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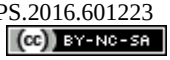
ISSN 2231-3354 

The impact of oxidative stress on Par2, Ptgs2 genes expression in rat duodenal epithelial cells under conditions of prolonged gastric hypochlorhydria and with administration of multiprobiotic

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ABSTRACT

Development of dysbiosis is a key consequence of long hypoacidity, in which there is colonization of the gastrointestinal tract by pathogenic microflora that forms source of sustainable endogenous infection and leads to inflammation. The prevalence of free-radical processes of antioxidant defense system is an important pathogenetic link in the origin and development of various disorders. Because reactive oxygen species are important for transcription of *Ptgs2* gene, the relationship between oxidative stress, antioxidant element and PAR2-mediated signaling pathways may take place under conditions of dysbiotic changes due to hypochlorhydria. To evaluate the intensity of free radical processes and their impact on *Par2, Ptgs2* genes expression in rat duodenal epithelial cells under conditions of prolonged gastric hypochlorhydria and with administration of multi-probiotic. Four experimental groups were created with 8 rats in each. One group was a control, while others were injected with acid suppressant omeprazole or multi-strain probiotic preparation or with both compounds simultaneously for 28 days. Protein oxidative modification products, free SH-groups, metallothioneins content were measured in villus and crypt epithelial cells with standard biochemical assays. Level of *Par2, Ptgs2* genes mRNA was determined with semi-quantitative RT-PCR. Prolonged inhibition of acid secretion in stomach was accompanied by significant elevation of free radical processes (intensification of protein oxidative modification, free SH-groups pool reduction, changes of metallothioneins concentration) and elevation of *Par2* and *Ptgs2* genes expression levels in rat duodenal epithelial cells. Reduction of free radical processes intensity; restoration of redox balance and the normalization of *Par2, Ptgs2* gene expression pattern in the duodenum upon the simultaneous administration of the multi-probiotic Symbiter were observed. *Par2* over-expression leads to activation *Ptgs2* on the background of free radical processes over the antioxidant defense system in the development of inflammation in the duodenum through dysbiotic changes in the conditions of prolonged hypochlorhydria.

INTRODUCTION

The gastric hypochlorhydria has a multifactorial etiology. She accompanies several diseases (atrophic gastritis, gastric cancer, autoimmune disorders) and is the result of pharmaceutical drugs administration - proton pump inhibitors of gastric parietal cells of the stomach, for example, widely representative omeprazole (Shin *et al*., 2008, Sundstrom *et al*., 2006, Johnson *et al*., 2013, Barrett *et al*., 2006). The development of dysbiosis - a key consequence of long hypoacidity, in which there is colonization of the gastrointestinal tract (GIT) by pathogenic microflora that forms source of sustainable endogenous infection. This leads to inflammation and is an additional factor which besides hypergastrinemy, promotes gastric carcinogenesis and the occurrence of sporadic tumors in other parts of the GIT and its associated organs (Barrett *et al*., 2006, Thomson *et al*., 2010).

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Par2 gene (encodes proteinase-activated receptor 2 (PAR2), subgroup of G-protein coupled receptor family) is highly expressed in different pathologies of GIT (Seo *et al*., 2011; Kamath *et al*., 2001; Ritchie *et al*., 2007). PAR2 is activated in response to the partial proteolytic cleavage of the extracellular domain by serine proteases under the influence of p38 MAP (mitogen-activated protein) kinase and by the NFκB signalling pathway, including the potentially divergent roles of the kinases inhibitory kappa B kinases (IKK)-α and –β (Ritchie *et al*., 2007). Inflammatory cytokines (IL-1α, TNF-α) increase mRNA expression of *Par2* in disorders (Seo *et al*., 2011; Kamath *et al*., 2001; Ritchie *et al*., 2007).

Also the increase in this gene expression is decided to consider as a protective mechanism of cells from premature activation of proteolytic enzymes (Gorelick, 2007). *Par2* modulates activation of proinflammatory genes and genes involved in carcinogenesis, for example, *Ptgs2* (encoding cyclooxygenase-2, COX-2) due to activation of G-protein, Src-kinase and also NFκB - signaling pathway. COX-2 is the inducible enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2, a precursor to other prostaglandins, prostacyclin and thromboxane A2) and activated under certain disorders in the body, including inflammation and carcinogenesis (Seo *et al*., 2011; Kamath *et al*., 2001).

The process of free radical oxidation is a component of the metabolic activity of cells that are constantly occurring in living organisms. The main mechanism of activation of free radical processes is to increase the formation of reactive oxygen species (ROS).

Their damaging effect in many cases due to subsequent stimulation of free radical oxidation that leads to oxidative stress (OS), which is manifested by the accumulation of toxic products that damage molecules (including activation of lipid peroxidation and intensification of protein oxidative modification (POM)), membranes of cells, tissues, organs and body in general. The limitation of the negative effects of free radicals is provided by activation of antioxidant system, including through free SH-groups of biothiols and metallothioneins (MT), which exhibit high antioxidant activity in neutralizing ROS and block further oxidative cell damage (Dvorshchenko *et al*., 2013, Wlodek, 2001). Prevalence of free-radical processes of antioxidant defense system is an important pathogenetic link in the origin and development of various diseases.

Because ROS are important for transcription of *Ptgs2* (Seo *et al*., 2011), the relationship between OS, antioxidant element and PAR2-mediated signaling pathways may take place under conditions of dysbiotic changes due to gastric hypochlorhydria.

Probiotic preparations, which play an important role in maintaining the overall homeostasis of the body by optimizing its microecological, are used for the correction of the structural and functional abnormalities in GIT (Culligan *et al*., 2009). Multi-strain probiotic preparations are more effective compared with monocomponent drugs, because they composed of a combination of different bacterial strains.

For our research we have chosen multi-probiotic Symbiter, which is a symbiosis of living cells biomass of 14 microorganism strains, which are physiological for mammalian intestinal and belong to 10 species: *Bifidobacterium bifidum, B. longum, Lactobacillus acidophilus, L. delbrueckii, L. helveticus, Propionibacterium freudenreichii, P. acidipropionici, Lactococcus lactis, Acetobacter aceti, Streptococcus salivarius* (Culligan *et al*., 2009, Iankovsky *et al*., 2008).

Considering the above, the aim of this research was to assess the intensity of free radical processes and their impact on *Par2*, *Ptgs2* genes expression in rat duodenal epithelial cells under conditions of prolonged gastric hypochlorhydria and with administration of multi-probiotic.

MATERIALS AND METHODS

Animal model

All experiments were carried out on white non-strain male rats with initial weight 180-200 g. Animals were divided randomly into four groups with 8 rats in each of them. Animals treated with 0.2 ml of normal saline i.p. and 0.5 ml of water per os were used as a control (first group). Rats of second group were treated only with multi-strain probiotic preparation "Symbiter® acidophilus concentrated" (manufactured by LLC "O.D. Prolisok", Ukraine; also referred to as Symbiter) orally in a dose of 140 mg/kg (1014 colony-forming unit per kg) during 28 days in order to estimate the influence of heterogeneous normal microbiota on healthy animals. Hypochlorhydria was evoked through intraperitoneal injection of omeprazole (14 mg per kg of weight once a day) during 28 days (Tsyriuk *et al*., 2007); this group represented model of hypochlorhydria associated with dysbiosis (third group). Animals of fourth group were simultaneously treated with omeprazole and Symbiter per os in the same dosage.

Isolation of biological material

After 28 days since the start of experimental animals were sacrificed via decapitation according to "AVMA Guidelines for the Eutanasia of Animals" (2013). The crypts and the villi of duodenal epithelial cells were extracted by the means of a rapid low-temperature (4°C) method, which includes intestine washing under the constant stirring in different buffers with the consequential gradual collection of villus or crypt fractions (Flint *et al*., 1991).

Measurement of protein oxidative modification products, free SH-groups, metallothioneins content

Determination of the OMP products were determined by reaction between carbonyl derivatives of proteins and schiff bases with 2.4-dinitrophenylhydrazine to form neutral and basic 2,4-dinitrophenylhydrazones (Dubinina *et al*., 1995). Determination of total, protein-bound and non-protein-bound SH-groups content

was conducted by the method of Elman (Ellman, 1959). The relative content of MT was evaluated in partially purified fractions of metalloproteins that received by chloroform - ethanol fractionation of villus and crypt epithelial cells in acidic conditions (Linde *et al*., 2006). Protein content was determined by the method of Lowry (Lowry *et al*., 1951). All indexes are listed at 1 mg of protein.

Semiquantitative RT-PCR

RNA was isolated following Chomczynski and Sacchi (Chomczynski *et al*., 1987); cDNA was synthesized in 20 µl of reaction mix containing 2 µg of RNA, 1 mM dNTP, 200 U of reverse transcriptase "RevertAid M-MLV", corresponding buffer, 20 U of ribonuclease inhibitor "Thermo Scientific RiboLock RNase Inhibitor" ("Thermo Scientific", Lithuania), 20 pmol of reverse primer.

Synthesis was carried out in the following conditions: 65°C – 5 min, further 45°C – 1 h. PCR was performed in 30 µl of reaction mix containing 3 µl of cDNA, PCR buffer, 200 µM of each dNTP, 30 pmol of each primer, 2.5 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase ("Thermo Scientific", Lithuania). PCR amplifications consisted of the initial denaturing step of 95ºC for 3 min, followed by 30 cycles (28 cycles for *Actb* – gene used as the internal control of reaction due to its constitutive expression) of 95ºC for 45 s, appropriate annealing temperature: 45ºC – 45 s for *Par2* (357 b.p.), 53ºC – 40 s for *Ptgs2* (123 b.p.), and 49ºC – 40 s for *Actb* (521 b.p.), the extending step at 72ºC for 1 min. And the final extension step was performed at 72ºC for 5 min. The following primers were used in reactions (designed using Primer-BLAST) – for *Par2*: forward – GAATGCACCGGGACCCAA, reverse – TCCCCATAGGTCCAGTCGTT; for *Ptgs2*: forward – TGCTGTTCCAACCCATGTCA, reverse – TGTCAGAAACTCAGGCGTAGT; for *Actb*: forward – TGGGACGATATGGAGAAGAT, reverse – ATTGCCGATAGTGATGACCT. Separation of PCR products was carried out electrophoretically in 1.6% agarose gel with 0.5x TBE buffer (Green *et al*., 2012). For semi-quantitative analysis of amplicons expression based on densitometry the Image J 1.48d program ("NIH", USA) was used. The indices of mRNA expression were calculated in relation to *Actb* for each sample following Konturek *et al*. (Konturek *et al*., 1998).

Ethics

Positive findings of ethical expertise were adopted on session of Bioethical Commission of ESC "Institute of Biology" of Taras Shevchenko National University of Kyiv on 26 June 2013. All animals were handled humanly according to rules outlined in "Guide for the Care and Use of Laboratory Animals" (2011), and Order of Ukraine №3447-IV" About defense of animals from abusive handling" from 21 February 2006.

Statistics

Statistical processing of experimental results was carried out in "GraphPad Prism 5" ("GraphPad Software Inc.", USA). Type of data distribution in groups was checked with Shapiro-Wilk test. As data were distributed normally ($p > 0.05$), two-way ANOVA was conducted to determine the significance of difference between means with Bonferroni post test. Difference between means was judged as statistically significant if $p \leq 0.05$. Mean (M) and standard deviation (SD) were calculated for each group. Pearson's linear correlation coefficient (r) was used to assess the relationship between specific series. Correlation was estimated as strong for $|r| > 0.5$, moderate – for $|r| = 0.3 – 0.5$, and small – for $|r| = 0.1 – 0.3$.

RESULTS

Content of protein oxidative modification products.

In animals subjected to prolonged inhibition of hydrochloric acid secretion in stomach the content of POM neutral products with absorption peak of 356 and 370 nm increased: in villus epitheliocytes - 1,7 ($p \leq 0.0001$) and 1,8 ($p \leq 0.0001$) times; in crypts - 2,1 ($p \leq 0.0001$) times compared to the control (Table 1a).

Table 1a: Content of protein oxidative modification neutral products in duodenal epithelial cells of rats under conditions of prolonged hypochlorhydria and with the multi-probiotic administration, CU / mg of protein (M ± SD, n = 8).

Group of animals / Analyzed parameter		POM basic products, absorption peak - 430 nm	POM basic products, absorption peak - 530 nm
Control	villi	$0.072 \pm 0.0071^{\div\div\div\div}$	$0.057 \pm 0.0053^{\div\div\div\div}$
	crypts	$0.111 \pm 0.0092^{\div\div\div\div}$	$0.080 \pm 0.0071^{\div\div\div\div}$
Symbiter	villi	$0.062 \pm 0.0052^{*/\div\div\div\div}$	$0.045 \pm 0.0042^{**/\div\div\div}$
	crypts	$0.095 \pm 0.0093^{*/\div\div\div\div}$	$0.063 \pm 0.0063^{**/\div\div\div}$
Omeprazole	villi	$0{,}138 \pm 0.0124^{****/\div\div\div}$	$0{,}107 \pm 0.0093^{****/\div\div\div\div}$
	crypts	$0{,}185 \pm 0.0182^{****/\div\div\div}$	$0{,}151 \pm 0.0131^{****/\div\div\div\div}$
Omeprazole + Symbiter	villi	$0.061 \pm 0.0063^{**/\#\#\#\#/\div\div\div\div}$	$0.041 \pm 0.0044^{***/\#\#\#\#/\div\div}$
	crypts	$0.144 \pm 0.0151^{**/\#\#/\div\div\div\div}$	$0.053 \pm 0.0052^{****/\#\#\#\#/\div\div}$

Remarks:*, **, ***, **** – $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ relative to the control; ##, #### - $p \leq 0.01$, $p \leq 0.0001$ relatively animals which only omeprazole treating; ÷÷, ÷÷÷, ÷÷÷÷ - $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ villi compared with crypts.

Table 1b: Content of protein oxidative modification basic products in duodenal epithelial cells of rats under conditions of prolonged hypochlorhydria and with the multi-probiotic administration, CU / mg of protein (M ± SD, n = 8).

Group of animals / Analyzed parameter		POM basic products, absorption peak – 430 nm	POM basic products, absorption peak – 530 nm
Control	villi	$0.072 \pm 0.0071^{\div\div\div\div}$	$0.057 \pm 0.0053^{\div\div\div\div}$
	crypts	$0{,}111 \pm 0.0092^{\div\div\div\div}$	$0.080 \pm 0.0071^{\div\div\div\div}$
Symbiter	villi	$0.062 \pm 0.0052^{*/\div\div\div\div}$	$0.045 \pm 0.0042^{**/\div\div\div}$
	crypts	$0.095 \pm 0.0093^{*/\div\div\div\div}$	$0.063 \pm 0.0063^{**/\div\div\div}$
Omeprazole	villi	$0{,}138 \pm 0.0124^{****/\div\div\div}$	$0{,}107 \pm 0.0093^{****/\div\div\div\div}$
	crypts	$0{,}185 \pm 0.0182^{****/\div\div\div}$	$0{,}151 \pm 0.0131^{****/\div\div\div\div}$
Omeprazole + Symbiter	villi	$0.061 \pm 0.0063^{**/\#\#\#\#/\div\div\div\div}$	$0.041 \pm 0.0044^{***/\#\#\#\#/\div\div}$
	crypts	$0{,}144 \pm 0.0151^{**/\#\#/\div\div\div\div}$	$0.053 \pm 0.0052^{****/\#\#\#\#/\div\div}$

Remarks:*, **, ***, **** – $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ relative to the control; ##, #### - $p \leq 0.01$, $p \leq 0.0001$ relatively animals which only omeprazole treating; ÷÷, ÷÷÷, ÷÷÷÷ - $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ villi compared with crypts.

Table 2: Content of SH-groups in duodenal epithelial cells of rats under conditions of prolonged hypochlorhydria and with the multi-probiotic administration, mmol / mg of protein (M ± SD, n = 8)

Group of animals / Analyzed parameter		Protein-bound	Non-protein	Total
Control	villi	2,28 ± 0,191$^{\div\div\div\div}$	0,77 ± 0.071$^{\div\div\div\div}$	2,95 ± 0,283$^{\div\div\div}$
	crypts	1,08 ± 0.092$^{\div\div\div\div}$	1,13 ± 0,112$^{\div\div\div\div}$	2,21 ± 0,221$^{\div\div\div}$
Symbiter	villi	2,05 ± 0,182$^{\div\div\div\div}$	0,69 ± 0.062$^{\div\div\div}$	2,68 ± 0,242$^{\div\div}$
	crypts	1,41 ± 0,133$^{***/\div\div\div\div}$	0,88 ± 0.073$^{**/\div\div\div}$	2,15 ± 0,213$^{\div\div}$
Omeprazole	villi	1,29 ± 0,124$^{****/\div\div\div}$	0,61 ±0.054$^{**/\div\div\div}$	1,92 ±0,163$^{****/\div\div\div\div}$
	crypts	0,81 ± 0.072$^{***/\div\div\div}$	0,44 ±0.042$^{****/\div\div\div}$	1,25±0,121$^{****/\div\div\div\div}$
Omeprazole + Symbiter	villi	1,84±0,143$^{**/\#\#\#\#}$	0,77±0.063$^{\#\#\#/\div\div\div\div}$	2,38±0,214$^{**/\#\#/\div\div\div}$
	crypts	1,67±0,161$^{**/**/\#\#\#\#}$	1,58±0,151$^{***/\#\#\#\#/\div\div\div\div}$	3,25±0,322$^{****/\#\#\#\#/\div\div\div}$

Remarks:**, ***, **** – p ≤0.01, p ≤ 0.001, p ≤ 0.0001 relative to the control; ##, ###, #### - p ≤0.01, p ≤ 0.001, p ≤ 0.0001 relatively animals which only omeprazole treating; ÷÷, ÷÷÷, ÷÷÷÷ - p ≤ 0.01, p ≤ 0.001, p ≤ 0.0001 villi compared with crypts.

In animals upon joint administration of omeprazole and Symbiter the content of POM neutral products with absorption peak of 356 and 370 nm decreased in villi: 2,4 (p ≤ 0.0001) and 2,5 times (p ≤ 0.0001) compared to the third experimental group; and – в 1,4 times (p ≤ 0.001) in comparison to the control one. In crypt cells these indexes were lower 2,4 (p ≤ 0.0001) and 2,8 (p ≤ 0.0001) compared to the third group, meanwhile the content of POM neutral products with absorption peak of 370 reduced 1,3 times (p ≤ 0.01) in comparison to the control animals.

In rats treated only with Symbiter the content of POM neutral products with absorption peak of 356 and 370 nm decreased: in villi: 1,1 (p ≤ 0.05) and 1,2 (p ≤ 0.01) times respectively, in crypts – 1,4 (p ≤ 0.001) and 1,3 (p ≤ 0.01) times, compared to the control group.

In animals treated with omeprazole the content of POM basic products with absorption peak of 430 and 530 nm increased: in villi - 1,9 (p ≤ 0.0001) and 1,8 (p ≤ 0.0001) times; in crypts - 1,9 (p ≤ 0.0001) times compared to the control (Table 1b).

These parameters in villi of rats from the third group were lower: 2,3 (p ≤ 0.0001) and 2,6 times (p ≤ 0.0001) compared to the third group; in the same time the content of POM neutral products with absorption peak of 530 decreased 1,4 times (p ≤ 0.001) in comparison to the control animals. In crypt cells the content of POM basic products with absorption peak of 430 nm decreased 1,3 (p ≤ 0.0001) times compared to the third group and was 1,3 times higher than the same parameter in the control group. The content of POM basic products with absorption peak of 530 nm decreased 2,8 times (p ≤ 0.0001) compared to the third group and 1,5 times (p ≤ 0.0001) to the first group. In rats treated only with Symbiter the content of POM basic products with absorption peak of 430 and 530 nm was lower: in villi: 1,2 (p ≤ 0.05) and 1,3 (p ≤ 0.01) times respectively, in crypts – 1,4 (p ≤ 0.001) and 1,3 (p ≤ 0.01) times, compared to the control group.

Content of total, protein-bound and non-protein-bound SH-groups

It was found that the content of total, protein-bound and non-protein SH-groups of rats with prolonged inhibition of gastric acid-function decreased: in the epithelium villi - 1,5 (p ≤ 0.0001), 1,8 (p ≤ 0.0001) and 1,3 (p ≤ 0.01) times, respectively, in the crypt - 1,8 (p ≤ 0.0001), 1,3 (p ≤ 0.001) and 2,6 (p ≤ 0.0001) times, accordingly, compared with control (Table 2).

Animals of the fourth group had higher content of total, protein-bound and non-protein SH-groups: in villi: – 1,2 (p ≤ 0.01), 1,4 (p ≤ 0.0001) and 1,3 (p ≤ 0.001) times in accordance, in crypts – 2,6 (p ≤ 0.0001), 2,1 (p ≤ 0.0001) and 3,6 (p ≤ 0.0001) times respectively, in relation to the control group. Besides this, the above mentioned parameters were 1,2 (p ≤ 0.01) lower in comparison to the control indexes in villi, meanwhile 1,5 (p ≤ 0.0001), 1,6 (p ≤ 0.0001) and 1,4 (p ≤ 0.001) times higher accordingly, compared with control. The content of protein-bound and non-protein SH-groups in crypts of animals treated only with Symbiter was 1,3 times (p ≤ 0.001) higher and 1,3 times (p ≤ 0.01) lower in relation to the control.

Metallothioneins content

In animals injected with omeprazole during 28 days the total MT concentration was: in villi – 2 times (p ≤ 0.0001) higher, in crypts – 1,7 times (p ≤ 0.0001) lower relatively to control (Table 3).

Table 3: Content of metallothioneins in duodenal epithelial cells of rats under conditions of prolonged hypochlorhydria and with the multi-probiotic administration, (M ± SD, n = 8).

Group of animals	Epithelial cells	µmol / mg of protein
Control	villi	0,198 ± 0.0183$^{\div\div\div\div}$
	crypts	0,285 ± 0.0261$^{\div\div\div\div}$
Symbiter	villi	0,159 ± 0.0102$^{**/\div\div\div\div}$
	crypts	0,491 ± 0.0443$^{****/\div\div\div\div}$
Omeprazole	villi	0,391 ± 0.0353$^{****/\div\div\div\div}$
	crypts	0,171 ± 0.0151$^{****/\div\div\div\div}$
Omeprazole + Symbiter	villi	0,210 ± 0.0194$^{\#\#\#\#/\div\div\div\div}$
	crypts	0,440 ± 0.0412$^{****/\#\#\#\#/\div\div\div\div}$

Remarks: **, **** – p ≤ 0.01, p ≤ 0.0001 relative to the control, #### - p ≤ 0.0001 relatively animals which only omeprazole treating; ÷÷÷÷ - p ≤ 0.0001 villi compared with crypts.

In villi of animals upon joint administration of omeprazole and Symbiter the total MT concentration decreased 1,9 times (p ≤ 0.0001) in comparison with the third group. In similar terms, this index in crypts increased: 2,6 times (p ≤ 0.0001) in relation to the third group of animals and 1,5 times (p ≤ 0.0001) relative to controls. MT content in rats injected only with Symbiter

was 1,3 times ($p \leq 0.01$) lower in villi and 1,7 times ($p \leq 0.0001$) higher in comparison with the control.

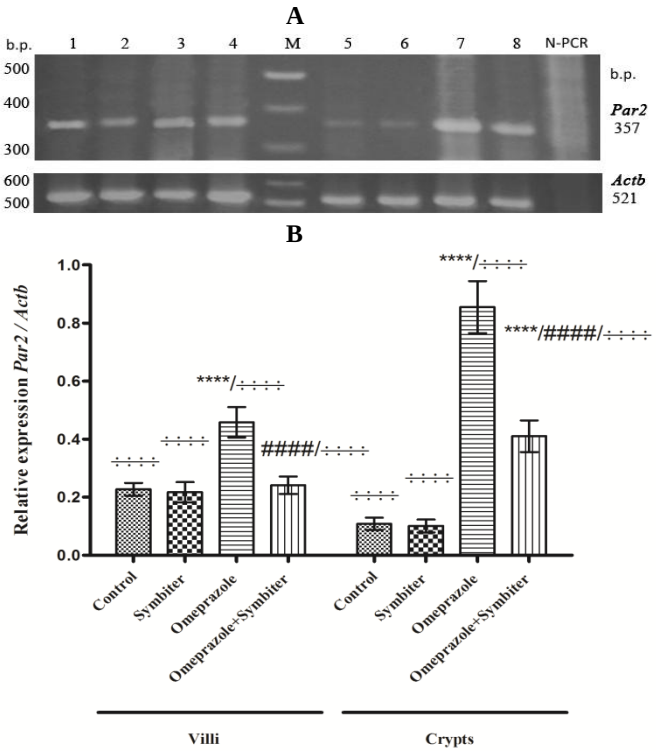

Fig. 1: Level of *Par2* gene mRNA in rat duodenum upon long-term hypoacidity and with the multi-probiotic administration. A – RT-PCR electrophoregram, B – relative expression histogram, M – molecular mass marker – "GeneRuler^{TM} 100bp DNA Ladder" ("Fermentas", Lithuania); villus epithelial cells: 1 – control; 2 – Symbiter; 3 – omeprazole; 4 – omeprazole + Symbiter; crypt epithelial cells: 5 – control; 6 – Symbiter; 7 – omeprazole; 8 – omeprazole + Symbiter; N-PCR – negative PCR control; **** - $p \leq 0.0001$ in relation to control; #### - $p \leq 0.0001$ compared to the animals treated with omeprazole; ÷÷÷÷ - $p \leq 0.0001$ villi in comparison with crypts.

Relative expression level of *Par2* gene

The level of *Par2* gene expression was 2 times ($p \leq 0.0001$) higher of the control value in villi and 7,9 ($p \leq 0.0001$) times higher in crypts of the animals treated only with omeprazole for 28 days (Figure 1, A,B). Upon simultaneous administration of the multi-probiotic this parameter was 1,6 times lower in villi compared to the third experimental group ($p \leq 0.0001$).

Upon simultaneous administration of the multi-probiotic this parameter was 1,9 times ($p \leq 0.0001$) lower in villi compared to the third experimental group. In crypts this index was 2,1 times lower than in the third group of animals ($p \leq 0.0001$) and remained 3,4 times ($p \leq 0.0001$) higher of the control value. In rats treated only with Symbiter there was no change of this value both in villus and crypt epithelial cells of duodenum in comparison to the control one.

Relative expression level of *Ptgs2* gene

We found, that the level of *Ptgs2* gene expression was 1,8 times ($p \leq 0.0001$) higher compared to the control values in villi and 4,3 times ($p \leq 0.0001$) higher in crypts upon long-term hypoacidity (Figure 2, A,B.)

In case of the simultaneous administration of the multi-probiotic the above mentioned parameter was 1,5 ($p \leq 0.0001$) and 2,3 times ($p \leq 0.0001$) lower in villi and crypts respectively than in the third group, but remained 1,9 times ($p \leq 0.0001$) higher of the control value in crypts. In rats treated only with Symbiter there was no change of this value both in villus and crypt epithelial cells of duodenum in comparison to the control one.

Also we found a strong positive correlation between *Par2* and *Ptgs2* gene expression in villi ($r = 0,9$, $p = 0.021$) and crypt epithelial cells ($r = 0,9$, $p = 0.015$) under long-term gastric hypochlorhydria and with administration of multi-probiotic.

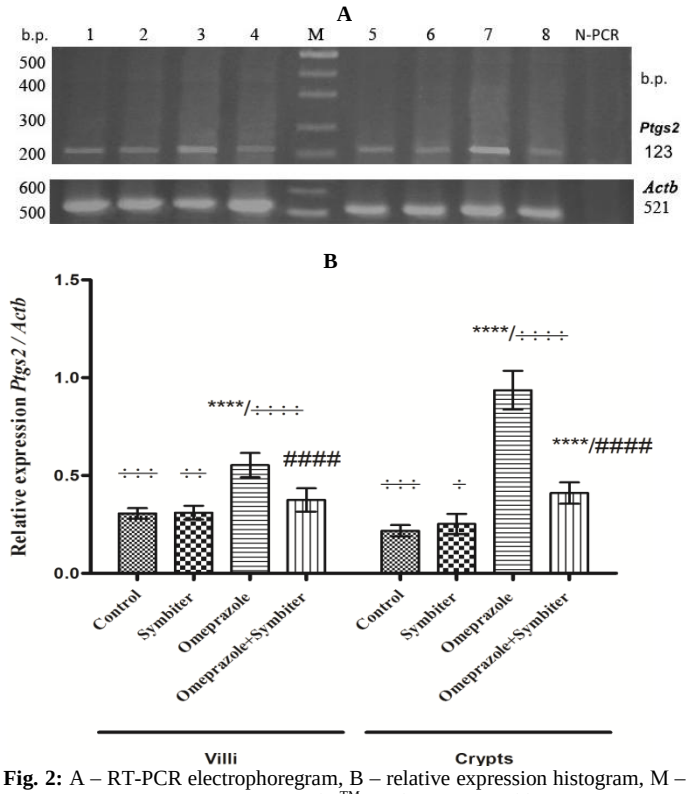

Fig. 2: A – RT-PCR electrophoregram, B – relative expression histogram, M – molecular mass marker – "GeneRuler^{TM} 100bp DNA Ladder" ("Fermentas", Lithuania); villus epithelial cells: 1 – control; 2 – Symbiter; 3 – omeprazole; 4 – omeprazole + Symbiter; crypt epithelial cells: 5 – control; 6 – Symbiter; 7 – omeprazole; 8 – omeprazole + Symbiter; N-PCR – negative PCR control; **** - $p \leq 0.0001$ in relation to control; #### - $p \leq 0.0001$ compared to the animals treated with omeprazole; ÷÷÷÷ - $p \leq 0.0001$, ÷÷÷ - $p \leq 0.001$, ÷÷ - $p \leq 0.01$, ÷ - $p \leq 0.05$ villi in comparison with crypts.

DISCUSSION

Obtained increase of POM neutral and basis products content is served as a conclusive evidence of intensification of free radical processes in rat duodenal epithelial cells under long-term gastric hypochlorhydria. Recent study showed, that intensification of POM was involved in protein misfolding, the loss of enzymatic activity, violation of membrane reception and assembly of protein complexes (Gamou *et al*., 1995). Thus, under these conditions is a

massive oxidation of proteins in duodenal villi and crypts with further generation of POM that have destructive properties and cause the development of OS.

In the body free SH-groups are mainly composed of cysteine residues of low molecular weight peptides (non-protein-bound SH-groups) or high molecular weight peptides (protein-bound SH-groups) and show high sensitivity to free radical oxidation. So, OS is correlated with reduced levels of free SH-groups. In current investigation we showed reduction of protein-bound SH-groups content under conditions of prolonged gastric hypoacidity, which may correlate with protein malfunction, loss of their function or degradation in the duodenal epithelial cells (Wlodek, 2002).

Decrease of non-protein-bound SH-groups under similar conditions indicated intensification of prooxidant component in duodenal villi and crypts, because the majority of these sulfhydryl groups were the part of the low molecular weight thiol - glutathione, which exhibits antioxidant properties (Meyer *et al*., 2005). A large number of protein SH-groups are accounted for metallothioneins, which are characterized by low molecular weight, ability to bind heavy metals and antioxidant effects on cells (Linde *et al*., 2006).

Obtained increase of MT content in duodenal villus epithelial cells under conditions of long hypochlorhydria may be due to the presence of specific sequences in the genes of their promoter, which are capable of binding stress transcription factors - Sp1, AP-1, AP-2 (Davis *et al*., 2000). In turn, MT reduction shown in crypts under these conditions reflects both the reduction of protein-bound SH-groups (which correlates with our data obtained in the analysis of the content of protein-bound SH-groups) and the weakening of antioxidant protection and development of OS.

The increase of *Par2* gene mRNA level under prolonged gastric hypochlorhydria in duodenal villi and crypts may indicate premature activation of proteolytic enzymes, including trypsin, which is the dominant activator of PAR2 (Kawabata *et al*., 2001). Thus, the obtained elevation of *Par2* expression level was consistent with our previous data (Vakal *et al*., 2015), where we demonstrated the growth of trypsin activity in rat pancreas homogenate under prolonged inhibition of gastric acid-function, since trypsin is one of the major natural activators of PAR2 along with mast cells tryptases.

Moreover, the found presence of pancreatic proteases activated cationic isoforms in pancreatic juice may also be due to enhanced *Par2* gene expression, as PAR2 activation promotes secretion of pancreatic juice with dissolved hydrolases in it (Vakal *et al*., 2015). In addition, activation of PAR2 can cause increased secretion of pancreatic juice with dissolved hydrolases from the duct to the duodenum. In addition, elevated levels of *Par2* expression under prolonged gastric hypochlorhydria in duodenal epitheliocytes may indicate the development of inflammation in the background of dysbiotic changes; because was shown, that PAR2 was also activated by mast cells tryptases (Bueno, 2008). Because, the impact of cellular or secretory components of pathogenic microorganisms and (or) load by proinflammatory molecules from endogenous sources of inflammation near duodenum lead to rapid development of inflammation in the organ. This is supported by intensified migration of leukocytes into tissue, followed by growth of proinflammatory cytokines content (TNF-α, IL-1, IL-8 and platelet-activating factor (PAF)), synthesis of cell adhesion molecules, generation ROS and development of OS in duodenum (Seo *et al.*, 2011, McClave, 2012). Recent studies demonstrate that *Par2* enhances *Ptgs2* expression by means of G-protein activation, Src-kinase in different disorders, for example, in *H. pylori*-associated gastric carcinogenesis and inflammation. Thus, we found a strong positive correlation between the levels of these genes expression in conditions of prolonged gastric hypoacidity and with the multi-probiotic administration. G_i protein is known to mediate the activation of NF-κB in carcinogenesis and the connection of Src kinase to NF-κB has been reported in toll-like receptor-activated signaling and epidermal growth factor-induced cell proliferation (Seo *et al*., 2011).

Since NFkB binding site is located in the promoter region of *Ptgs2*, G-protein and Src-kinase may be associated with activation of this gene expression by NF-kB - signaling pathway in addition to *Par2* - mediated signaling pathway. As *Ptgs2* gene is responsible for many mitogenic and inflammatory stimuli and ROS are important for transcription of *Ptgs2*, obtained *Ptgs2* upregulation is accompanied by COX-2 and prostaglandin E2 production, accelerated cell proliferation and may be linked with further development of the inflammatory process in the background of dysbiotic changes in duodenal cells (Seo *et al*., 2011, Kamath *et al*., 2001).

So, the obtained changes in *Par2* gene expression in villus and crypt epithelial cells under prolonged gastric hypochlorhydria indicate the development of pathological processes in the duodenal tissue, including inflammation. Different levels of change in analyzed genes expression in villi and crypts are due to structural and functional features of duodenal cells: presence of poorly differentiated clones of proliferating cells as part of crypts cells, which are less resistance to OS compared to differentiated postmitotic cells. It was also observed, that for the first crypt epithelial cells of the small intestine, including the duodenum were damaged in the early stages of inflammatory diseases, due to migration of neutrophils from blood vessels into the lumen of the crypts, leading to the formation of crypt abscesses (Barrett *et al*., 2006).

Thus, the results of current research may indicate that the increase of *Par2* expression leads to activation of *Ptgs2* on the background of free radical processes prevalence of the antioxidant defense system in the development of inflammation in the duodenum on account of dysbiosis in conditions of prolonged hypochlorhydria.

After administration of Symbiter decrease of POM neutral and basis products content, elevation of total, protein- and non-protein-bound SH-group, MT concentration recovery were also observed in current investigation due to the antioxidant and

anti-inflammatory effect of the multi-strain probiotic preparation. Normalization of *Par2* and *Ptgs2* gene expression using multi-probiotic preparation, on the one hand, indicates a lack of prematurely activated protease, on the other - can point to the important role dysbiotic-inflammatory way in the development of damage in duodenum epithelial cells in conditions of prolonged hypochlorhydria, because we know that PAR2 receptor is also activated by bacterial proteases (Bueno, 2008). Regarding the possible mechanisms of multi-probiotic Symbiter influence on gene expression in the duodenum, above all, we should note its ability to eliminate bacterial colonization in the GIT and dysbiosis, which removes from the GIT and associated organs loads from the pathogenic microflora (Dvorshchenko *et al*., 2013, Culligan *et al*., 2009, Iankovsky *et al*., 2008). Also, the waste products of bacterial strains that are represented in the multi-strain probiotic preparation Symbiter (vitamins, exopolysaccharides, short chain fatty acids, immunomodulators and so on) enhance the barrier function of the intestinal wall, anti-bacterial invasion, have antioxidant properties, etc., so they can restore normal flora of the GIT, inhibit the development OS and reduce the intensity of the inflammatory and destructive processes in the duodenum (Dvorshchenko *et al*., 2013, Iankovsky *et al*., 2008, Tsyriuk *et al*., 2007, Lutgendorff *et al*., 2008). Further study should be performed to elucidate the specific molecular mechanisms of pathogenic organisms influence on the duodenum.

CONCLUSION

Current study demonstrates, that prolonged experimental inhibition of acid secretion in stomach was accompanied by the activation of free radical processes (intensification of protein oxidative modification, free SH-groups pool reduction, changes of metallothioneins concentration) and elevation of *Par2* and *Ptgs2* genes expression levels in rat duodenal epithelial cells. Reduction of free radical processes intensity; restoration of redox balance and the normalization of *Par2*, *Ptgs2* gene expression pattern in the duodenum upon the simultaneous administration of the multi-probiotic Symbiter were observed. Our data indicate that *Par2* overexpression leads to activation *Ptgs2* on the background of free radical processes over the antioxidant defense system in the development of inflammation in the duodenum through dysbiotic changes in the conditions of prolonged hypochlorhydria.

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