidant protection disorder of the small intestine.

Table 1

The indices of free radical oxidation and NANA content in the small intestinal mucosa, M±m

Indices	Groups of the experimental animals		
	I (n=10)	II (n=10)	III (n=10)
Catalase, μmol/g	5.005±0.1839 95% CI 4.589-5.421	3.315±0.1561 95% CI 2.962-3.668	17.42±0.2789 95% CI 16.79-18.05
p	p_1=0.002	p_2=0,002	p_3=0,002
TBA reactants, nkat/g	17.30±0.9149 95% CI 15.23-19.37	12.07±1.344 95% CI 9.029-15.11	8.211±0.2951 95% CI 7.544-8.879
p	p_1=0.0059	p_2=0.002	p_3=0.0059
NANA, μmol/g	52.41±2.978 95% CI 45.67-59.14	42.80± 1.776 95% CI 38.78-46.82	31.64±0.5672 95% CI 30.35-32.92
p	p_1=0.0137	p_2=0.002	p_3=0.002

Note: in this table and thereafter the significant difference between: p_1 ($p<0.05$) – indices of subgroups I and II; p_2 ($p<0.05$) – indices of subgroups I and III; p_3 ($p<0.05$) – indices of subgroups II and III.

In Group II rats with simulated NASH, administration of doxorubicin was accompanied by a 1.4-fold increase in the concentration of TBA reactants in the small intestine homogenate (p=0.0059) with a simultaneous 5.2-fold decrease in catalase activity compared to the controls (p=0.002). Oxidative damage of MM of the small intestine in Group II rats with NASH and overweight caused 1.4-fold increase in concentration of NANA in the small intestinal mucosa compared to the control group (p=0.002) (table 1).

Generally, in Group II rats with overweight, the degree of oxidative damage was characterized by a smaller increase in the indices compared to Group I. Thus, the level of TBA reactants and catalase activity in the homogenate of the small intestine in rats of Group II was lower by 1.5 (p=0.0059) and 1.4 times (p=0.002), respectively, compared to Group I. Moreover, the NANA concentration in the small intestinal mucosa of rats of Group II was by1.2 times (p=0.00137) lower compared to Group I, which indicates a less pronounced lesion of the mucous membrane (table 1).

In Group I rats, no significant changes in catalase activity and the amount of TBA reactants in the homogenate of the large intestine have been found compared to the control group. In Group II rats with overweight, the level of TBA reactants was by 1.8 times (p=0.0059) higher than in the control group and no significant changes in catalase activity was noted (table 2). The increased amount of TBA reactants is primarily a consequence of doxorubicin-induced oxidative stress, which leads to damage to enterocytes of the large intestine and a decrease in its resistance, as evidenced by an increase in NANA levels in MM of the large intestine in Group I rats by 1.7 times (p=0.002) in the absence of significant changes in rats of Group II, compared to the controls.

In overweight rats, the level of TBA reactants was by 1.6 times (p=0.0059) higher than in rats of Group I in the absence of significant changes in catalase activity (table 2).

Table 2

The indices of free radical oxidation and NANA content in the large intestinal mucosa, M±m

Indices	Groups of experimental animals		
	I (n=10)	II (n=10)	III (n=10)
Catalase , μmol/g	4.191±0.4483 95% CI 3.177-5.205	3.644±0.2521 95% CI 3.074-4.215	3.370±0.2395 95% CI 2.828-3.912
p	p_1>0.05	p_2>0.05	p_3 >0.05
TBA reactants, nkat/g	15.87±0.5586 95% CI 14.60-17.13	26.16±2.116 95% CI 21.37-30.95	14.66±0.6746 95% CI 13.14-16.19
p	p_1=0.0059	p_2>0.05	p_3=0,0059
NANA, μmol/g	5.857±0.478 95% CI 4.776-6.938	4.104±0.7153 95% CI 2.486 -5.722	3.445±0.5147 95% CI 2.280-4.609
p	p_1=0.0273	p_2=0.002	p_3>0.05

The findings indicate a higher level of oxidative stress in the mucous membrane of the large intestine in overweight rats compared to Group I. Admittedly, no more severe damage to MM has been noted, as evidenced by a 1.4-fold increase (p=0.0273) in the concentration of NANA in the mucous membrane of rats of Group I, compared to Group II.

Therefore, generally, administration of doxorubicin led to the development of doxorubicin-induced oxidative stress in the mucous membrane of the small and large intestine of rats of both groups. A characteristic feature of doxorubicin-induced intestinal lesions, regardless of the rats' body weight, was more severe damage to MM of the small intestine, which may be associated with more pronounced metabolic activity of proximal intestinal enterocytes. In addition, overweight rats had less pronounced intestinal lesions

compared to the rat with normal body weight. The findings can be assigned to the so-called "obesity paradox". Currently, it is hypothesized that excessive amount of the adipose tissue, which is usually roughly expressed in the body mass index, is associated with a lower survival rate in oncology treatment. However, some studies have proved that overweight and early obesity in patients was associated with better survival rates than in patients with normal body weight. This phenomenon, which characterizes the differences in the impact of obesity on the survival and treatment outcomes of patients, is called the "obesity paradox". Thus, the "obesity paradox" has been described in the treatment of numerous forms of oncology, namely, colorectal cancer [13], kidney cancer [7], in the treatment of acute myeloid leukemia in elderly patients [5], and in patients with lymphoma who underwent autologous hematopoietic stem cell transplantation [11].

Sinicrope FA, Foster NR [14] in the study of the prognostic value of body mass index in 25.291 patients with colon cancer of stage II and III who took 5-fluorouracil as adjuvant therapy, proved that the survival rates of patients with body mass index 25.0-34.9 kg/m^2 were better compared to patients who had a normal weight.

The explanation for the higher survival rate of overweight patients can be the so-called hibernation hypothesis, which considers the adipose tissue as an energy depot that during stress, which is actually anticancer therapy, may give an advantage in survival. This theory is similar to the hibernation theory in evolutionary biology, in which animals set aside energy reserves in anticipation of difficult times ahead [9].

Conclusions

1. In rats without NASH, administration of doxorubicin at a cumulative dose of 15 mg/kg leads to oxidative damage to the MM of the small intestine, which was characterized by an increase in the amount of TBA reactants by 2.1 times (p=0.002), a decrease in catalase activity by 3.4 times (p=0.002) and an increase in the level of NANA by 1.6 times (p=0.002) in its homogenate compared to the control group.

2. In rats with NASH and overweight, administration of doxorubicin at a cumulative dose of 15 mg/kg leads to activation of free radical lesions of the MM of the small intestine, which was characterized by a 1.4-fold increase in the concentration of TBA reactants (p=0.0059) concomitant with decreased catalase activity – by 5.2 times (p=0.002), and an increase in NANA concentration – by 1.4 times compared to the control group (p=0.002).

3. In MM of the small intestine of rats with NASH, administration of doxorubicin caused less pronounced oxidative damage compared to the rats with normal body weight, as evidenced by lowering the level of TBA reactants by 1.5 times (p=0.0059), catalase activity – by 1.4 times (p=0.002) and NANA concentration – by 1.2 times (p=0.00137).

4. In MM of the large intestine of rats with NASH and overweight, administration of doxorubicin caused a more pronounced activation of FRO, as evidenced by a 1.6-fold higher (p=0.0059) level of TBA reactants compared to rats with normal weight and by 1.8 times higher (p=0.0059) compared to the control group. Admittedly, no more severe damage to MM was noted, as evidenced by a 1.4-fold increased (p=0.0273) concentration of NANA in normal weight rats compared to overweight rats.

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