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The distribution of 4-hydroxynonenal-modified proteins in gastric mucosa of duodenal peptic ulcer patients

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Abstract

This study used monoclonal antibody specific for 4-hydroxynonenal (HNE)-histidine to evaluate immunohistochemical distribution of HNE–protein adducts in gastric mucosa biopsies of 52 peptic ulcer patients (all positive for *H. pylori*) and of 20 healthy volunteers (eight positive and 12 negative for *H. pylori*). HNE-modified proteins were present in glandular epithelium in all subjects, both patients with duodenal peptic ulcer and healthy subjects. Hence, the presence of HNE did not appear to be related to the presence of *H. pylori*. However, in patients with duodenal peptic ulcer accumulation of HNE-protein adducts was frequently observed also in nuclei, while in the control group such subcellular distribution of HNE was not observed at all. This study shows physiological presence of HNE in human gastric mucosa, but also suggests its role in pathology of gastric dysfunction in duodenal peptic ulcer patients manifested by accumulation of HNE-protein adducts in particular in nuclei of gastric glandular epithelium.

Keywords: *Lipid peroxidation, 4-hydroxynonenal, oxidative stress,* Helicobacter pylori, *peptic ulcer, gastric mucosa*

Introduction

Helicobacter pylori infection has been proved to be an important risk factor of many gastrointestinal diseases, such as chronic atrophic and non-atrophic gastritis, peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue lymphoma [1–3]. The prevalence of *H. pylori* among the adult population in different countries varies between 20–40% in developed countries and exceeds 90% in developing countries [4,5].

Bacterial pathogenicity of *H. pylori* have been linked to its virulence factors such as cytotoxin-associated gene A (CagA), vacuolating toxin A (VacA), urease and adherence factors [4], which stimulate chemotaxis of neutrophiles and macrophages with subsequent 'oxidative burst' and excessive production of reactive oxygen species (ROS) [6–10]. Moreover, phagocytes are able to produce hypochloric acid in myeloperoxidase reaction, which in presence of ammonia, produced by microbial urease, generate chloramines—extremely reactive

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and toxic lipophilic agents capable of damaging mucous membrane and inflammation enhancement. Thus, persistence of *H. pylori* causes oxidative stress and development of chronic inflammation, which are important pathogenic mechanisms of ulcerogenesis and malignant transformation [7,11–14].

Furthermore, this micro-organism possesses several levels of defense against ROS damage. First of all, membrane structures of *H. pylori* contain predominantly saturated and monounsaturated fatty acids, which are known to be more resistant to ROS damage, compared to polyunsaturated. The latter were absent or were detected in extremely low concentrations in *H. pylori* cell wall [15]. Besides, *H. pylori* was demonstrated to have antioxidant enzymes (catalase, superoxide dismutase, peroxiredoxins), which protect it from oxidative damage and, thus, play an important role in its survival and ability to colonize gastric mucosa [16–18]. *H. pylori* upregulates the host inducible nitric oxide synthase (iNOS) and simultaneously produces an arginase, which siphons L-arginine away from the competing host iNOS, thereby limiting the production of bactericidal NO [19]. Moreover, by its periplasmatic enzyme gamma-glutamyltranspeptidase *H. pylori* can consume glutathione and glutamine from the host mucosa and use them for glutamate and NH_3 production. Thus, extracellular glutathione is used for *H. pylori* metabolism and environment alkalinization, which, on the one hand, promotes its persistence and, on the other hand, lowers the mucosal antioxidant capacity [20]. To sum up, the property of *H. pylori* to cause mucosa lesions is further facilitated by its ability to protect itself from oxidative damage. Nevertheless, most of *H. pylori* infected subjects remain asymptomatic, which points to the importance of the host antioxidant defense, namely its ability to control ROS production and eliminate harmful consequences of oxidative stress [14]. ROS cause damage to major macromolecules (nucleic acids, proteins and lipids) which, together with decline in host antioxidant capacity, could lead to pathological aberrations.

In particular, accumulation of the lipid peroxidation products such as 4-hydroxy-2,3-nonenal (HNE), denoted also as a 'second messenger of free radicals' could be important in carcinogenesis [21–24]. HNE originates from ω-6 polyunsaturated fatty acids oxidation [21] and its biological effects are defined by interactions with proteins, phospholipids and nucleic acids and modulation of their functional activity [22].

Being in high concentrations cyto- and genotoxic, in the physiological concentrations HNE was found to be an important regulatory molecule in many processes, including regulation of proliferation, differentiation and apoptosis [23–28]. Moreover, low concentrations of HNE are chemoattractant for macrophages and neutrophiles, while its high concentrations (>10 μM) upregulate cycloxygense-2 expression with subsequent proinflammatory prostaglandins synthesis [29–31]. Recent studies demonstrated that in physiological concentrations HNE acts as a potent nuclear peroxisome proliferator-activated receptor (PPAR) agonist and is involved in regulation of energetic metabolism and inflammation [27,30,32]. Because gastroprotective and ulcer healing properties of PPAR agonist were demonstrated [33], it is attractive to speculate that HNE-involving mechanisms could be exploited in new strategies of duodenal peptic ulcer (DPU) treatment by modulation of peroxisomal oxidation intensity. However, so far there were no studies on possible involvement of HNE in the *H. pylori* and DPU pathology.

Therefore, this study evaluated the presence and distribution of HNE-protein adducts in gastric mucosa of DPU patients as a potentially important factor in the pathogenesis of *H. pylori* associated diseases.

Materials and methods

Subjects

Patients at the age of 18–44 were selected from the subjects consulted in Lviv National Medical University and Novoyavorivsk Regional Hospital No. 1 because of abdominal complaints. All of them underwent upper gastrointestinal endoscopy. In 52 of them (mean age $\pm$ SEM, 32.0 ± 0.8 years, 34 males and 18 females) *H. pylori* associated DPU was diagnosed. The control group consisted of 20 healthy volunteers (mean age $\pm$ SEM, 29.7 ± 1.4, 13 males and seven females) without any gastrointestinal complaints and other registered health problems. Out of these patients eight were positive and 12 were negative for *H. pylori* presence. None of them had taken H2 receptor antagonists, proton pump inhibitors, bismuth, antibiotics, non-steroidal anti-inflammatory drugs or corticosteroids within at least 1 month before enrolment. The design of the study was approved by local Ethics Committee of Lviv National Medical University and informed consent was obtained from all patients and healthy volunteers.

Histology and assessment of H. pylori *infection*

Two biopsy specimens were obtained endoscopically from antrum and two from corpus of the stomach in all cases for histological examination. Namely, it is known that *H. pylori* is living in gastric mucosa predominantly in antrum and less likely in corpus of the stomach. Because of that, the specimens used for analytical and diagnostic purposes are taken from antrum and corpus. In duodenum this micro-organism is rarely observed, while the risk of complications (perforation, haemorrhage) is much higher. Therefore, biopsies of duodenum are considered as relative

risk and are not performed without good medical indices.

Biopsy specimens were immediately fixed in buffered 10% formalin, then dehydrated in ethanol and embedded in paraffin, cut into 5-μm thick sections and examined with haematoxylin-eosin and modified Giemsa staining. Histologically, *H. pylori* infection was considered as negative if *H. pylori* was absent in all biopsies obtained from one patient and positive if it was found in at least one sample. Additionally, one antral biopsy specimen was taken for rapid urease test. Inflammation, contamination of *H. pylori*, glandular atrophy and intestinal metaplasia were classified qualitatively as negative (0) or positive (1); and semi-quantitatively into four grades according to updated Sydney System as follows: 0, none; 1, mild; 2, moderate and 3, severe.

Representative paraffin blocks were further used for immunohistochemical staining: one slice of each specimen was cut into 5-μm thin sections, mounted on the slide coated with 3-aminopropyl-triethoxy silane, deparaffinated in xylene and rehydrated trough a series of ethanol.

Immunohistochemistry for HNE-modified proteins was carried out using monoclonal antibodies obtained from culture medium of the clone 'HNE 1g4', produced by a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALB-c mouse immunized with HNE-modified keyhole limpet hemocyanine. The antibody is specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates and gives only 5% cross-reactivity with HNE-lysine and 4% with HNE-cysteine [34].

Immunohistochemistry was done in a three step procedure as described before [23,35] using LSAB kit (DAKO, Denmark) where the first step was incubation with anti-HNE monoclonal antibodies (dilution 1:10) during 2 h in humid chambers at room temperature. The second step was incubation with biotinylated secondary goat anti-mouse and anti-rabbit immunoglobulins (AB2) during 30 min. The third step was incubation with streptavidin peroxidase during 30 min. Finally, the reaction was visualized by a DAB (3,3-diaminobenzidine tetrahydrochloride in organic solvent) after 10 min. Negative control was done on one histological slice of the same tissue, without application of HNE-histidine specific monoclonal antibodies. Intensity and distribution of the HNE-immunostaining in the gastric mucosa from antrum and corpus of the stomach were evaluated semi-quantitatively. The absence of immunopositivity in cytoplasm and nuclei of glandular epithelial cell was marked with (0), while with (1) we marked weak immunopositivity in less than 25% of the cells, with (2) medium immunopositivity in 25–50% of cells and finally with (3) strong immunopositivity in more than 50% of cells. In superficial and foveolar epithelium, as well as in lamina propria of gastric mucosa, we distinguished only positive (1) and negative (0) immunostaining. All immunohistochemical analyses were done by an pathologist experienced in the HNE immunohistochemistry without prior knowledge of the study group design.

Statistical analysis

The prevalence of *H pylori* infection, inflammation, glandular atrophy and intestinal metaplasia, as well as incidence of HNE in gastric tissues, was evaluated by Chi-square test after classifying the marker as positive or negative. Possible difference in *H. pylori* contamination and intensity of HNE staining were done by Mann-Whitney U-test, using numerical description of positivity corresponding to respective standard grading of positivity as described above. Calculations were carried out using statistical software SPSS 9.0.

Results

The presence of HNE-protein adducts in gastric mucosa was found not only in the biopsies of DPU patients but also in healthy subjects (Figure 1). We did not observe any particular differences in the HNE-immunopositivity between *H. pylori* positive and negative controls. Distribution of HNE-protein adducts in different parts of gastric mucosa revealed similarities between healthy controls and DPU patients, yet specific pattern of the tissue accumulation of the HNE-protein adducts was found for the DPU patients (Table I). HNE-positivity in superficial gastric epithelium was observed only in two controls (10.0%) and 13 (25.0%) DPU patients (difference between groups not significant at $p = 0.160$). Similarly, in foveolar epithelium HNE-immunopositivity was observed in one control subject (5.0%) and 13 (25.0%) patients ($p = 0.055$). In lamina propria of gastric mucosa accumulation of HNE was much more frequent and was predominantly associated with inflammatory cells (neutrophiles, macrophages and in part lymphocytes). HNE-immunopositivity was observed in lamina propria of seven (35%) subjects of control group and of 30 (57.7%) of DPU patients (difference between groups was significant at $p < 0.001$). Furthermore, almost all controls (19 of 20 subjects), as well as all DPU patients, revealed the presence of HNE-protein adducts in cytoplasm of gastric glandular epithelium. Opposite to that, nuclei of the glandular epithelium were negative for all healthy subjects and for the majority of DPU (Table II). However, distribution of studied subjects by the grade of immunopositivity in cytoplasm and nuclei of glandular epithelium (Table II) revealed significant difference in incidence of nuclear immunopositivity ($p = 0.0143$) between healthy controls and DPU patients. While there was no healthy

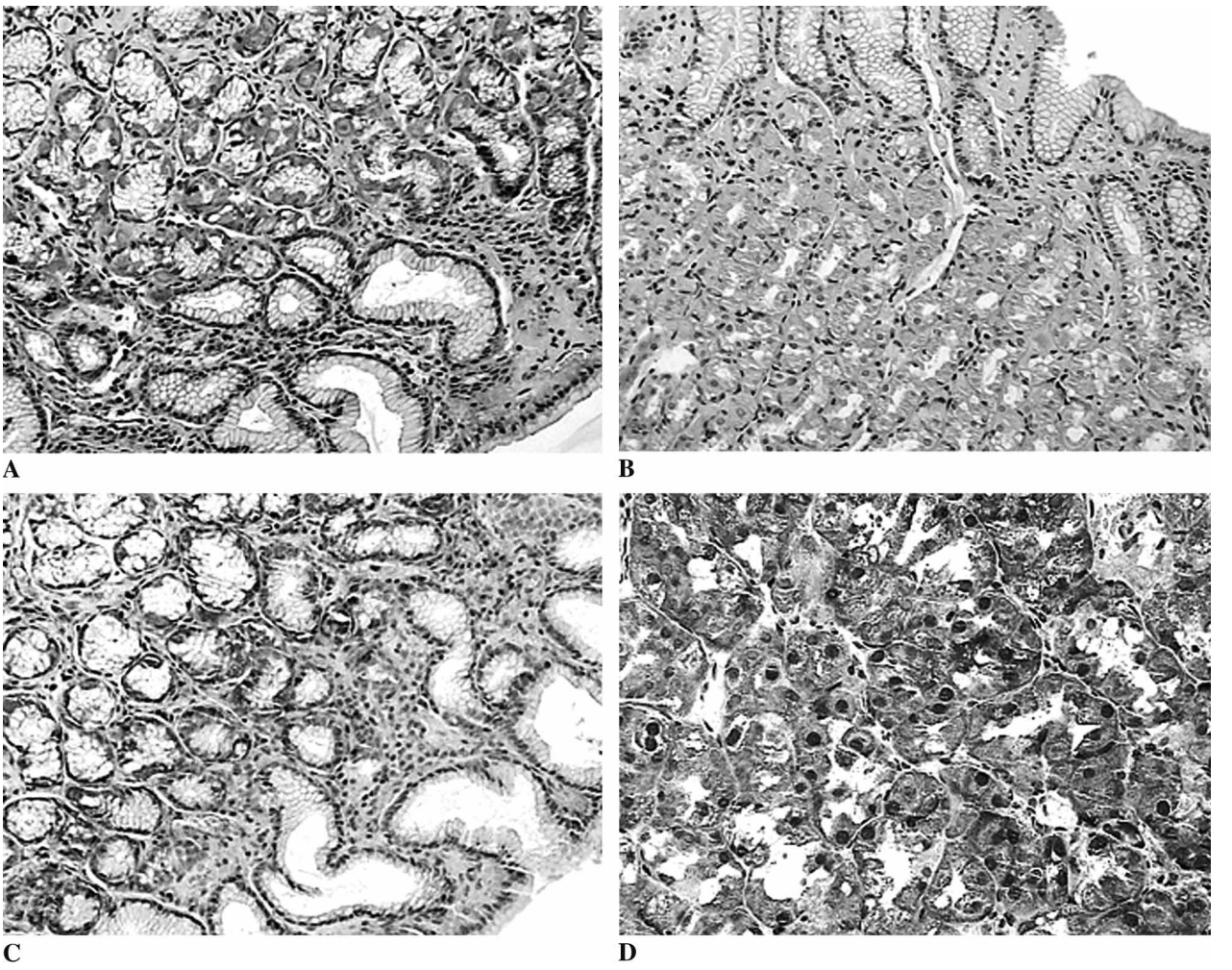

Figure 1. Immunohistochemical appearance of HNE in gastric mucosa, magnification 200×; (A) *H. pylori* negative control subject—occasional HNE positivity of glandular epithelium cytoplasm, the other cells are HNE negative; (B) *H. pylori* positive asymptomatic volunteer—mild positivity of most glandular epitheliocytes, superficial and foveolar epithelium, magnification 200×; (C) *H. pylori* positive duodenal peptic ulcer patient with occasionally mild to strong HNE positivity in glandular epithelium, other layers are negative, magnification 200×; (D) *H. pylori* positive duodenal peptic ulcer patient with strong HNE positivity in glandular epithelium (cytoplasms and nuclei), magnification 400×.

subjects with evident HNE-protein adducts in the nuclei of the gastric mucosa epithelium, nuclear HNE-immunopositivity in DPU patients group was predominantly moderate (eight cases, 16.3% from all DPU patients). Only one patient was found to be severely positive (1.9%) and five (9.6%) revealed mild HNE-positivity.

Table I. HNE-immunohistochemistry of gastric mucosa in healthy subjects and in patients with duodenal peptic ulcer (DPU).

	Controls	DPU patients	Significance* (p)
Superficial epithelium	2 (10.0)**	13 (25.0)	0.160
Foveolar epithelium	1 (5.0)	13 (25.0)	0.055
Lamina propria	7 (35)	30 (57.7)	<0.001
Glandular epithelium	19 (95.0)	52 (100)	>0.1

*Chi square test; **relative incidence values are given in brackets (%).

Finally, it should be mentioned that in three patients with pronounced presence of the HNE protein adducts in glandular nuclei atrophic changes in gastric mucosa was noticed as well.

Table II. Subcellular HNE-immunohistochemistry of gastric mucosa in healthy subjects and in patients with duodenal peptic ulcer (DPU).

	Grade of positivity*	Controls	DPU patients
Glandular cytoplasm	0	1 (5.0)**	5 (9.6)
	1	5 (25.0)	5 (9.6)
	2	6 (30.0)	18 (34.6)
	3	8 (40.0)	24 (46.1)
Glandular nuclei	0	20 (100.0)	38 (73.1)
	1	0 (0.0)	5 (9.6)
	2	0 (0.0)	8 (15.4)
	3	0 (0.0)	1 (1.9)

*Semiquantitative staging as described in Materials and methods section; **relative incidence values are given in brackets (%).

Discussion

In our study *H. pylori* infection appeared to be closely associated with DPU, however *H. pylori* negative cases of DPU also were found, which is in agreement with the fact that asymptomatic *H. pylori* bearing is frequently observed in subjects referred to as 'healthy volunteers' since gastric and duodenal mucosa is colonized by this Gram-negative bacteria in about half of the population [1,4,5]. We also observed that *H. pylori* presence even in healthy subjects was nearly always associated with at least moderate signs of chronic inflammation, but, in contrast to gastric ulcers, it did not correlate with the severity of gastritis, as was also observed in another recently published study [36]. These findings suggest that for DPU development the fact of *H. pylori* infection is probably more important than the grade of contamination and the outcome would depend upon microbial virulence factors and the host defense or susceptibility to pathogen [3].

Chronic gastritis associated with *H. pylori* was usually manifested by mononuclear infiltration in gastric mucosa, while neutrophiles were found less frequently. Atrophy, metaplasia and dysplasia were found in a relatively low number of DPU patients (data not presented). These results are in agreement with other histological studies [36,37]. Relatively low prevalence of atrophy, metaplasia and dysplasia is probably due to the following factors: predominantly young age of studied subjects; peptic ulcer is commonly associated with normal or increased acidity; and, finally, more seldom association between DPU and subsequent development of gastric cancer [36–38]. Therefore, it cannot be stated that the observed presence of HNE is associated with the malignant transformation of the gastric epithelium, although this possibility seems probable due to genotoxicity of HNE and its effects on regulation of various cellular functions including cell cycle signalling, proliferation, differentiation, apoptosis, regulation of energy metabolism, modulation of the inflammatory pathways, etc. [24–27,32,39]. Because gastric mucosa can be considered as a stressed tissue due to the very acidic environment and high enzymatic activities further influenced by food ingredients (toxins, alcohol, crude materials, etc.), the presence of HNE observed in gastric mucosa of nearly all studied subjects does not seem surprising. Moreover, it is possible that exogenous HNE, originating from predominantly fried oils, could accumulate in gastric mucosa. Concentrations of HNE, determined as major lipid peroxidation product in fried plant oils [40], seem to be too low to cause strong cytotoxicity. Therefore, HNE might accumulate in gastric epithelium, in particular if bound to the proteins of gastric mucosa. Accumulation of HNE-protein adducts could be mostly expected in case of frequent and continuous intake of fried food. This could perhaps lead to tissue damage and/or transformation of the cells, similar as it is assumed that exogenous toxic compounds in the air contribute to the damage and malignant transformation of the respiratory epithelium.

On the other hand, the finding of HNE in gastric mucosa of apparently healthy subjects indicates a certain physiological role of HNE in glandular epithelium. Namely, mechanical and enzymatic digestion of the food under extreme pH normally occurring in the stomach could be interpreted as intense and chronic metabolic stress for the gastric epithelium leading to continuous tissue damage and renewal. Since HNE is known as a proapoptotic and growth regulating factor it might be possible that it acts not only as a toxic product of the lipid peroxidation but also as a factor regulating homeostasis of the gastric epithelium, which should be further studied.

Immunohistochemistry revealed important peculiarities in the intensity and distribution of HNE between apparently healthy persons and patients. In DPU patients the presence of HNE-protein adducts was observed not only in cytoplasm of glandular epithelium, but also in nuclei, which was not at all observed in the healthy control group. HNE concentration in cells is determined by intensity of its production and activity of utilization pathways. Glutathione S-transferase, alcohol dehydrogenase, aldo-keto reductase and aldehyde dehydrogenase requiring glutathione, NADH, NADPH and NAD+, respectively, are involved in the HNE metabolism [26]. Therefore, the rate of HNE decay is highly dependent on the intensity of redox reactions in the cell. *H. pylori* exhaust glutathione pool in gastric mucosa [20], promote oxidative damage, cause insufficiency of coenzymes and could reduce the HNE elimination in DPU patients with consequential accumulation of the aldehyde not only in cellular cytoplasm, but also in the region of nucleus, as was observed in gastric glandular epithelium of our patients. Since HNE is a highly reactive aldehyde and easily binds with proteins and nucleic acids, it seems probable that such accumulation of HNE could be relevant for malignant transformation of the cells, which will be further studied.

Normally the nucleus is well protected by antioxidant systems and accumulation of lipid peroxidation products is rarely observed in nuclear and perinuclear spaces [35,41]. Therefore, we assume that the accumulation of HNE in nucleus or perinuclear space, which we could not distinguish by immunohistochemistry, indicates low antioxidant capacity of glandular epithelial cells and development of oxidative stress. As revealed by immunoelectronmicroscopy of the rat inflammatory cells analysed *ex vivo*, HNE seems to have the affinity to accumulate near cellular membranes, most likely due to its lipophilicity [35]. Similarly, recent findings obtained for

murine hippocampal neuron HT22 cells indicate that the highest concentration of oxidized proteins appears in the cytosolic region near the cell membrane [41]. However, the highest protein oxidation (protein carbonyl formation) is taking place in the cytosol, while nuclear proteins seem to be very well protected by high proteasome concentrations [42]. Therefore, it is more likely that the accumulation of the HNE-protein adducts observed in our study in gastric epithelium was in the perinuclear space linked to the proteins associated to the nuclear membrane. HNE-modified proteins usually undergo proteasomal degradation, however, high intracellular concentration of HNE inhibits this process and shifts the cell into apoptosis [24]. This is certainly an important mechanism in pathogenesis of DPU manifested by the cellular decay. The further increase in intracellular HNE concentration leads to destruction of cellular structures and could cause necrosis, as well as cancer transformation.

It is known, that chronic inflammation is closely related to permanent oxidative stress, leading to damage of epithelial cells and gradual degradation of mucosa (atrophy). *H. pylori* associated atrophic gastritis, as well as metaplasia and dysplasia, are considered as pre-cancerous conditions [3], similarly to colon carcinogenesis in which accumulation of reactive aldehydes, namely, HNE and acrolein, was shown to be associated with cancer progression [43,44]. It should be mentioned that in the case of acrolein, the recent study revealed also nuclear accumulation of this lipid peroxidation product in some cases of high-grade colon villotubular adenomas and early stage adenocarcinomas. This supports involvement of lipid peroxidation in tumour initiation and even its transition from benign into malignant [44]. It was shown that gastric cancer frequently develops even after *H. pylori* eradication, especially in aged patients.

Therefore, invasion of gastric mucosa by *H. pylori* is important, but not the single risk factor for malignant gastric transformation [45,46], while long-term outcome of *H. pylori* associated diseases may depend on oxidative status of an organism.

The onset of lipid peroxidation and excessive accumulation of HNE in the gastric mucosa cells of DPU patients, described in this study, further point to the importance of oxidative stress and lipid peroxidation in the pathology of digestive system and gastric carcinogenesis.

Conclusions

This study shows physiological presence of HNE in human gastric mucosa, but also suggests its role in the pathology of gastric dysfunction in DPU patients manifested by accumulation of HNE protein adducts in particular in nuclei of gastric glandular epithelium.

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