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EXPRESSION OF *CHGA* GENE IN RAT DUODENAL EPITHELIAL CELLS UPON LONG-TERM GASTRIC HYPOACIDITY AND WITH ADMINISTRATION OF MULTIPROBIOTIC SYMBITER

The increasing of Chga gene's expression in rat's duodenal villus and crypt epithelial cells upon gastric hypoacidic conditions were shown. The level of Chga mRNA was similar to the control value both in villus and crypt epitheliocytes upon treatment of hypoacidic rats with multiprobiotic Symbiter.

Key words: gastric hypoacidity, duodenal, rats, Chga gene expression, multiprobiotic.

Introduction. In recent decades, proton-pump inhibitors (PPI) of gastric parietal cells, such as omeprazole, remain the most effective therapeutic agents against acid-related disorders [18, 19]. Development of dysbiosis is one of the key consequences of long-term hypoacidity. Colonization of gastrointestinal tract (GIT) by opportunistic microbiota forms stable sources of endogenous infection and besides the effect of hypergastrinemia additionally promotes gastric carcinogenesis and tumorigenesis both in other parts of GIT and associated organs [1, 20].

Chromogranin A (encoded by *Chga* gene) – is the protein of granin's family, it expresses in neuroendocrine cells of APUD–system (for example, endocrine cells if GIT), which is the integrated control diffuse neuroendocrine system of human organism [8, 11, 14, 22, 23]. Expression of this gene is regulated exactly by gastrin and chromogranin A is necessary for processing of histamine propeptide in the gastric epithelial cells [5]. In small intestines, in particular in duodenum, chromogranin are produced by different types of enteroendocrine cells: P/D1, EC (enterochromaffin cell), D, G, S, N and so on [1, 11].

Analysis of scientific literature showed, that chromogranin A is a potentially useful approach for identification of inflammatory intestinal disorders: unspecific ulcerative colitis and Crohn's disease [14, 22, 23].

It was found more than 20 types of neuroendocrine cells (pancreatic islets, cells of APUD-system and so on) which can develop into respective malignancies under progression of cancers (nesidioblastoma, glucagonoma, gastrinoma, somatostatinoma, carcinoids of GIT and so on) [6, 8, 11, 14, 22, 23]. Thus, it was shown overexpression of *Chga* gene, focal hyperplasia of ECL-cells (enterochromaffin-like cells) with future development of malignant carcinoid from these cells upon long-term uninterrupted administration of PPIs from omeprazole, lansoprazole and other groups [5, 10, 13].

Overexpression of *Chga* gene mRNA is demonstrated in carcinomas with metastatic tissue and in GI tumors, in particular in duodenal carcinoid (extremely rare tumor which biological behavior has not been fully elucidated) [22]. Chromogranin A is found in neuroendocrine tumors, which whether secrete or do not extract hormones and amines, what is especially important for early diagnosing of their concealed functional activity, when characteristic clinic manifestations of disorder are absent as overexpression of appropriate hormones, but the tumor is located. So, the determination of *Chga* mRNA level is a useful tumor marker for the monitoring of gastrointestinal neuroendocrine carcinoids [14].

It was proved in clinical trials that probiotics were able not only to cure dysbiotic states, but also to instantly reduce damage ratio of GIT [3, 7]. Multiprobiotics of "Symbiter® acidophilic" concentrated group (hereinafter referred to as Symbiter) are characterized by complexity, wide array of bioactivity, high level of safety for organism and composition that is maximally close to nature microbial populations of human and animals [7, 12].

Analysis of scientific literature showed lack of data on the pattern of above mentioned gene expression in duodenal upon experimental or natural hypoacidity. Data about effect of probiotics on *Chga* gene expression in duodenal upon these conditions are also absent.

Consequently, the aim of current investigation was to determine the expression of *Chga* gene in rat duodenal epithelial cells upon long-term gastric hypoacidity by means of injection of omeprazole (and thereafter upon the excess of gastrin in blood) and with administration of Symbiter.

Materials and methods. The International recommendations on performance of medical and biological investigations with the use of animals according to European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes were followed. Experiments were carried out on white non-strain male rats with initial weight around 180-200 g.

All animals were divided into four groups. The rats injected abdominally with 0,2 ml of physiological solution and 0,5 ml of water for injections orally were used as a control (first group). Animals of second group were treated with Symbiter (manufactured by LLC "O.D. Prolisok") orally (0,14 ml/kg) during 28 days. Hypoacidity (third group) was modeled by everyday intraperitoneal injection of omeprazole (14 mg/kg) during 28 days [21]. Fourth experimental group simultaneously with omeprazole obtained Symbiter in the same dose. Number of animals in each experimental group was 6. Crypts and villi of duodenal epithelial cells were extracted by means of low-temperature method [9]. RNA was isolated following Chomczynski and Sacchi [2]; cDNA was synthesized in 20 μl of reaction mix containing 2 μg of RNA, 1 mM dNTP, 50 U of reverse transcriptase "MultiScribeTM Reverse Transcriptase", corresponding buffer, 20 U of ribonuclease inhibitor "RNase Inhibitor" ("Applied Biosystems", США), 20 pmol (1,0 μM) of reverse primer. Synthesis was carried out in the following conditions: 37° C – 2 hour. Polymerase chain reaction was performed in 30 μl of reaction mix containing 10 μl of cDNA, PCR buffer, 200 μM of each dNTP, 30 pmol (1,0 μM) of each primer, 2,5 mM of MgCl2 and 1,5 U of Taq DNA polymerase ("iTaqTM", "Bio-Rad", США).

PCR amplifications consisted of an initial denaturing step of 95ºC for 3 min, followed by 35 (28 for Actb – gene used as internal control of reaction due to its constitutive expression) cycles of 95ºC for 45 s, the annealing step (with optimal annealing temperature): *Chga* (620 b.p., 59ºC – 45 s) and *Actb* (521 b. p., 49º C – 40 s); the extending step at 72º C for 1 min 15 s (for *Chga*) or 1 min (for *Actb*). Final extension step was performed upon 72º C for 5 min.

Such primer sequences were used in reactions: for *Chga* – forward – GGCCCAGCAGCCGCTGAAGCAGCA and reverse – CTCTGCGGTTGGCGCTGCCCTCCT; for Actb – forward – TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGACCT. Reproducibility of the amplification results was evaluated in parallel experiments by the repetition of the PCR reactions with all ani-

mals and each primer at least three times. Separation of PCR products was performed electrophoretically in 1,6 % agarose gel with 0,5 x TBE buffer following Sambrook et al. [17]. For semi-quantitative analysis of amplicons expression based on densitometry the ImageJ 1.45s program was used. Indices of mRNA expression were calculated for each sample following Konturek et al. [15].

Mathematical and statistical processing of experimental data was performed using GraphPad Prism 4.03 ("GraphPad Software Inc.", USA). The normal Gaussian distribution of the data was verified by the Shapiro-Wilk normality test. Two-way analysis of variance (two-way ANOVA) and Bonferroni post tests were performed on obtained data. Statistical significance was set at $p \leq 0,05$. The data are expressed as means and standard deviations.

Results and discussion. PCR analysis of cDNA samples generated in the rat's duodenal villous and crypt epithelial cells indicated the presence of a specific signal with the expected length (620 b. p.) for *Chga* gene both in the control and second (animals treated only with Symbiter) groups of investigated animals (Fig. 1.).

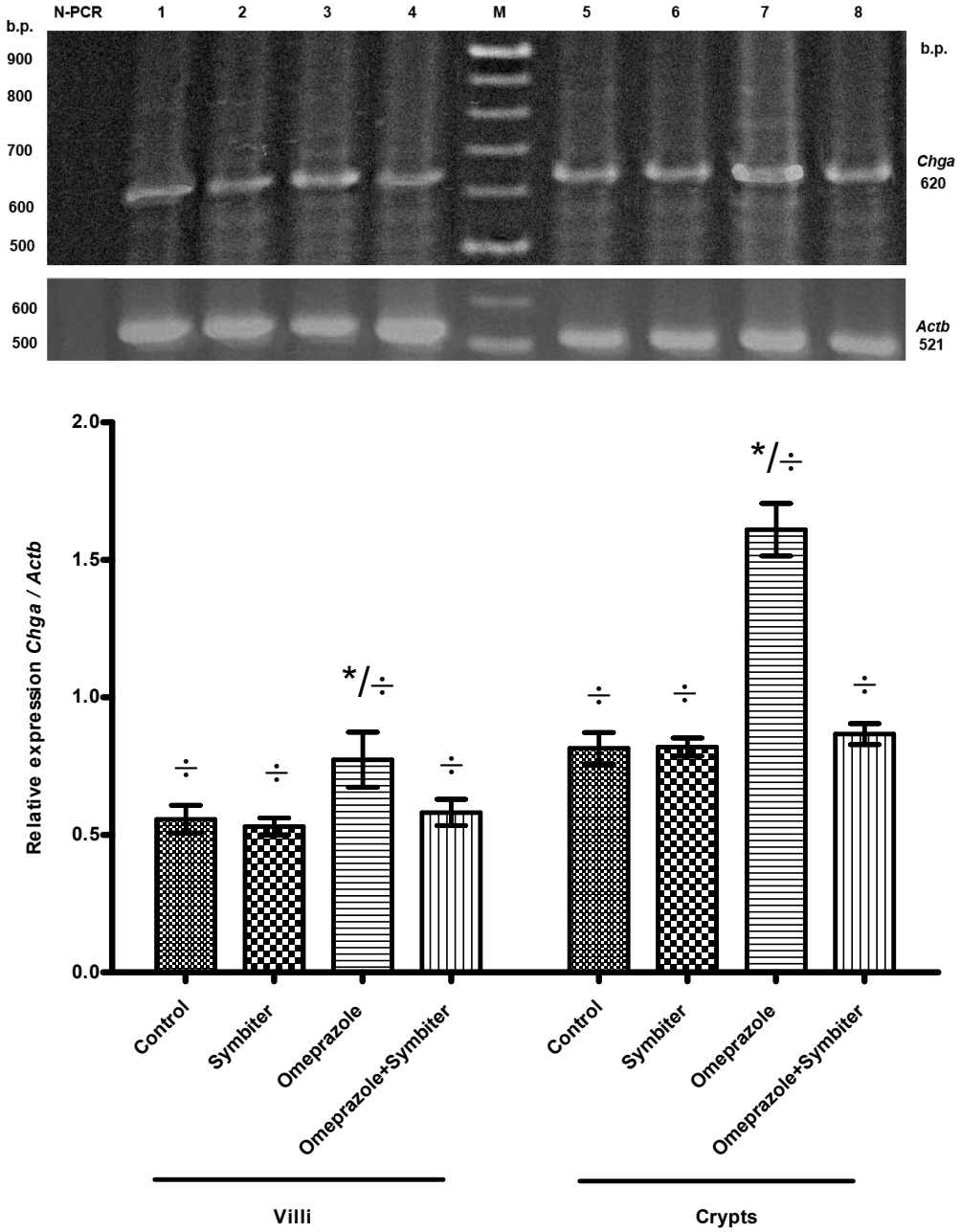

Fig. 1. Level of *Chga* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic Symbiter.
M – molecular mass marker; villus epithelial cells: 1 – control; 2 – Symbiter; 3 – omeprazole;
4 – omeprazole + Symbiter; crypts epithelial cells: 5 – control; 6 – Symbiter; 7 – omeprazole;
8 – omeprazole + Symbiter; N-PCR – negative PCR control; * – $p \leq 0,0001$ in relation to control; ÷ – $p \leq 0,0001$ villi in comparison with crypts

It was established that the levels of *Chga* gene's expression did not significantly differ in the control and second groups from one another both in villus epitheliocytes and crypts comparing with the types of epithelial cells. While, the levels of this gene mRNA in duodenal samples of animals injected with omeprazole during 28 days and rats of fours group (Omeprazole + Symbiter) as significantly differentiate both in villous epithelium and crypt cells as between analyzed types of epitheliocytes ($p \leq 0,0001$) (Fig. 1., Table. 1.).

Table 1. **Level of *Chga* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic Symbiter (m ± SD, n = 6)**

Groups of animals	Typo of epithelial cells	Relative expression *Chga / Actb*
Control	villi	0,557 ± 0,0481 ÷
	crypts	0,815 ± 0,0543 ÷
Symbiter	villi	0,530 ± 0,0292 ÷
	crypts	0,819 ± 0,0312 ÷
Omeprazole	villi	0,773 ± 0,0951* ÷
	crypts	1,61 ± 0,0912* ÷
Omeprazole + Symbiter	villi	0,581 ± 0,0452 ÷
	crypts	0,866 ± 0,0365 ÷

Notes: SD – standard deviation;
** – p ≤ 0,0001 in relation to control;*
÷ – p ≤ 0,0001 villi in comparison with crypts.

As we see from the *Table 1*., the level of *Chga* gene's expression was higher than control values in 1,4 times in villi and approximately in 2 times in crypts of animals upon long-term gastric hypoacidity (p≤0,0001). At the same time upon simultaneous administration of multiprobiotic Symbiter this parameter was in 1,3 and about 1,9 times lower than in animals of third group (p≤0,0001).

It is well known, *Chga* gene is expressed by different duodenal enteroendocrine cells. Message of *Chga* are expressed at ≈ 1000 × the level in malignant gastro-intestinal carcinoids compared with normal mucosa. Detectable levels of *Chga* in normal mucosa (small intestine/gastric) reflects the presence of *Chga*-expressing endocrine cells in these tissues and further emphasizes the sensitivity of the technique since it detects neuroendocrine cells, which represent approximately 1 per 2000 epithelial cells in duodenum (there are more enteroendocrine cells in crypts then in villi, although the functional activity of these cells are approximately the same). Since endocrine cells constitute ≈ 1 % by volume of the gastro-intestinal mucosa, the detection in normal mucosa further confirms the sensitivity of PCR as an identification tool and emphasizes its ability to detect disease at a cellular level (1, 11, 14). In our experiment we demonstrated the elevation of *Chga* mRNA level in villi and crypts upon hypoacidic conditions (Fig. 1., Table. 1.). It can be assumed not only about intensification of inflammatory processes in duodenal [4], but about possible neoplasia in epithelial cells on later stages of pathology process development [4]. Thus, determination of *Chga* gene's level of expression could be used as one of the most sensitive marker of neuroendocrine tumors [8, 14, 22, 23].

Thus, the obtained changes in expression of *Chga* gene in rat duodenal villus and crypts epithelial cells upon hypoacidic conditions should point out the development of teratoid displacements in duodenal tissue. Different rates in alterations of above mentioned gene's expression in villus and crypts epithelial cells is determined by their structural and functional characteristics (1, 11, 14). Besides this, according to a literature, it is well known, that an early feature of some inflammatory diseases, including pathological disorders in intestines, is the formation of crypt abscesses, which are composed of neutrophils that have migrated across the epithelium and into the crypt lumen [1].

Among probable mechanisms of Symbiter's action on gene expression in rat duodenal, firstly, it should be pointed out its ability to liquidate dysbiosis and bacterial colonization of GIT. As a result, the burden of pathogenic microbiota is removed from GIT and associated organs [3, 7]. Furthermore, multicomponent probiotic Symbiter is able to increase de novo synthesis of the main low-molecular cellular antioxidant – reduced glutathione and, thus, to raise its content both in GIT and duodenal. Besides this, the products of Symbiter's bacterials vital functions (vitamins, exopolysaccharides, short chain fat acids, immune-response modulating agents and so on) are characterized by antioxidant properties, on the basis of what they can delay the development of oxidative stress and decrease the intensity of inflammation and pathological actions in duodenal [4, 6, 12, 16]. The reduction of gastrin level in the blood upon prolonged administration of Symbiter has recently been observed [7]. On the basis of binding studies, it may be suggested that observed effects of Symbiter are linked not only with normalization of GIT microbiota, but also with restriction of hypergastrinemia effects [7, 16]. But final acceptance or rejection of this suggestion requires further investigations, which will allow us to distinctly distinguish the consequences of hypergastrinemia and bacterial colonization of GIT by use of selective antagonists of gastrin receptor.

Conclusion. In summary, we have shown, that long-term experimental hypoacidity is accompanied by changes in expression of *Chga* gene in rat duodenal epithelial cells. While upon simultaneous administration of multiprobiotic Symbiter the expression pattern of this gene is similar to control both in villous and crypt ephiteliel cells. Based on the obtained data, it can be assumed, that analyzed gene is involved in the development of pathological processes in duodenal and there is some potential risk of duodenal carcinogenesis upon long-term use of omeprazole (and probably other PPIs).

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ЕКСПРЕСІЯ ГЕНА CHGA В ЕПІТЕЛІОЦИТАХ ДВАНАДЦЯТИПАЛОЇ КИШКИ ЗА УМОВ ТРИВАЛОЇ ГІПОАЦИДНОСТІ ШЛУНКА ТА ПРИ ВВЕДЕННІ МУЛЬТИПРОБІОТИКА СИМБІТЕР

Показано зростання рівня експресії гена Chga в епітеліоцитах ворсинок та крипт дванадцятипалої кишки щурів за гіпоацидних умов. При введенні мультипробіотика Симбітер за тих самих умов патерн експресії вищезазначеного гена в епітеліоцитах як ворсинок, так і крипт був подібний до контролю.

Ключові слова: шлункова гіпоацидність, дванадцятипала кишка, щури, експресія гена Chga, мультипробіотик.

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ЭКСПРЕССИЯ ГЕНА *CHGA* В ЭПИТЕЛИОЦИТАХ ДВЕНАДЦАТИПЕРСТНОЙ КИШКИ КРЫС ПРИ ДЛИТЕЛЬНОЙ ЖЕЛУДОЧНОЙ ГИПОАЦИДНОСТИ И ПРИ ВВЕДЕНИИ МУЛЬТИПРОБИОТИКА СИМБИТЕР

Показано увеличение уровня экспрессии гена Chga в эпителиоцитах ворсинок и крипт двенадцатиперстной кишки крыс в гипоацидных условиях. При введении мультипробиотика Симбитер в тех же условиях содержание мРНК Chga в эпителиоцитах как ворсинок, так и крипт было на уровне контрольных значений.

Ключевые слова: желудочная гипоацидность, двенадцатиперстная кишка, крысы, экспрессия гена Chga, мультипробиотик.

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THE CHANGES IN FUNCTIONING OF MUCUS BARRIER OF STOMACH IN CONDITIONS OF LONG-TERM HYPOACIDITY AND THEIR CORRECTION

Increase in the duration of hypoacidity of gastric juice evoked by daily injection of blocker of gastric acid secretion omeprazole from 7 to 28 days accompanied by substantial rise of the level of oxiproline, fucose, N-acetylneuraminic acid and hexuronic acids in gastric mucus in rats. It is witness of intensification of degradation collagenic and noncollagenic proteins in gastric mucus. Injection of multiprobiotic Symbiter against the background of hypoacidity evoked by omeprazole led to decrease the level of studied parameters to control values in all terms of investigations.

Key words: gastric mucus, omeprazole, multiprobiotic.

Introduction. The basis mucous layer of the stomach are polymerized structural glycoproteins of mucus. Due to its polymer structure and hydrophobic properties gel mucus protects gastric mucosa from direct contact with xenobiotics, endogenous nitroso compounds, free radicals, bacterial toxins. Long-term hypoacidity of gastric juice and anacidity are risk factors for carcinogenesis in stomach. In conditions of hypoacidity in stomach dysbiosis develops [5], this can lead to structural changes of mucus. Dysbiosis [9] and disturbance of structure of gastric mucus [4] in turn accelerate the development of neoplastic changes in stomach.

In connection with this the aim of our work was to investigate effect of multiprobiotic "Symbiter® acidophilic" as drug for prophylaxis of dysbiosis, on state of mucus barrier in stomach in conditions of hypoacidity of different duration evoked by omeprazole.

Materials and methods. The study was done on white rats which were divided into 12 group. To the rats of 4 groups during 7, 14, 21, 28 days consequently were injected blocker of gastric acid secretion omeprazole ("Sigma", USA) (14 mg/kg intraperitonealy once a day). To the rats of others 4 groups during the same terms simultaneously with omeprazole we injected multiprobiotic "Symbiter® acidophilic" (Symbiter) (limited company "O.D. Prolisok") (0.14 ml/kg per os once a day). To the rats of 4 control groups were injected during 7, 14, 21 and 28 days 0.2 ml H_2O intraperitonealy and 0.5 ml H_2O per os. Symbiter is concentrated fluid biomass of bioplasts of symbiosis of 14 microorganisms strains. The composition of one dose (10 ml) of Symbiter is concentrated biomass of bioplasts of bacterium's symbiosis CFU/cm^3, no less: Lactobacillus and Lactococcus – $6.0x10^{10}$, Propionic bacterium – $3.0x10^{10}$, Bifidobacterium – $1.0x10^{10}$, Acetic bacterium – $1.0x10^{6}$. For assessment of mucus barrier state in stomach in a day of last injection of drugs in parietal mucus we determined the levels of oxiproline using method as described earlier [8] ,