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Glutathione transferase activity and reduce glutathione content in the cytosol of rat gastric mucosa cells under carcinogen N-methyl-N'-nitro-N-nitrosoguanidine treatment

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Aim. *To determine the activity of glutathione transferase (GT) and the content of reduced glutathione (GSH) in the cytosol of the gastric mucosa cells in experimental gastrocarcinogenesis.* ***Methods***. *The activity of GT was determined spectrophotometrically, the content of GSH was measured spectrofluorimetrically. Gastrocarcinogenesis was initiated by 10-week replacement of drinking water by 0.01 % solution of carcinogen N-methyl-N'-nitro-N-nitrosoguanidine, at the same time the rats were given a diet containing 5 % NaCl.* ***Results***. *It was establi- shed that at the end of the 4th and 6th weeks of consumption of carcinogen and NaCl, the activity of GT increased by 26 and 94 %, whereas the content of GSH increased by 135 and 85 %, respectively. After 12 weeks there was a decrease in the activity of GT by 50 % and the maximum decrease in the GSH concentration by 69 %. At the end of the 18th and 24th weeks it was recorded the increase in the activity of GT by 44 and 47 % and the decrease in the GSH content by 55 and 52 %.* ***Conclusions***. *The changes in the activity of GT and GSH-content are evi- dence of the violation of glutathione homeostasis, which may cause the delay as well as initiation of development of the pathology. The reduction of GSH is established at the early stages of tumors formation.*

Keywords: glutathione transferase, reduced glutathione, gastric cancer.

Introduction. The glutathione system has a unique role in the formation of organism resistance to different chemical and physical factors. This is the most important defence mechanism of the cell, involved in biotransformation of exogenous foreign compounds and a number of endogenous substances, for instance, hydroperoxides of polyunsaturated fatty acids – linole ic and arachidonic acid, the products of peroxide oxidation of lipids – 4-hydroxy-2-enal, cholesterol-oxide, and others. This system performs antioxidative functions, facilitates redox-homeostasis; it includes glutathione-dependent enzymes (glutathione transferase and glutathione peroxidase) as well as the enzymes of aldehydes elimination (glyoxalase I, formaldehyde dehydrogenase). However, the highest diversity and significance are attributed to the functions of glutathione transferase (GT) [1] and reduced glutathione (GSH) which directly or indirectly related to all the detoxication stages in both extra-microsomal and microsomal media [2]. Similar to thiotransferases [1], GT is capable of reducing some disulfides demonstrating expressed glutathione peroxidase activity, while reducing organic hydroperoxides [2]. Moreover, some GT isoenzymes demonstrate higher activity regarding thymine and DNA peroxides compared to glutathione peroxidases [3].

GTs (EC 2.5.1.18) are a group of isoenzymes of the second phase of detoxication, metabolizing the majority of exogenous and endogenous hydrophobic

electrophilic compounds due to conjugation with glutathione, which results in an increase in their solubility in water and facilitation of exocytic release via special ATP-dependent transport systems [5]. GTs are known to destroy over 3,000 organic compounds of almost all classes: alkenes, arenes, aralkenes, halogen compounds and oxygen-containing compounds, derivatives of sulphur, nitrogen, phosphorus, which are toxic, carcinogenic, and mutagenic substances, cytostatics, pesticides, dyes, medicines, etc. In addition to the biotransformation of xenobiotics, GTs perform important functions in the endogenous metabolism of leukotriens and prostagladins [1]. In the organism GTs bind such non-substrate ligands as bilirubin, biliary and fatty acids, steroids, heme derivatives [1, 6], and thyroid hormones [7]. GTs of classes м and p are the regulators of MAP-kinase cascade, related to the signalling of cell survival or death [7].

Due to the presence of a reactive sulfhydryl group, the tripeptide glutathione takes part in numerous metabolic reactions [4]. In particular, it sustains the functional activity of membranes, participates in the protein synthesis as a storing and transporting form of cystein, is involved in transpeptidation of amino acids, the synthesis of DNA predecessors (reaction of reducing ribonucleoside diphosphates to desoxyribonucleoside diphosphates) and the simulation of the conformation of protein molecules and the regulation of enzyme activity [2]. It is the main redox-buffer of the cell [4]. A very important function of GSH is to eliminate peroxide compounds using glutathione peroxidases and detoxication of xenobiotics and organic peroxides, implemented by GT.

Intensification of research of this enzyme in carcinogenesis, in gastric carcinogenesis, in particular, is caused by numerous data on the increase in the GT activity [1] and expression [7] (some of the isoforms, in particular) for most cases of malignant growth as well as recently discovered capability of GTs of some classes to simulate the work of signalling pathways, related to the apoptosis and cell proliferation [7, 8]. In the breakdown of cancer morbidity and mortality gastric cancer is rated the second after lung cancer [9, 10]. The frequency of diagnosing early forms of gastric cancer is less than 10–20 %, and regional metastases are found in 83 % of patients with primary cancer [11]. At the same time the study of the early stages of pathology may be a basis for understanding pathogenesis and, even more, for the improvement of treating patients with gastric cancer.

Taking the abovementioned into account and considering the oxidative stress as a key factor in the pathogenesis of gastric cancer [10, 12], we have studied the activity of GTs and the content of main low-molecular thiol – GSH – in cytosol of the gastric mucosa cells under conditions of experimental gastric carcinogenesis.

Materials and Methods. The experiments were conducted using white male rats ($n = 80$) with the initial weight of 100 ± 20 g.

Gastric carcinogenesis was initiated by replacement of drinking water with 0.01% solution of carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 10 weeks while the rats were given a diet, containing sodium chloride (5% NaCl of dry weight). After this period the animals were fed with standard vivarium diet till the end of the 24th week [13]. Then they were killed by the cervical vertebrae dislocation. The mucous membrane of the extracted stomach was turned outward and fixed in neutral 10 % solution of formalin for histological investigations, the mucous membrane cells were extracted from other stomachs for biochemical analysis. The sampling of experimental material was performed at the end of the 4th, 6th, 8th, 10th, 12th, 18th, and 24th weeks. The control group of animals was fed with the standard diet.

The development of investigated pathology was diagnosed visually and histologically. The extracted stomach was fixed in 10 % neutral buffered formalin, and embedded with paraffin after the standard histological treatment. The cuts of 5–7 мм were stained with Boehmer hematoxylin and subsequent addition of eosine and orange G [14]. The obtained preparations were analyzed at the light-optical level using Olympus BX-41 microscope (*Olympus Europe GmbH*, Japan); the micropictures were obtained with Olympus C-5050 Zoom digital camera (*Olympus Europe GmbH*); the morphometry was performed using WCIF ImageJ software.

The cells of gastric mucosa (mucous membrane) were isolated by the method, based on the enzymatic disaggregation of cells using pronase [15, 16]. This

method presupposes turning the mucous membranes of the isolated stomachs outward using ligatures, filling them with the pronase solution (1 mg/ml), incubating (30 min, 37 °C) at intense stirring in the medium, saturated with 95 % O_2 and 5 % CO_2, and harvesting the disintegrated cells. To obtain cytosol the isolated cells were homogenized on ice in a small teflon Potter-Elvenjem homogenizer. The homogenate was centrifuged at 20,000 *g* for 15 min (4°C) at Sigma centrifuge (USA). GT-activity and GSH content were revealed in the supernatant. At last, 0.01 M of formic acid was added to homogenate (1:1) to precipitate the proteins [17].

The GT activity was determined by the optic density of S-(2,4-dinitrophenil)-glutathione (the product of glutathione conjugation with 1-chlor-2,4-dinitrobenzol) which is characterized by the adsorption maximum at $\lambda = 346$ nm [18]. The reaction mixture contained 1.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.2 ml of 10 mM of GSH, 0.1 ml of the supernatant. The reaction was launched by the addition of 0.02 ml of 0.1 M 1-chlor-2,4-dinitrobenzol. The surplus of the optic density was registered for 4 min at $\lambda = 346$ nm and expressed in the conjugate nanomoles per 1 ml of cytosol protein for 1 min.

The GSH content was registered using orthophthalic aldehyde, the reaction of the latter with GSH results in the formation of highly fluorescent products ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 420$ nm) [17]. The final mixture for the analysis consisted of 100 мl of the supernatant, diluted tenfold with 0.1 M phosphate buffer with 5 mM EDTA (pH 8.0), 1.8 ml of phosphate-EDTA buffer and 100 мl of ortho-phthalic aldehyde (1 mg/ml in methanol). The fluorescence intensity was measured at 420 nm with the activation of 350 nm after 15 min incubation at room temperature. The calibration curve was built to determine GSH concentration.

The protein concentration was registered by Bradford's method [19]. The measurements were performed using Ultrospec 1100 pro spectrophotometer (*Amersham Biosciences*) and RF-510 spectrofluorometer (*Shimadzu*).

The experimental data were processed by common methods of the variance analysis with 10 repeats. The reliability of discrepancies between two samplings was determined using Student's criterion. The results are presented in the values of the arithmetic mean and mean square error, $M \pm m$ [20].

The investigations are in good agreement with the main requirements to keeping and working with laboratory animals in accordance to the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) as well as with the ethic norms specified in the Ukrainian legislation.

Results and Discussion. The carcinogen MNNG, inducing in rats the formation of tumors, morphologically and histologically similar to those, diagnosed in humans, was used to induce the experimental gastric carcinogenesis [21].

This carcinogen was chosen as an inducer of malignant transformation because people are affected by carcinogenic substances, similar to MNNG, during intragastric nitrosylation of such natural guanidine compounds as L-arginine and creatinine by food nitrates in the presence of hydrochloric acid [22]. High salt concentration impairs the mucus barrier in the stomach, causes the inflammation, diffuse erosion and degradation. It is highly probable that the consumption of high amounts of salt increases the risk of gastric cancer in humans [21].

The investigators use 0.01 % MNNG solution to create the experimental model of precancerous changes in the stomach [23]. It was established that the consumption of MNNG solution for 4 and 6 weeks causes an increase in the GT activity by 26 and 94 % respectively, compared to the control (Fig. 1). These changes may be explained by intensification of the metabolic inactivation of the carcinogen by the gastric mucous membrane cells due to the conjugation of the xenobiotic and GSH, the level of which also increased (by 135 and 85 %) in the same periods of investigation (Fig. 2). The latter proves the literature data, indicating GT as an inducible enzymes. The activity of these enzymes considerably increases when introducing different xenobiotics, including carcinogenic substances [1].

GTs destroy xenobiotics, inactivating them chemically and physically. Physical inactivation consists in binding xenobiotic molecules with ligandin sites of GTs which results in temporary decrease in their concentration in the cell. GTs are assumed to transport

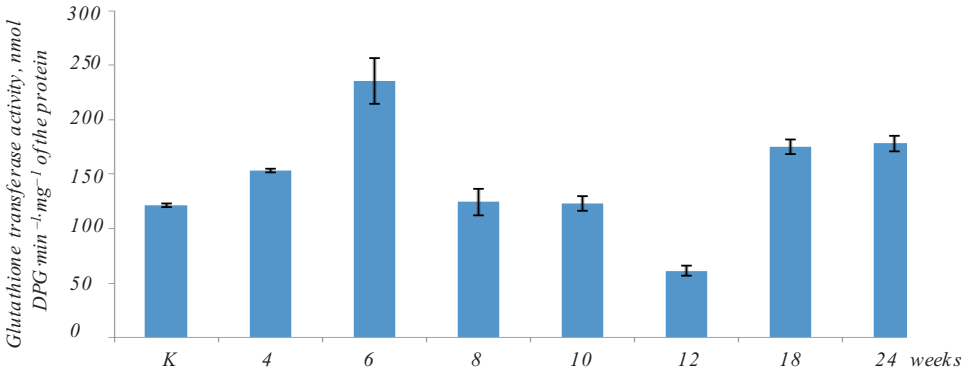

Fig. 1 Glutathione transferase activity in the cytosol of gastric mucous membrane cells. *DPG – S-(2.4-dinitrophenil)-glutathione; **p <0.05 (difference reliability compared to the indices of the control group of rats). The axis of abscissas – the period of observing (weeks) MNNG effect

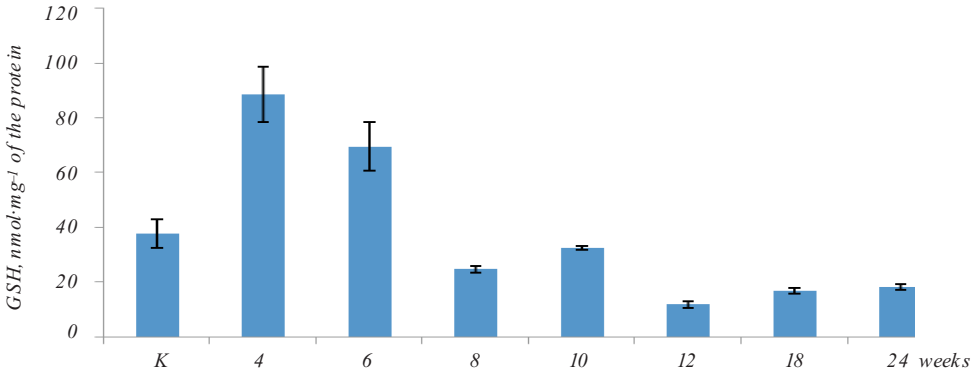

Fig. 2 The content of reduced glutathione (GSH) in the cytosol of the gastric mucous membrane cells. *p < 0.05 (difference reliability compared to the indices of the control group of rats). The axis of abscissas – the period of observation of MNNG effect (weeks)

xenobiotics and transfer them to the enzymes of the first phase of detoxication – P450 cytochrome, and monooxygenases, while the oxidized product is used in the transferase reaction. Chemical inactivation is implemented via the catalysis of two types of reactions: nucleophilic binding of the GSH thiol group to the substrate electophilic center and nucleophilic substitution for glutathione of the electrophilic atom of carbon, nitrogen, sulphur or phosphor [8].

Therefore, GTs bind numerous hydrophobic substances, but chemically destroy only those with the electrophilic center [1]. Most reactions, catalyzed by GTs, result in the formation of thioethers which are glutathione conjugates; GSH is irreversibly lost. Then the thioethers are transported by the blood flow into the liver, where they are transformed into cysteic conjugates. The latter are N-acetylated and transformed into non-toxic mercapturic acids, excreted with urine [24].

It seems logically to assume that GT induction at the MNNG introduction is a defence mechanism of the organism. However, the metabolic activation of some xenobiotics and their transformation into strong alkylators, capable of destroying DNA, were registered for GT. Alkylnitrosoguanidines [1], the structure of which is similar to that of MNNG, are an example of such compounds. In this case the stimulating effect of MNNG in the pathogenesis of chemically induced gastric cancer is conditioned by the activation of this compound by glutathione transferase.

Taking the abovementioned into consideration, it is difficult to determine a role of GT activation in the development of the pathology in question, as it can be both inhibitory (via intensification of carcinogen evening-out) and stimulation due to the metabolic activation of the carcinogen.

In acidic medium of the stomach, MNNG is transformed into N-methyl-N'-nitroguanidine with the release of nitrous acid. This active form of carcinogen is capable of alkylating DNA and RNA purine bases of epithelial cells with the formation of 7-methylguanine and 3-methyladenine [25] and the occurrence of mutations [26], as well as conditioning the methylation of proteins [23] and generation of active forms of oxygen [27].

A decrease in the thickness of the mucous membrane and in the depth of the intestinal crypts was discovered after four weeks of MNNG effect. At the same time the area of parietal and main cells and their nuclei increased by 20–30 % (Fig. 3, *b,* see the insert). The latter may testify to the enhancement of the functional activity of cells, the main function of which is known to be the secretion of the hydrochloric acid into the gastric lumen and the production of mucus, probably for the protection against the damaging factor. At the end of the 6th week of MNNG effect there were changes in the morphofunctional state of the gastric mucous membrane, which is evidenced by the extension of gland lumens, the extension and plethora of vessels, the desquamation of epithelial cells, and the increase in the mucus production (Fig. 3, *c,* see the insert).

At the stage of the 8th and 10th weeks of the study on experimental gastric carcinogenesis the level of GT-activity corresponded to that of the control animals.

The decrease in GT-activity to the control indices compared to previous periods may be related to the reliable decrease in GSH content (by 34 %) during the 8th week. Here the participation of GTs in the processes of chemical detoxication may be impaired. However physical destruction is also probable and it consists in noncovalent binding to ligandin sites of the enzyme of hydrophobic substances, including nitroso compounds [1, 2].

In conditions of the oxidative stress some GT represntatives (for instance, GTP1-1 of p family) are significant for the self-defence mechanism. When the glutathione pool is exhausted, the nitrosylated glutathione nitrosylates Cys47 and Cys110 residues of one of GT subunits. Due to the negative cooperation the reactionary active residue Cys47 of another subunit is absconded from nitrosylation and retains its capability of a typical transferase reaction [8], which may be the reason of retaining GT activity during the 8th week in the range of control values.

After the MNNG effect for 8 weeks the gastric mucosa demonstrates the extension of gland lumens, the extension and plethora of vessels as well as the features of inflammation, the atrophic changes of the surface epithelium (Fig. 4, *a* see the insert). Some samples demonstrated gastric erosion and ulcers. The consumption of MNNG by animals for 8 weeks causes reliable thinning of the mucous membrane and decrease in the depth of gastric pits.

At the next stage of investigation (the 10th week) the intragastric content of GSH corresponded to the control values. In conditions of physiological values of GSH content the nitrosylated glutathione is transformed into dinitrosyl-diglutathionyl-iron complex (DNDGIC) with extremely high affinity to GT; the catalytic activity of the subunit, related to the complex formation, is lost. In these conditions the conformation of the other subunit changed with the decrease in the affinity to DNDGIC, however, it does not prevent the occurrence of its transferase activity via the involvement of the abovementioned self-defence mechanism [8]. At the end of the 10th week of MNNG effect there were considerable morphofunctional changes in the gastric mucosa (mucous membrane), its thickening, the extension of the gland lumens, the inflammation, some metaplastic and atrophic changes in the surface epithelium (Fig. 4, *b,* see the insert). There are areas with hyperplastic changes, some cells with atypia and hypertrophic modifications.

The decrease in GT activity by 50 % as well as maximal decrease in GSH concentration by 69%, compared to the control values, were registered after 12 weeks. The registered decrease in the enzymatic activity may be related to the formation of conjugates, capable of inhibiting GT [1].

In conditions of long-term GSH deficiency the third function of GT – covalent binding of strong electrophils, active metabolites of carcinogen – can be performed completely. This interception of alkylators inactivates the enzyme, but it is an additional defence mechanism of the cell. This capability of the enzyme is promoted by its high affinity to hydrophobic substances and large amount in the cell [2, 8].

It should be noted that the signs of malignant transformation were revealed on histological preparations of the gastric mucosa as early as during the 12th week of the experimental gastric carcinogenesis (Fig. 4, *c,* see the insert). At the end of the 12th week the gastric mucosa undergoes significant changes, there are the extension of gland lumens, inflammation signs, atrophic and metaplastic modifications, sites with hyperplastic impairments, and some cells with atypia. Some rats had gastric erosions

Figures to article by Tymoshenko M. O. et al.

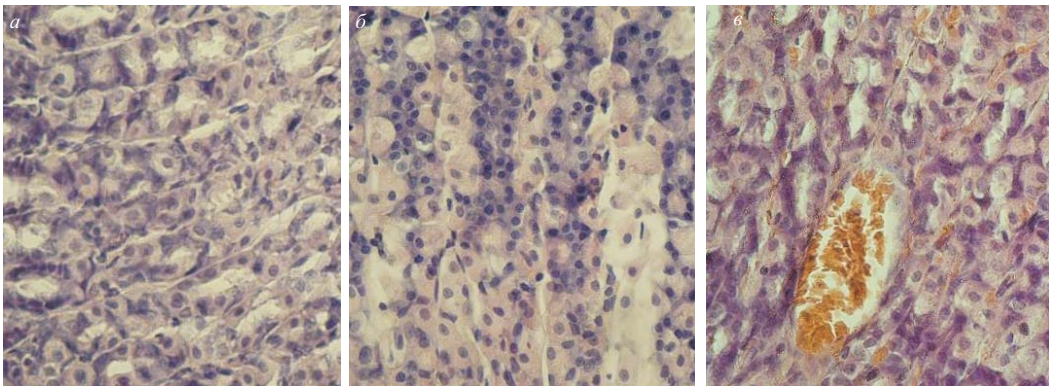

Рис. 3. Мікрофотографії зрізів слизової оболонки шлунка за умов експериментального гастроканцерогенезу: *а* – контроль; *б* – 4-й тиждень; *в* – 6-й тиждень. Забарвлення гематоксиліном та еозином; × 600

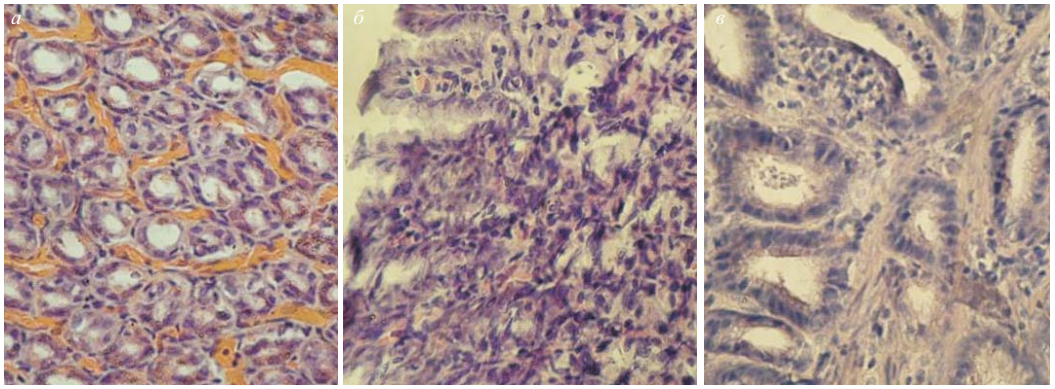

Рис. 4. Мікрофотографії зрізів слизової оболонки шлунка за умов експериментального гастроканцерогенезу: *а* – шлункові ямки (8-й тиждень); *б* – метапластичні зміни (10-й тиждень); *в* – аденома (12-й тиждень). Забарвлення гематоксиліном та еозином; ×600

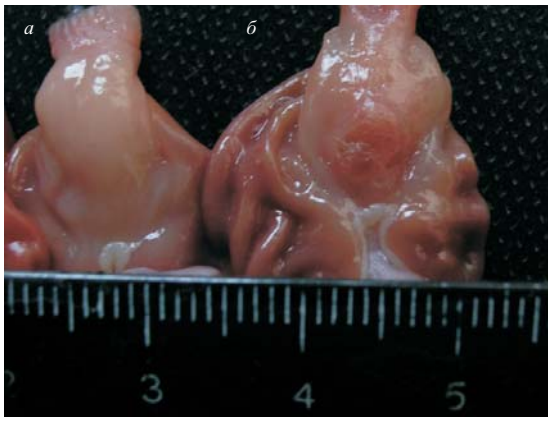

Рис. 5. Макроскопічні зміни слизової оболонки шлунка через 18 тижнів експериментального гастроканцерогенезу: *а* – контроль; *б* – 18-й тиждень гастроканцерогенезу (*1* – пухлина)

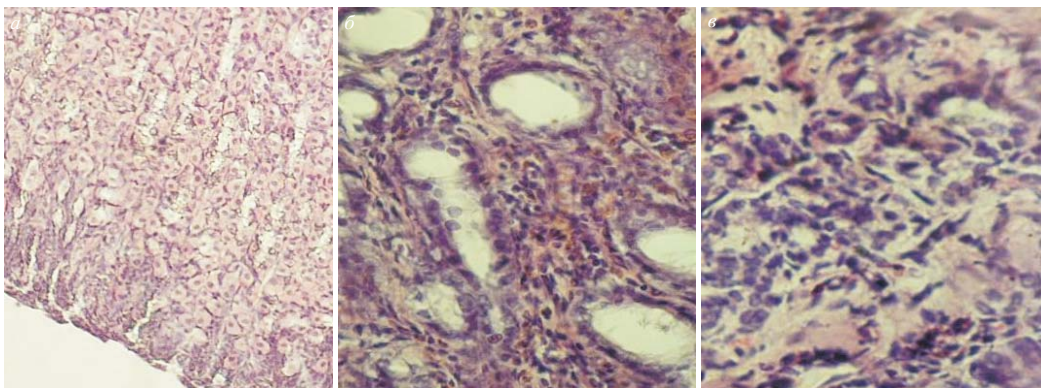

Рис. 6. Мікрофотографії зрізів слизової оболонки шлунка за умов експериментального гастроканцерогенезу: *а* – контроль; *б* – аденома (18-й тиждень); *в* – аденокарцинома (18-й тиждень). Забарвлення гематоксиліном та еозином; × 600

and ulcers. Some animals had adenomas and one adenocarcinoma.

At the end of the 18th and 24th weeks of the experimental gastric carcinogenesis the GSH concentration in the gastric mucosa decreases by 55 % and 52 % respectively. The increase in GT activity by 44 % and 47 % compared to the control was registered in the same periods of the study. This increase may be caused by the increase in the number of endogenous substrates as the stimulation with carcinogen-xenobiotic was terminated at the 10th week. It may be related to the induction of the enzyme by the products of the peroxide oxidation of lipids. The result of the reactions of reducing organic peroxides to alcohols by glutathione transferases is oxidized glutathione which is capable of being reduced by glutathione reductase later.

Starting from the 18th week 70 % of rats had the neoplasms in the pyloric part of the stomach, which were in the form of local dish-like thickenings of the gastric mucosa with a bowl in the center (Fig. 5, see the insert), the histological studies demonstrated their similarity to adenoma and adenocarcinoma (Fig. 6, *b, c,* see the insert). The cuts of gastric mucosa had visualized deformed glands, dysplastic changes, cell atypia and regenerative hyperplasia with erosions and ulcers.

Taking into consideration the complicated process of regulating GT activity as well as the impact of MNNG xenobiotic and its derivatives, it is difficult to determine unambiguously the significance of this enzyme at each stage of MNNG-stimulated gastric carcinogenesis. However, there is no doubt that GTs and the system of their functioning play an important role in the cell transformation not only due to defence from free radicals, degradation and removal of exo- and endogenous electrophilic compounds from the organism, however, this system can be also involved in the stimulation via the generation of additional metabolically aggressive derivatives of the carcinogen.

Conclusions. The changes in glutathione transferase activity and concentration of reduced glutathione testify to the impairment of glutathione homeostasis, which may be related to both delay and initiation of the pathology development. The decrease in GSH content at the early stages of tumor formation has been discovered.

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Глутатіонтрансферазна активність і вміст відновленого глутатіону в цитозолі клітин слизової оболонки шлунка щурів після впливу канцерогену N-метил-N'-нітро-N-нітрозогуанідину

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Summary

Мета. *Визначити глутатіонтрансферазну (ГТ) активність і вміст відновленого глутатіону (GSH) у цитозолі клітин слизової оболонки шлунка за умов експериментального гастроканцерогенезу.* ***Методи***. *ГТ-активність визначали спектрофотометричним методом, вміст GSH – методом спектрофлуориметрії. Гастроканцерогенез ініціювали 10-тижневою заміною питної води на 0,01 %-й розчин канцеро- гену N-метил-N'-нітро-N-нітрозогуанідину з одночасним переведенням щурів на корм, який містить 5 % NaCl.* ***Результати***. *По закінченні 4- і 6-тижневого споживання щурами канцерогену і NaCl ГТ-актив- ність зростала відповідно на 26 і 94 %, тоді як вміст GSH – на 135 і 85 %. Через 12 тижнів спостерігали зниження активності ГТ на 50 % і максимальне зменшення концентрації GSH на 69 %. Після 18 і 24 тижнів зафіксовано зростання ГТ-активності на 44 і 47 % та зниження вмісту GSH на 55 і 52 %.* ***Висновки***. *Зміни ГТ-активності і концентрації GSH свідчать про порушення глутатіонового гомеостазу, що може призводити як до затримки, так і ініціації розвитку патології. Встановлено зниження вмісту GSH на ранніх стадіях формування пухлин.*

Ключові слова: глутатіонтрансфераза, відновлений глутатіон, рак шлунка.

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Глутатионтрансферазная активность и содержание восстановленного глутатиона в цитозоле клеток слизистой оболочки желудка крыс после воздействия канцерогена N-метил-N'-нитро-N-нитрозогуанидина

Резюме

Цель. *Определить глутатионтрансферазную (ГТ) активность и содержание восстановленного глутатиона (GSH) в цитозоле клеток слизистой оболочки желудка в условиях экспериментального гастроканцерогенеза.* ***Методы***. *ГТ-активность определяли спектрофотометрическим методом, содержание GSH – методом спектрофлуориметрии. Гастроканцерогенез инициировали 10-недельной заменой питьевой воды на 0,01 %-й раствор канцерогена N-метил-N'-нитро-N-нитрозогуанидина с одновременным переведением крыс на корм, содержащий 5 % NaCl.* ***Результаты***. *По окончании 4 и 6 недель потребления крысами канцерогена и NaCl ГТ-активность увеличилась соответственно на 26 и 94 %, тогда как содержание GSH – на 135 и 85 %. Через 12 недель наблюдали снижение ГТ-активности на 50 % и максимальное уменьшение концентрации GSH на 69 %. Спустя 18 и 24 недели зафиксировано возрастание активности ГТ на 44 и 47 %, а также снижение содержания GSH на 55 и 52 %.* ***Выводы***. *Изменения ГТ-активности и концентрации GSH свидетельствуют о нарушении*

гомеостаза глутатиона, что может вызывать как задержку, так и инициацию развития патологии. Установлено снижение содержания GSH на ранних стадиях формирования опухолей.

Ключевые слова: глутатионтрансфераза, восстановленный глутатион, рак желудка.

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