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Role of Gut Microbiota in the Development of Pancreatitis-Like Biochemical Changes upon Long-Term Suppression of Gastric Acid Secretion in Rats.

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ABSTRACT

Long-term suppression of gastric acid secretion is sometimes associated with development of pancreatitis-like biochemical changes. Few possible mechanisms can be involved: dysbiosis development, hypergastrinemia or direct action of acid suppressants on pancreas. Our aim was to evaluate the role of gut microbiota alterations in the development of pancreatitis-like biochemical changes upon suppression of gastric secretion in rats. Four experimental groups were created with 10 rats in each. One group was a control, while others were injected with acid suppressant omeprazole or multi-strain probiotics preparation or with both compounds simultaneously for 28 days. Alpha-1-antitrypsin and reduced glutathione levels, trypsin, lipase and amylase activities were measured in blood serum and pancreatic tissue with standard biochemical assays. Pancreatic juice proteolytic profile was assessed with contact zymography. Level of *Par2* gene mRNA was determined with semi-quantitative RT-PCR. Significant deviation of most studied parameters from the control was observed upon treatment with acid suppressant. Active cationic proteases were identified in pancreatic juice samples of rats upon hypochlorhydria. Most of studied parameters didn't differ from control values upon treatment with multi-strain probiotics preparation. Alterations of gut microbiota have a crucial role in development of pancreatitis-like biochemical changes upon long-term suppression of gastric acid secretion in rats.

Keywords: Dysbiosis; hypochlorhydria; microbiota; pancreatitis; proton pump inhibitors

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INTRODUCTION

Intragastric pH value and acid-producing function of the stomach play important role in the pathophysiology of acid-related disorders, such as peptic ulcer disease, gastroesophageal reflux disease, Zollinger-Ellison syndrome etc. The most effective pharmacological blockers of acid secretion to date are proton pump inhibitors (PPI) [1]. Although PPI safety is estimated as one of the best among all classes of drugs, their prolonged usage is associated with a number of adverse effects, including malabsorption of vitamins and minerals, decreased bones density, change of pharmacokinetic and pharmacodynamic properties of coadministered drugs, increased susceptibility to infections etc [2].

During last two decades new data on potential association between long-term use of PPI and increased risk of acute pancreatitis development have emerged [3, 4, 5, 6]. Moreover, in recent research of Dvorshchenko et al. [7] the development of pancreatitis-like biochemical changes upon long-term suppression of gastric acid secretion in rats was observed, including violation of prooxidative-antioxidative balance and oxidative stress development in pancreatic cells.

Whereas pathogenic mechanisms of PPI adverse effects are not fully understood in most cases, three hypothetical mechanisms connecting low gastric acidity with the development of pancreatitis-like biochemical changes (or even pancreatitis) can be proposed basing on analysis of available scientific literature:

- Disturbance of gut microbiota composition (dysbiosis) owing to loss of gastric juice bactericidal properties with further colonization of gastroduodenal region by pathogenic microbiota;
- Effects of increased serum gastrin level on pancreatic cells;
- Direct action of acid-suppressing drugs on pancreatic cells.

As far as disturbance of gastric microbiota composition was shown for chosen experimental model [8], the aim of this research was to determine the role of gut dysbiosis in development of pancreatitis-like biochemical changes upon long-term suppression of gastric acid secretion in rats. Activities of pancreatic hydrolases, level of antiproteolytic response and content of reduced glutathione in blood serum and pancreatic tissue were selected as main indices of pancreas state. If maintenance of normal gut microbiota would counteract the development of pancreatitis-like biochemical changes upon suppression of gastric acid secretion, than estimation of contribution of MSPP antioxidant properties to such beneficial effects should also be performed.

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 10 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). Hypochlorhydria was evoked through intraperitoneal injection of omeprazole (14 mg per kg of weight once a day) during 28 days [9]; this group represented model of hypochlorhydria associated with dysbiosis (second group). Animals of third group were simultaneously treated with omeprazole and multi-strain probiotic preparation (MSPP) “Symbiter® acidophilus concentrated” (manufactured by LLC “O.D. Prolisok”, Ukraine) orally in a dose of 140 mg/kg (10^{14} CFU per kg) during 28 days on a day-to-day basis; this group represented model of hypochlorhydria without dysbiosis. Rats of fourth group were treated only with MSPP per os in the same dosage in order to estimate the influence of heterogeneous normal microbiota on healthy animals. The experiment had blind design, since staff that treated animals and measured biochemical indices didn’t know the identity of groups.

Isolation of biological material

After 28 days since the start of experiment animals were sacrificed via decapitation according to “AVMA Guidelines for the Eutanasia of Animals” (2013). The whole pancreas was quickly removed, weighed and used for isolation of cytosolic-microsomal fraction (CMF) of pancreatic cells through differential centrifugation. Blood serum was separated through centrifugation of whole blood upon 1500×g during 15 min.

Samples of pancreatic juice were collected by a modified method of Waynforth and Flecknell [10] from 6 rats of each group.

Determination of α-1-antitrypsin (A1AT) level

The level of A1AT in rat blood serum was measured by immunoturbidimetric assay on account of turbidity changes in samples at the wavelength λ = 340 nm, and represented as g / l of serum [11].

Pancreatic enzymes assays

Amylase activity of pancreatic CMF and blood serum was determined based on accumulation of 2-chloro-4-nitrophenol that arises upon hydrolysis of 2-chloro-4-nitrophenyl-α-galactic maltoside [12]. Monoclonal antibodies to salivary isoform of α-amylase ("Erba Lachema", Czech Republic) was used in order to measure the activity of pancreatic α-amylase isoform separately. Amylase activity was given as U / l of serum (for blood serum), and U / g of tissue (for CMF of pancreatic cells). One unit of amylase activity will produce 1 μmol of product per 1 min.

Lipase activity of pancreatic CMF and blood serum was measured by titrimetric assay with glyceryl trioleate emulsion as a substrate [13], and represented as U / g of tissue or U / l of serum, respectively. One unit of lipase activity corresponds to the volume (ml) of NaOH used for neutralization of fatty acids released upon lipase action during 2 h of incubation.

Trypsin activity was estimated based on ethanol release during cleavage of synthetic substrate N_{α}-benzoyl-L-arginine ethyl ester [14] and represented as U / g of tissue (for CMF of pancreatic cells) or U / l of serum (for blood serum). One unit will produce the increase in sample extinction on 0.001 per 1 min for 25°C upon pH = 7.6.

Visualization of proteases in pancreatic juice

The presence of active proteases in pancreatic juice samples was determined by contact print zymography technique following Paech et al. [15]. Immediately after the end of native protein electrophoresis gel was removed and pressured with two gelatin-coated radiographic films in sandwich-like manner. This construction was placed between two glass tiles and tightly fixed with office clips, and then placed into thermostat for 30 min upon 38°C. In order to visualize spots of proteolytic activity all films were steeped into hot water (60-70°C) and shaken to remove hydrolyzed gelatin. Films were air-dried, and then gelatin was removed from the back (not exposed) side by sponge wetted with solution of S. griseus pronase. Upper and lower films were linked together in order to superpose profiles, than photographed against light background and archived.

Semiquantitative RT-PCR

RNA was isolated following Chomczynski and Sacchi [16]: 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, 20 pmol (1.0 μM) of reverse primer. Synthesis was carried out in the following conditions: 70°C – 5 min, further 37°C – 5 min, 42°C – 1 h. PCR 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 $MgCl_2$ and 1.5 U of Taq DNA polymerase ("MBI Fermentas", Lithuania). PCR amplifications consisted of the initial denaturing step of 94ºC for 4 min, followed by 35 cycles (28 cycles for Actb – gene used as the internal control of reaction due to its constitutive expression) of 94ºC for 45 s, appropriate annealing temperature: 50ºC – 45 s for Par2 (357 b.p.) and 49ºC – 40 s for Actb (521 b.p.), the final extension step at 72ºC for 1 min 15 s (for Par2) or 1 min (for Actb). Terminal fill-in of PCR products was performed at 72ºC for 5 min. The following primers were used in reactions – for Par2: forward – GAATGCACCGGGACCCAA, reverse – TCCCCATAGGTCCAGTCGTT; for Actb: forward – TGGGACGATATGGAGAAGAT, reverse – ATTGCCGATAGTGATGACCT. Separation of PCR products was carried out electrophoretically in 1.6% agarose gel with 0.5x TBE buffer. For semi-quantitative analysis of amplicons expression based on densitometry the ImageJ 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. [17].

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Measurement of reduced glutathione (GSH) content

GSH content was measured in protein-free CMF of pancreatic cells based on formation of highly fluorescent product of reaction between GSH and ortho-phthalic aldehyde [18], and given as µmol / g of tissue.

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

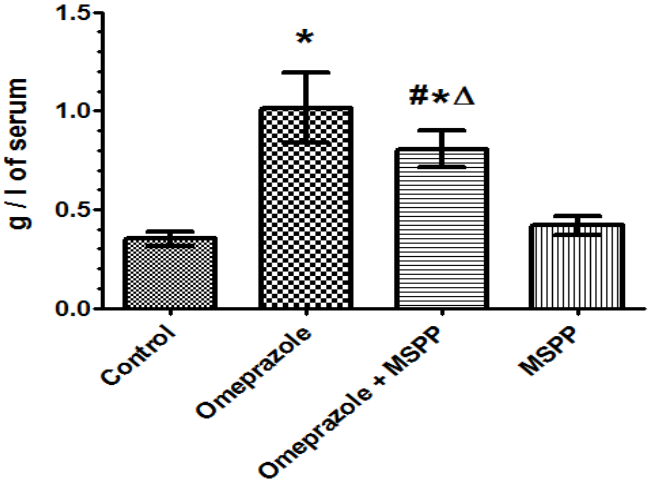
Statistical processing of experimental results was carried out in "IBM® SPSS Statistics 20" (USA) and "GraphPad Prism 5" (USA). Mean (M) and standard deviation (SD) were calculated for each group. Type of data distribution and presence of outliers in groups were checked with Shapiro-Wilk test and z-score (the relation between standardized values of skewness and kurtosis). As data were distributed normally ($p > 0.05$ according to Shapiro-Wilk, z-score within ± 2.58), one-way ANOVA was conducted to determine the significance of difference between means. In the case of homogeneity of variances (according to Levene's test) Tukey post-hoc test was performed; when homogeneity of variances was violated Welch ANOVA with Games-Howell post-hoc test were used. Difference between means was judged as statistically significant if $p < 0.05$. 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 $|r| = 0.1 – 0.3$ [19].

RESULTS

Level of α-1-antitrypsin in rat blood serum (Figure 1)

Violation of homogeneity of variances in groups was established ($p < 0.001$; Levene). Statistically significant difference between means was observed ($p < 0.001$; Welch ANOVA). Level of A1AT in blood serum of animals injected with omeprazole was equal to 0.352 ± 0.038 g / l of serum, thus 2.9 times surpassing the value of control group ($p < 0.001$; Games-Howell). In animals of the third group this parameter was 1.3 times lower in comparison with second group ($p = 0.027$), but 2.3 times higher than control value ($p < 0.001$). In animals treated only with MSPP the level of A1AT in blood serum reached 0.420 ± 0.047 that was 1.9 lower than in animals of the third group ($p < 0.001$), but didn't differ significantly from the control ($p = 0.480$).

Figure 1: Level of α-1-antitrypsin in rat blood serum, g / l of serum (M ± SD, n = 10).



Remarks: MSPP – multi-strain probiotic preparation; * – $p < 0.001$ in comparison with control animals; # – $p < 0.001$ in comparison with animals treated with omeprazole only; Δ – $p < 0.001$ in comparison with animals treated with multi-strain probiotic preparation only.

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Activity of pancreatic hydrolases in rat blood serum (Table 1).

Homogeneity of variances was detected (p = 0.724; Levene). At the same time, no statistically significant difference between group means was observed (p = 0.054; one-way ANOVA).

Table 1: Activity of pancreatic hydrolases in blood serum and cytosolic-microsomal fraction of rat pancreas (M ± SD, n = 10)

Group / Parameter	Control	Omeprazole	Omeprazole + MSPP	MSPP
Blood serum, U / l of serum				
Amylase activity	21.4 ± 2.5	24.3 ± 3.1	23.0 ± 2.4	20.9 ± 2.5
Lipase activity	8.6 ± 0.8	13.4 ± 2.6**	11.7 ± 1.4$^{*\Delta}$	9.1 ± 1.5
Trypsin activity	4.6 ± 0.7	5.3 ± 0.9	5.2 ± 0.8	4.4 ± 0.8
Pancreas, U / g of tissue				
Amylase activity	47.9 ± 5.4	61.9 ± 6.8**	49.2 ± 6.5$^{\#}$	44.5 ± 5.0
Lipase activity	26.7 ± 2.4	35.7 ± 2.9**	25.2 ± 29$^{\#\Delta\Delta}$	17.8 ± 2.7**
Trypsin activity	10.5 ± 2.0	16.9 ± 1.9**	11.2 ± 1.8$^{\#}$	10.7 ± 1.8

Remarks: MSPP – multi-strain probiotic preparation; * – p < 0.01 and ** – p < 0.001 in comparison with control animals; # – p < 0.001 in comparison with animals treated with omeprazole only; Δ – p < 0.01 and ΔΔ – p < 0.001 in comparison with animals treated with multi-strain probiotic preparation only.

In the case of lipase activity of blood serum, the assumption of homogeneity of variances was violated as assessed by Levene's test (p = 0.002). Lipase activity was statistically significantly different between studied groups (p < 0.001; Welch ANOVA). Lipase activity in blood serum of control rats reached 8.57 ± 0.79 U / l of serum. In animals treated with omeprazole this parameter was 1.6 times higher comparing with the control (p = 0.001; Games-Howell). Lipase activity was equal to 11.7 ± 1.4 U / l of serum upon joint administration of omeprazole and MSPP (p = 0.307 in comparison with the second group; p < 0.001 in relation to the control; p = 0.004 comparing with the fourth group). Lipase activity in blood serum of rats treated only with MSPP didn't differ significantly from the control (p = 0.767).

The differences between mean trypsin activity in blood serum of rats from different groups was not statistically significant (p = 0.064; one-way ANOVA).

Activity of hydrolases in cytosolic-microsomal fraction of rat pancreatic cells (Table 1). Presence of low enzymatic activity of α-amylase, lipase and trypsin in CMF of pancreatic cells of animals from control group was established. There was homogeneity of variances, as assessed by Levene's test for all three enzymes (amylase: p = 0.908; lipase: p = 0.870; trypsin: p = 0.970).

Amylase activity was statistically significantly different between experimental groups (p < 0.001; one-way ANOVA). Amylase activity in CMF of pancreatic cells of control rats was equal to 47.9 ± 5.4 U / g of tissue. In animals injected with omeprazole this parameter exceeded control value in 1.3 times (p < 0.001; Tukey). Amylase activity in pancreatic CMF of rats from the third group reached 49.2 ± 6.5 U / g of tissue, which was lower than value of animals from the second group in 1.3 times (p < 0.001), but didn't differ from values of animals from the first and fourth groups (p = 0.967 and p = 0.312, respectively). In animals treated only with MSPP this index didn't significantly differ from the control (p = 0.575).

One-way ANOVA revealed that lipase activity in CMF of pancreatic cells was substantially different in studied groups (p < 0.001). Lipase activity in pancreatic tissue of animals injected with omeprazole reached 35.7 ± 2.9 U / g of tissue, thus 1.3 times exceeding control value (p < 0.001; Tukey). Animals of the third group had 1.4 times lower lipase activity in comparison with the second group (p < 0.001), but it was 1.4 times higher than in animals treated with MSPP only (p < 0.001). There was no significant difference in pancreatic lipase activity between animals of the first and third groups (p = 0.613). At that, lipase activity in pancreatic tissue of animals from the fourth group was equal to 17.8 ± 2.7 U / g of tissue, which is 1.5 times lower in comparison with the control (p < 0.001).

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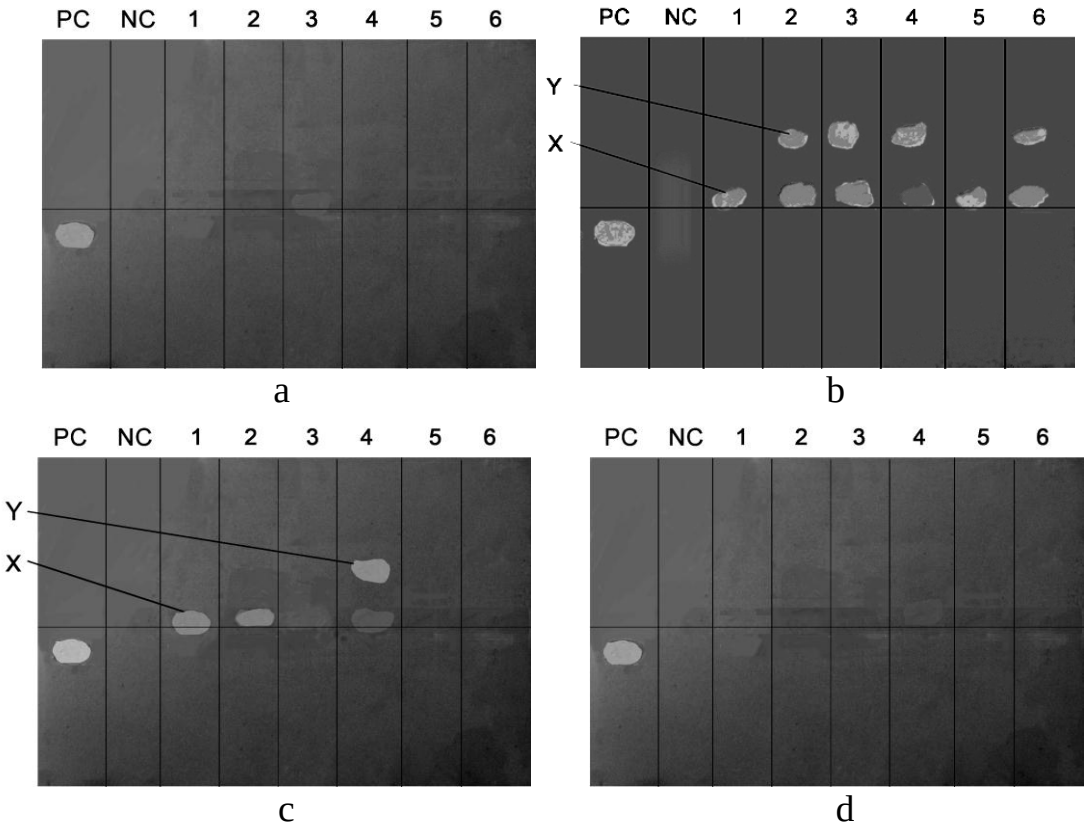
Pancreatic trypsin activity was statistically significantly different between studied groups ($p < 0.001$; one-way ANOVA). Trypsin activity in CMF of pancreatic cells of control rats was equal to 10.5 ± 2.0 U / g of tissue. Substantially higher value of trypsin activity was shown for rats injected with omeprazole ($p < 0.001$ in relation to the control; Tukey). In rats jointly treated with omeprazole and MSPP this parameter was 1.5 times lower than in the second group ($p < 0.001$) and didn't differ significantly from the first and fourth groups ($p = 0.835$ and $p = 0.958$ respectively). There also was no substantial difference in pancreatic trypsin activity between animals treated with MSPP and control rats ($p = 0.987$).

Proteolytic profile of rat pancreatic juice (Figure 2)

Absence of active proteases in pancreatic juice samples of rats from the first and fourth groups was shown by contact print zymography technique (Figures 2a and 2d). Presence of two spots of proteolytic activity in the majority of samples from rats treated with omeprazole was revealed (Figure 2b). It should be noted, that these spots are in the upper part of film meaning cationic character of discovered proteases, since migration towards the top of gels upon native electrophoresis are due to positive charge of these proteins upon selected conditions.

Figure 2: Zymograms of proteases in rat pancreatic juice (n = 6).



Remarks: a – control; b – omeprazole; c – omeprazole + multi-strain probiotic preparation; d – multi-strain probiotic preparation; PC – positive control; NC – negative control; 1-6 – samples from separate animals; X and Y – spots corresponding to separate proteases in samples.

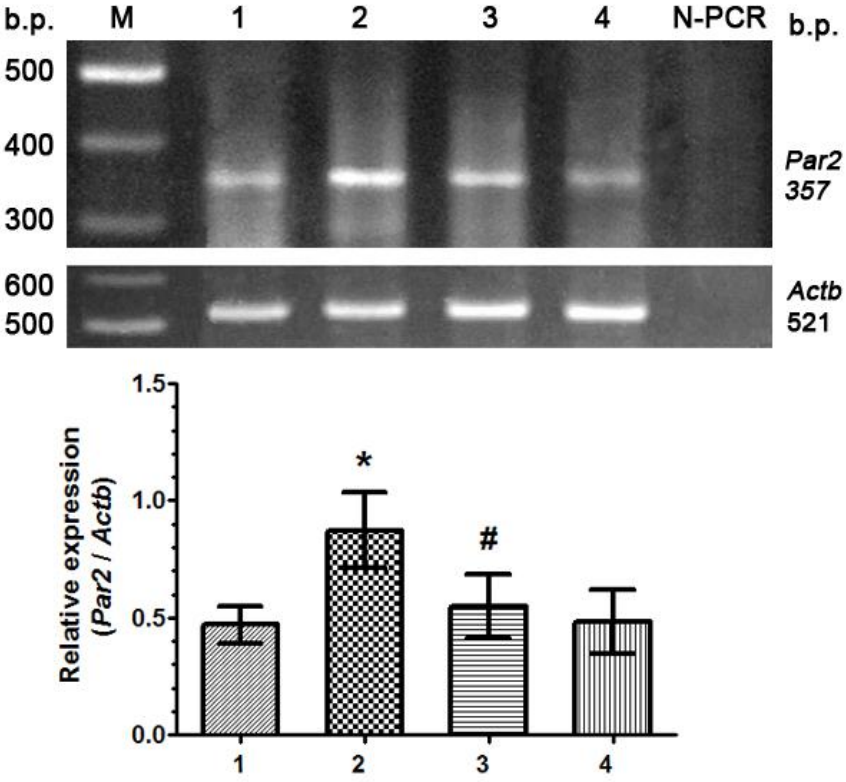
Single spot of proteolytic activity was revealed in three samples of pancreatic juice from animals treated with omeprazole and MSPP simultaneously (Figure 2c). Indicated spots are also located in the upper part of film suggesting cationic character of these proteases. Electrophoretic separation was performed with pH equal to 8.4 suggesting that isoelectric point (pI) of these proteases is higher than 8.4. According to Uniprot database, only trypsin-3 (P08426), chymotrypsin-like elastase-1 (P00773) and elastase-2 (P00774), and carboxypeptidase N (Q9EQV8) have pI value above 8.4. Isoelectric point values are equal to 8.43, 8.80, 9.01 and 8.61, respectively. Moreover, carboxypeptidase Z (O54858) also has very similar pI value – 8.34. Based on these data, it can be suggested that first spot (indicated as X on Figures 2b and 2c) can correspond to trypsin-3 or carboxypeptidase Z, whereas second spot (marked as Y on Figures 2b and 2c) fits chymotrypsin-like

elastase-1/2 and carboxypeptidase N. Accurate identification of proteases corresponding to revealed spots requires further additional investigations.

Level of Par2 gene mRNA in rat pancreatic tissue (Figure 3)

There was homogeneity of variances for all studied groups ($p = 0.248$; Levene). Level of Par2 gene mRNA was statistically significantly different between groups ($p < 0.001$; one-way ANOVA). Relative content of Par2 gene mRNA in pancreatic tissue of control rats was equal to 0.470 ± 0.080 in relation to Actb. Animals injected with PPI omeprazole had 1.9 times higher level of Par2 mRNA in comparison with the control ($p < 0.001$; Tukey). The level of Par2 gene mRNA in pancreatic tissue of rats jointly treated with omeprazole and MSPP was substantially different from values of the first ($p < 0.001$), second ($p < 0.001$) and fourth group ($p < 0.001$). This parameter reached 0.482 ± 0.136 in relation to Actb in pancreatic tissue of animals treated with MSPP that wasn't highly different from the control ($p = 0.655$).

Figure 3: Relative content of Par2 gene mRNA in rat pancreas (M ± SD, n = 8).



Remarks: M – molecular mass marker; 1 – control; 2 – omeprazole; 3 – omeprazole + multi-strain probiotic preparation; 4 – multi-strain probiotic preparation; N-PCR – negative PCR control; * – $p < 0.001$ in comparison with control animals; # – $p < 0.001$ in comparison with animals treated with omeprazole only.

Content of reduced glutathione in rat pancreas (Figure 4).

Statistically significant difference between means of studied groups was observed ($p < 0.001$; one-way ANOVA). The level of GSH in CMF of pancreatic cells of control rats was equal to 1.98 ± 0.23 µmol / g of tissue. Animals injected with omeprazole during 28 days have 2.1 times lower content of GSH in comparison with the control ($p < 0.001$; Tukey). Animals of the third group had 1.5 times higher content of GSH in comparison with the second group ($p = 0.001$), but 1.8 times lower in relation to the fourth group ($p < 0.001$). The level of GSH in animals treated only with MSPP reached 2.54 ± 0.30 µmol / g of tissue, thus 1.3 exceeding control value ($p < 0.001$).

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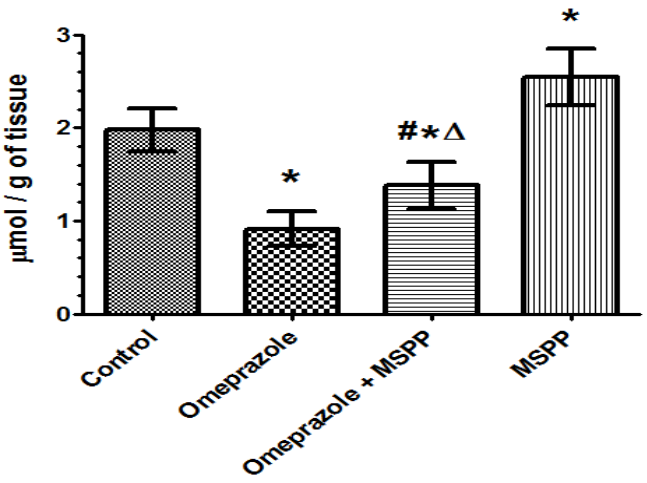
Figure 4: Level of reduced glutathione in rat pancreas, µmol / g of tissue (M ± SD, n = 10)



Remarks: MSPP – multi-strain probiotic preparation; * – $p < 0.001$ in comparison with control animals; # – $p < 0.001$ in comparison with animals treated with omeprazole only; Δ – $p < 0.001$ in comparison with animals treated with multi-strain probiotic preparation only.

Results of correlation analysis (Table 2).

Pearson's correlation analysis was performed in order to test two hypotheses:

Table 2: Pearson's correlation coefficients (r) for selected biochemical indices of rat blood and pancreas

Dependent variables	$Trypsin_P$		GSH	
	r	P	r	p
A1AT	0.422*	0.012	-0.526**	0.002
$Trypsin_P$	1.000	<0.001	-0.729***	<0.001
$Amylase_P$	0.757***	<0.001	-0.614**	0.003
$Lipase_P$	0.774***	<0.001	-0.729***	<0.001
$Trypsin_B$	0.341*	0.024	-0.316*	0.019
$Amylase_B$	0.316*	0.029	-0.364**	0.008
$Lipase_B$	0.561**	0.003	-0.617**	0.002
Par2	0.733***	<0.001	-0.605***	<0.001

Remarks: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$ (significance levels of correlation coefficient); GSH – content of reduced glutathione in pancreatic tissue; A1AT – level of α-1-antitrypsin in blood serum; $trypsin_P$, $amylase_P$ and $lipase_P$ – trypsin, amylase and lipase activities in pancreatic tissue; $trypsin_B$, $amylase_B$ and $lipase_B$ – trypsin, amylase and lipase activities in blood serum; Par2 – content of Par2 gene mRNA in pancreatic tissue.

- Trypsin activity in pancreatic tissue of rats positively correlates with activity of other hydrolytic enzymes, as well as with level of antiproteolytic response – both in pancreas and blood serum.

- Content of GSH in rat pancreatic tissue negatively correlates with activity of pancreatic hydrolases, as well as with the rate of antiproteolytic response – both in blood serum and pancreas.

Preliminary analysis revealed the linear character of interrelation between variables, absence of outliers, and normal distribution of data in groups ($p > 0.05$; Shapiro-Wilk). Strong positive correlation between trypsin activity in pancreatic CMF and lipase activity both in blood serum and pancreas was determined. Moreover, strong positive correlation of trypsin activity with the level of Par2 gene mRNA and amylase activity in pancreatic tissue was also revealed. Besides that, moderate positive correlation between trypsin activity in pancreatic tissue and A1AT content, lipase and amylase activities in blood serum was shown. Based on these data, first hypothesis may be judged as confirmed.

Strong negative correlation between GSH level and pancreatic hydrolases activity, as well as with Par2 gene mRNA content in rat pancreatic tissue was observed. Besides that, strong negative correlation between GSH level in pancreatic tissue and A1AT content, and lipase activity in blood serum was also revealed.

Moderate negative correlation between GSH level and blood trypsin and amylase activities was also shown. Above mentioned data indicate that second hypothesis is also confirmed.

DISCUSSION

Elucidation of gut microbiota role in the development of pancreatitis-like biochemical changes upon long-term suppression of gastric acid secretion in rats was based on the idea that administration of normal microbiota with MSPP will be capable of counteracting dysbiosis development that often sequels hypochlorhydria – the state of low gastric acidity. The precedent research of Abdulahad et al. [8] confirmed the efficacy of such approach. They have shown the qualitative and quantitative changes (dysbiosis) of rat gastric microbiota upon 28-day long administration of omeprazole. The stomach was colonized by Klebsiella, Proteus and Candida genera, as well as by E. coli, S. aureus, S. epidermidis species with contemporary depletion of lactobacilli. Composition of gastric microbiota was similar to the control upon joint administration of omeprazole and MSPP "Symbiter® acidophilic concentrated", but was associated with much higher content of lactobacilli and lower amount of bacteria from genus Citrobacter.

Results of current research revealed a series of pancreatitis-like biochemical changes in pancreatic tissue and blood serum upon 28-day long suppression of gastric acid secretion. In particular, increased amylase, lipase and trypsin activities, as well as enhanced expression of Par2 gene contrasted with depletion of GSH in pancreatic CMF were discovered. Moreover, presence of active proteases in pancreatic juice, as well as increased content of A1AT and elevated lipase activity in blood serum were also determined. At the same time, in case of normal gut microbiota maintenance by MSPP administration upon injection of omeprazole, majority of studied indices didn't significantly differ from control values suggesting that disturbance of gut microbiota are indeed involved in the development of pancreatitis-like changes upon current experimental conditions.

Let us consider the possible mechanisms connecting dysbiotic changes in gut with pancreatic damage. Pancreatic cells are known to express few subtypes of Toll-like receptors sensing bacterial cell wall components [20]. Activation of Toll-like receptors and initiation of inflammation are probable events upon bacterial colonization of gastroduodenal region. This initial inflammation is an example of the so called "sentinel event" [21] upon hypochlorhydria-associated dysbiosis. It's also possible that action of cellular or secretory components of pathogenic microbiota and (or) inflammatory cytokines from the source of endogenous inflammation near the pancreas leads to rapid development of pancreatic inflammation [21]. At that, release of proinflammatory cytokines, such as TNF-α, IL-1, IL-8 and platelet-activating factor (PAF) with simultaneous depletion of anti-inflammatory IL-10 [22] are taken place, as confirmed by recent results of Dvorshchenko et al. [23]. These cytokines stimulate expression of cell adhesion molecules (CAM) on plasma membrane of neutrophils and endothelial cells leading to migration of neutrophils from pancreatic microcirculation vessels into periacinar space [24]. Activation of neutrophils leads to further increase in proinflammatory cytokines release, rise of CAM expression, elevation of vessels permeability and generation of reactive oxygen species (ROS) in acinar cells [20, 22]. Release of cytokines and increased expression of CAM favour even greater migration of neutrophils into acini – inflammatory cycle emerges [22]. Net effect of above mentioned events is neutrophilic infiltration into pancreatic tissue with generation of ROS and oxidative stress development [25]. Indeed, elevation of ROS, products of free-radical oxidation of lipids and proteins, as well as decreased activity of antioxidant enzymes were shown in recent research of Dvorshchenko et al. [7]. Decrease of GSH level was also observed in current investigation (Figure 4), thus indicating depletion of antioxidant system and oxidative stress progression upon long-term suppression of gastric acid secretion. Furthermore, elevated content of A1AT in blood serum (Figure 1) upon these conditions can also indirectly suggest the presence of inflammation with leukocytic infiltration, since A1AT is an effective inhibitor of few hydrolases from azurophilic granules of neutrophils [26].

With the progression of free-radical processes and oxidative damage of zymogen granules and lysosomal membranes, their further colocalization and fusion occurs, thus leading to intrapancreatic activation of trypsinogen [24]. We have established the increase of trypsin activity in pancreatic tissue and presence of active proteases in pancreatic juice; it's in agreement with observed high expression level of Par2 gene (Figure 3) that encodes receptor sensing premature activated proteases [27]. Trypsin activates other zymogens (confirmed by data in Table 1) through limited proteolysis, thus promoting resultant pancreatic damage. Indeed, strong positive correlation between trypsin activity and activity of other hydrolases in CMF of

pancreatic tissue was revealed (Table 2). Apparently, enhanced paracellular penetration of hydrolytic enzymes into blood through duodenal wall can take place at that [28]. This idea is partially confirmed by observed increase in lipase activity and high content of protease inhibitor A1AT in blood serum of rats, but extent of duodenal wall permeability for different enzymes should be investigated further in order to test this hypothesis. It should also be noted that amylase and trypsin activities in blood serum only moderately correlated (Table 2) with hydrolytic activities in pancreatic tissue, suggesting that these indices are not very representative on the initial stages of pancreatic pathology.

Selected for our experiment MSPP «Symbiter® acidophilus concentrated» is a complex bacterial community consisting of 14 different strains of normal mammalian gut microbiota that belong to 10 species: Bifidobacterium bifidum, B. longum, Lactobacillus acidophilus, L. delbrueckii, L. helveticus, Propionibacterium freudenreichii, P. acidipropionici, Lactococcus lactis, Acetobacter aceti, Streptococcus salivarius.

Besides maintenance (or restoration) of normal gut microbiota probiotics have a set of activities, including enhancement of gut wall barrier function, release of bacteriocins, immune response modulation, counteraction to bacterial invasion etc [29, 30]. Prominent immune modulating effects of MSPP «Symbiter® acidophilus concentrated» were revealed in recent investigation of Dvorshchenko et al. [23]: animas treated with MSPP had much higher level of anti-inflammatory IL-10 and lower amounts of pro-inflammatory IL-1, IL-6 and TNF-α in comparison with control and hypochlorhydric animals. Moreover, increased content of GSH in pancreatic CMF upon administration of MSPP was observed in current research (Figure 4). Furthermore, there is strong negative correlation between GSH level in pancreatic tissue and majority of analyzed indices according to correlation analysis data (Table 2). Hence, it follows that the higher the level of GSH in pancreatic tissue, the lower the manifestation of pancreatitis-like biochemical changes, including lower activity of pancreatic hydrolases in the pancreas and fewer active proteases are there in pancreatic juice. Recently Lutgendorff et al. [31] also revealed that multicomponent probiotics are capable of increasing de novo synthesis of GSH and augment its content in pancreas. Using experimental model of acute pancreatitis they elicited that rate of oxidative stress and inflammation development, as well as degree of pancreatic damage were reduced upon preliminary therapy with probiotic bacteria. These data are in agreement with our results and justifies elevated level of GSH (Figure 4) in pancreatic tissue upon administration of MSPP. Based on the above stated it can be suggested that beneficial effects of MSPP revealed in our research are related not only with gut microbiota maintenance, but also with antioxidant and immune modulating properties of the preparation.

Thus, it was revealed that disturbance of normal gut microbiota plays significant role in the initiation of pancreatitis-like biochemical changes upon long-term suppression of gastric acid secretion in rats, considering that administration of multicomponent probiotic preparation for the purpose of gut microbiota maintenance wasn't associated with pathologic biochemical changes or only slight tendency was observed. Moreover, obtained data indicate that discovered beneficial effects of MSPP are in part due to increase in intrapancreatic level of GSH that enhances antioxidant capacity of pancreatic cells and counteracts oxidative damage. More detailed investigations are required to elucidate specific molecular mechanisms of cross-talk between pathogenic gut microbiota and pancreatic cells.

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