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O. ZAYACHKIVSKA, I. PSHYK-TITKO, N. HRYCEVYCH, M. SAVYTSKA

NEW INSIGHT INTO ESOPHAGEAL INJURY AND PROTECTION IN PHYSIOLOGICALLY RELEVANT ANIMAL MODELS

Department of Physiology, Lviv National Medical University, Lviv, Ukraine

Chronic diseases of lifestyle (CDL), the most common chronic group of non-infectious and non-transmissible diseases worldwide, which share the similar risk factors of unhealthy lifestyle, have become most recognized as a serious trigger in the genesis of oesophageal injury. Non-erosive lesions (NEOL) are found more frequently than erosive or ulcer lesions in patients with reflux oesophagitis (RO) related to CDL. They also have restricted healing options, which often leads to carcinogenesis. Therefore, developing a physiologically relevant animal model of NEOL remains an urgent priority. One of triggers of CDL, postprandial hyperglycemia (PHG), which is characterized by hyperglycemic spikes, and overloading nitro-compounds leading to oxidative stress may predispose to NEOL. The present study was designed to set up a model of RO related to CDL in rodents to understand mechanisms of oesophageal pre-ulcerogenic injury under such conditions as food-associated long-term PHG, restrained water-immersion stress (WIS), and imbalance of entero-salivary nitrites recirculation (ESNR). Beneficial effects of L-tryptophan (L-Try) have already been described by many activities in kynurenine and melatonin (Mel) synthesis, redox reactions, which play a key role for cytoprotection and proliferation. Nevertheless, the effect of L-Try and Mel on NEOL under PHG is still unknown. An extract of *Cucurbita maxim sweet* seed (eCSE), which contains a high amount of antioxidants, also appear to play an important role in foregut cytoprotection. Thus, the second aim was to observe the effects of eCSE on oesophageal mucosa (OEM) in modification of ESNR (mESNR). Rats were used with without/with pre-treatment L-Try, Mel during WIS and PHG. In the second series of experiments rats were used with without/with CSE pre-treatment in mESNR; oral and OEM lesions were determined by histology; inflammation of OEM by lectin histochemistry; esophageal NO_2^-, cNOS and iNOS *via* bioassays; interleukin 1β (IL-1β), interleukin-8 (IL-8) *via* ELISA. PHG caused destructive lesions in the OEM accompanied by the up-regulation of iNOS and down-regulation of cNOS expressions, excessive NO_2^- while COX significantly aggravated the severity of these lesions; L-Try prevented ulcerogenic response to PHG with potent up-regulation of cNOS but did not affect synthesis NO_2^-. Mannose (Man)-containing specific α-DMan glycoconjugates labelled by lectins GNA, PSL, LCA, ConA and fucose (Fuc)-rich Fuc-α1 glycoconjugates - PFA, LABA are contributed in OEM integrity. It was shown that the changes of subepithelial and epithelial structures labelled by GNA, PSL, LCA displayed their highest exposure in the surface layer, whereas in the intima of microvasculature and nerve fibres of serosa membrane of the oesophagus by ConA during PHG NEOL. Also, the overexpression of Fuc glycans was present in OEM pre-epithelial and epithelial layers labelled by LABA and in the epithelial-glial-endothelial activity by PFA. Thus, initial changes in endothelial metabolism *via* iNOS and eNOS can be diagnostic and prognostic markers of NEOL in RO. Our mESNR studies also documented an early increase in pro-inflammatory mediators in the initial stage of oesophageal ulcerogenesis and repair and it can be a model for both proximal and distal oesophageal reflux diseases, as determinate by NEOL in oral mucosa and OEM. These findings suggest that endothelial metabolism is deeply involved in pathogenesis of NEOL. These models may be useful for detecting a new therapeutic strategy NERD, testing anti-ulcer drugs against RO and impaired healing OEM. Our results suggest that L-Try and Mel prevent OEM damage inducted by PHG and oesophagoprotective effect *via* modulation NO/NOS activity. The anti-inflammatory effect of eCMS could be used to protect oral mucosa and OEM against mESNR.

Key words: *oesophagus, animal model, postprandial hyperglycaemia, entero-salivary nitrites recirculation, L-tryptophan, Cucurbita Maxima sweet seed extract, nitric oxide, nitric oxide synthase, inflammation, interleukin-1β, GRO/CINC-1, mannose-, fucose-specific glycoconjugates*

INTRODUCTION

Gastroesophageal reflux disease (GERD), one of the common groups of the acid-related diseases, still has been described as chronic and recurring disorders with a major global social and economic impact (1). It often leads from unknown causes to long-term complications, as Barrett's oesophagus, peptic stricture, oesophageal carcinoma, the most malignant cancer of gastrointestinal tract worldwide (2-4). Also, latest data showed that GERD is often associated with chronic diseases of

lifestyle (CDL), one of the most widely distributed chronic groups of non-infectious and non-transmissible diseases worldwide, which share similar risk factors of unhealthy lifestyle and, moreover, have come to be recognized as a serious clinical problem. The key factors in the development of CDL are life style, diet, environment and para-mechanisms (5-7). The para-mechanisms include genetic, metabolic, environmental background and aging, the functioning of gut-brain axis, gut microbiota and immune response disorders (8, 9). Numerous latest data show that food and numerous dietary factors are related to CDL, as well as irregular dietary habits, gastric distention of upper stomach have an impact on the onset of GERD, demonstrating a key role of refluxate influence during postprandial acid reflux, proximal acid pocket and shift in redox system (10-13). GERD has many faces, which could be divided into the organic (endoscopic positive) subgroup, the functional and extraoesophageal subgroup, including asthma, reflux laryngitis, and oral cavity complications: periodontitis and dental erosions (endoscopic negative), which represent nonerosive reflux disease (NERD) that is twice higher than endoscopic positive disorders (9, 12). Foregut injury was considered as isolated distal and proximal as well as a combined type with both distal and proximal reflux due to the structure and functional activities (7, 12). Most of CDLs related with daily wave-shaped postprandial hyperglycemia (PHG), a typical dominating condition of dominating high intake of sugar or sugar-containing foods in a standard modern diet, reflecting oxidative stress with the hyperproduction of reactive oxygen spices (ROS), stimulates pancreatic β-cell to hypersecretion insulin, keeping fasting plasma glucose concentrations above the normal range for several years (13-15). The prevalence of abnormal glucose metabolism is a global trend and WHO treats it as non-inflectional epidemic and forecasts that by 2030 it will affect over 430 million people, and mostly by type 2 diabetic mellitus (T2DM) in adults (16). Moreover, PHG induces several gastrointestinal disorders: hyperacidic gastric secretion, hypersecretion of bile acids, decreased bicarbonate secretion, increased frequency of transient lower oesophageal sphincter relaxations (TLESRs) and hypokinetic type of oesophageal and gastric dysmotility (5, 8, 10, 17). These facts were background to developing a new approach to classification of different forms of GERD, highlighting NERD as characterized by pre-ulcer lesions of oesophagus. In 2009, an international workgroup completed clinical research focused on the problem of NERD and presented its results during a symposium in Vevey (Switzerland) (18). A new diagnostic classification was proposed, as an extension for the Los Angeles classification of grading oesophagitis, adding two new categories: grade M – minimal endoscopic lesions, as edema, hyperemia and friability, and grade N – absence of a visible oesophageal mucosal injury (6, 18-20). In this context, a

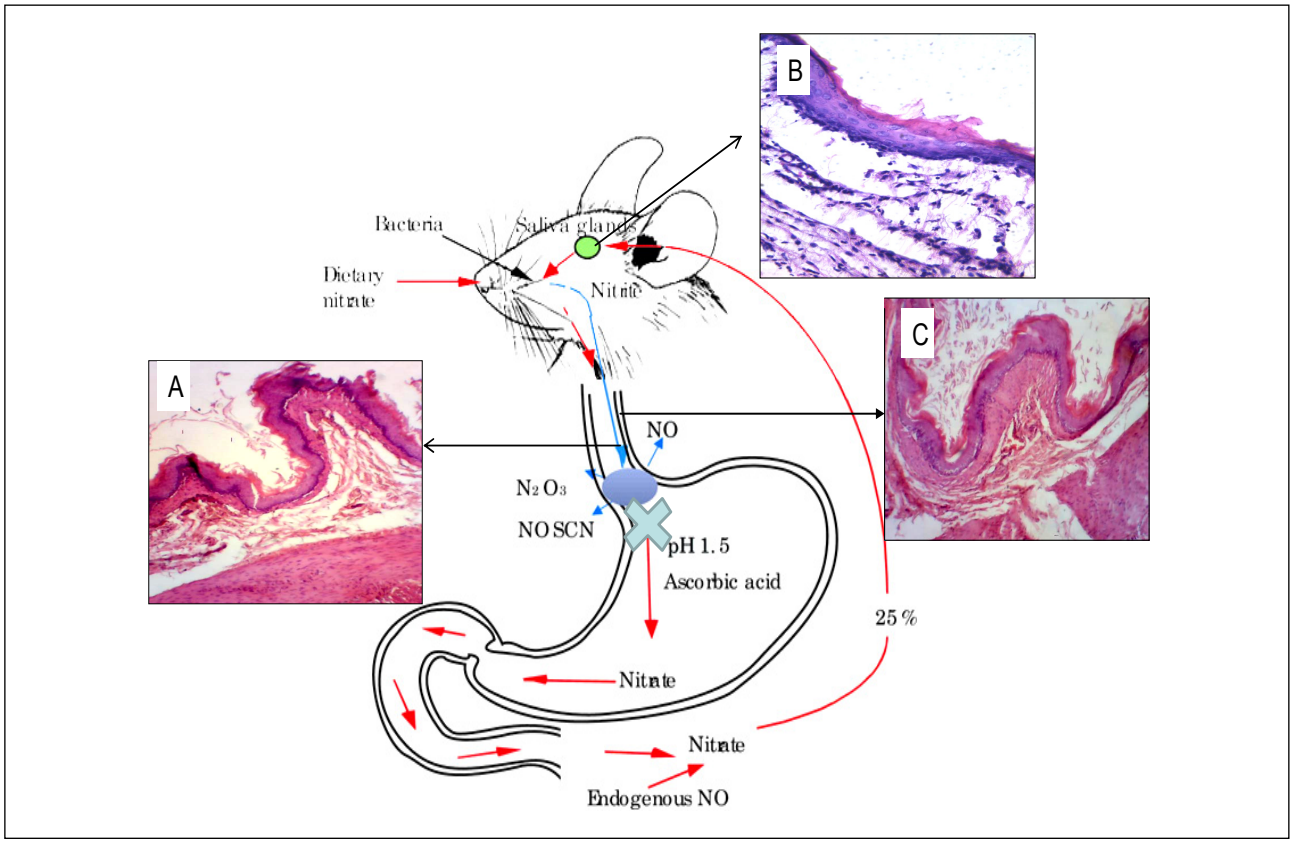

Fig. 1. Schematic illustration showing experimental imbalance of entero-salivary nitrites recirculation by combined blocking of cholinergic, histaminergic and nitroergic regulation after ranitidine, atropine and L-NAME application in rats with microphotos of sections of non-erosive oral (*A*) and oesophageal (*B*, *C*) mucosal lesions and after an injection of a *Cucurbita Maxima sweet* seed extract(*eCMS*): (*A*) – oral mucosa destruction of corneal layer, slight keratosis with single hyperchromatic nuclei in basal epithelial cells, subepithelial edema due to slightly increased vascular permeability, HE×150; (*B*) – pre-ulcerative changes of oesophageal mucosa (OEM), edema of subepithelial layer, submucosal vascular dilation, moderate leukocyte intraepithelial infiltration, basal hyperplasia, keratosis phenomena, HE×150; (*C*) – after administration of eCMS, OEM with minor keratosis, HE×150. NO, nitric oxide; N_2O_3, dinitrogen trioxide; NOSCN, nitrosothiocianate.

good experimental model of RO is important for understanding the M and N grades. An experimental study demonstrated that long-term fructose feeding (PHG) accelerates glycation and elevates oxidative stress conditions, both spontaneous chemical reactions that are implicated in cell damage and cytoprotection (21). Therefore, despite progress in endoscopic capsule techniques and other instrumental diagnostic methods, the search for physiologically relevant and effective animal models for NEOL related with CDL can bring new approaches to a new mechanistic dimension to our understanding of a specific pathogenesis mechanism of NERD.

Alterations in modern nutrition are frequently accompanied by changes in food that contains a certain amount of inorganic nitrates (NO_3^-), which are most commonly found in mineral fertilizers. The nitrates find their way into the human body through natural resources such as green vegetables, beetroot, strawberries, grapes (22-24). Nitrites (NO_3^-) in the form of sodium nitrite are used in the preservation of meat, fish, cheese, and are also sometimes found in drinking water (25, 26). The absorption of nitrates takes place in the small intestine. Apart from the circulating nitrates, one must also count a lesser amount of endogenously formed nitrites and the end product of enzyme-synthesized nitrite oxide. Approximately 25% of all nitrates are actively absorbed through salivary glands and secreted into the oral cavity, forming entero-salivary recirculation (27, 28). *Fig. 1* schematically presents the entero-salivary nitrites recirculation (ESNR), which is a large pool of potential NO bioactivity and plays an important role in cytoprotection, correct functioning of NO/NOS signaling, natural defensive properties of tissue restitution and resistance, including mucus and bicarbonate secretion and quantity and quality of saliva production, as well as the volume, frequency and aggressive components of the refluxate (29-33).

Hyposalivation, being a result of modern anti-acidic treatment of GERD and NERD, is strongly associated with increased viscosity of saliva, pH changes to more acidic values, and shifts in specific microbial components in foregut (29). It was proven in some of our previous studies that experimental hyposalivation after sialoadenectomy produces a significant injury in OEM (34). Thus, in the present study we investigated the role of modification of ESNR (mESNR) on the development of RO and tested novel therapeutic alternatives to the acid-oriented approach to the induction and healing NEOL. Recently, it was shown that using innovative biomarkers, such as small compound screening from metabolomics, provides novel ways to visualize and analyze complex physiological and disease processes in a whole organism, organ, and cellular or molecular network levels. Finally, glycoconjugates, carbohydrate-containing compounds, which are diverse members of glycome and expressed at the cell surface, can be ligands for several signalling pathways which have important multiplayer roles in discovering many physiological and pathological processes, including degeneration, inflammation, atherosclerosis and cancer (35-37).

Since modern data had shown that long-term anti-acid therapeutic approach is ineffective and cannot be proposed in patients with NERD, we also wanted to examine the effects of alternative pathogenesis-based substances. The main focus of our research has been on drugs that influence vascular factors in oesophageal injury and repair; especially since oesophageal enteroendocrinocyte's melatonin (Mel) and its precursor L-tryptophan (L-Try) are implicated in oesophagoprotection (36-40). On the other hand, the search for a novel anti-acid therapeutic approach based on natural and safe plant-originated substances may also play an important role. Our previous studies showed a potent cytoprotective effect of several phytochemical substances in the gastroenterology system through protection against oxidation by terminating free radicals (41-43). Moreover, the latest research data from other scientific groups have introduced new approach in experimental models (44, 45) and shown positive effects of plant-originated substances such as rikkunshito and curculiginis rhizome for the treatment of RE (46, 47). Meanwhile phenolic compounds extracted from oilseeds are major compounds with potentially health-promoting effects. A previous study had shown cytoprotective and anti-inflammatory effects of kavbuzol produced from a novel hybrid from family *Cucurbitaceae* (pumpkin and watermelon), extract of *Cucurbita Maxima sweet* seed (eCSE), selected by the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine (NASU), a plant-derived substance rich in fatty acids (linoleic, oleic, palmitic, and stearic - up to 95%) and with a high tocopherol content, as well as rich in flavanoids, tannins, phenolics and ascorbic acid (48). It is a source of carotene as well as magnesium, potassium, phosphorus ions and is rich other trace minerals (calcium, sodium, manganese, iron, zinc, and copper) (49, 50). Tocopherol homologues are phenolic antioxidants that occur naturally in CSE and are also known for their antitumor, radioprotective, antiatherosclerotic and hypoglycaemic effects (42). However, there has been no study of its possible effect on cytoprotection and repair of foregut.

Therefore, this study is aimed at introducing experimental rat models of NERD associated with CDL *via* the induction PHG and at verifying whether mannose- (Man), fucose- (Fuc) containing glycoconjugates in OEM are involved in response to injury. We investigated the efficacy of exogenous and endogenous Mel derived endogenously from L-Try on NEOL and the activity of nitric oxide (NO)/NO syntases (NOS) induced by PHG. The present study also compared the combined effect of mESNR *via* blocking cholinergic, histaminergic and nitroergic pathways to individual effects on rat oral mucosa, OEM integrity and pro-inflammatory levels.

MATERIAL AND METHODS

Animals

All experiments were carried out on rats weighing 180–220 g in accordance with the norms of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986), as well as the Committee on Bioethics of Lviv National Medical University (protocol No 5, 17.05.2010). Animals were maintained under a constant 12 h light and dark cycle and an ambient temperature of 21–23°C and were fed by standard diet. All animals were kept in raised mesh-bottom cages to prevent coprophagy. Six to seven rats were used in each group.

Experimental model on nonerosive oesophageal injury by long-term postprandial hyperglycemia

In the first series of experiments to study the impact of a sugar-overloaded diet a PHG animal model induced by Kozar *et al*. was used while male rats had *ad libitum* accesses to 30% fructose 200 g/L during 28 days *versus* to the control group with tap water access (51). The initial and final body weights of the various groups were recorded; the blood glucose concentration was measured from the tail vein by glycometr (Achtung TD-4207, Germany) every day. Acute oesophageal lesions were induced by the restraint water-immersion stress (WRS) by Takagi *et al*. (38, 39, 52), when the animals were placed in restraint cages and immersed vertically to the level of the xiphoid process in a water bath of 23°C for 3.5 hours. For determining the influence of the NO/NOS mechanism on oesophageal lesions rats were used with modification COX activity by the non-selective blocker of cyclooxygenase indomethacin ("Health", Ukraine) pre-treatment (COX) in dose 10 mg/kg, intraperitoneally (i.p.)

before 2 hours of experiment versus control rats, which received vehicle (1 ml of 0.9% NaCl solution, i.p.).

Experimental model of non-erosive oesophagitis via imbalance of entero-salivary nitrites recirculation

Our second series of the study have addressed the effect of mESNR on RO formation, evaluating oral mucosa and OEM, which was induced *via* blocking gastric acid and saliva production by histamine H-2-receptor antagonist ranitidine (R), antagonist of the muscarinic acetylcholine receptors, atropine (A) given separately or in combination with the non-selective blocker NOS L-nitro-arginine methyl ester (L-NAME) on the mucosal integrity of foregut. The reproducible mucosal damage of oesophagus was done by selecting an optimal time for mESNR in three time periods. The scheme of the experiment stipulated the division of rats into the following groups: the first group comprised animals (control) injected with vehicle at a dose 1 ml, i.p.; the second group of animals was subdivided into three subgroups and rats were sacrificed after administration of R (Ranitidine, "Darnycya Ltd", Ukraine) at a dose of 100 mg/kg i.p., or over two days, or over three days drug applying, after which actions euthanasia under ether narcosis was performed. The third group of animals was similarly subdivided and A (Atropine, "Darnycya Ltd", Ukraine), at a dose of 3 mg/kg, i.p., was administered in the same time and doses, with euthanasia after each; The fourth and fifth groups divided as mentioned were administered R with A, and R with A and L-NAME (in dose 10 mg/kg, i.p.) accordingly with euthanasia after each. All animals received 0.5 ml of vehicle orally. In another series of experiments eCMS produced by the Institute of Molecular Biology and Genetics of NASU was introduced *per os* in the dose of 0.5 ml/200g/day.

Macroscopic and microscopic structural examinations

Macroscopic lesions were defined as round or linear mucosal defects of at least 0.1 mm in diameter. For grading macroscopic changes, the lesion score system was used from 0 to 3. According to this macroscopic scoring, oral mucosa and esophagus had the score of 0 for normal shimmering mucosa; 1 – for hyperemic or edematous mucosa with focal hemorrhagic spots; 2 – for multiple erosions with hematin attached; 3 – for ulcer.

Oral and oesophageal mucosa histology

The oral mucosa and OEM from the lower third of oesophagus were fixed in 10% buffered formalin and embedded in paraffin, cut at a thickness of 5 μm on a microtome, placed on slides and stained with hematoxylin and eozin. The histomorpho-functional analysis changes in sections were estimated *via* grading of epithelial loss (0 – none, 1 – pre-ulcerative minimal changes and splitting, 2 – erosion, 3 – ulceration), and with evaluation vascular index (VI) used to grade vascular changes (0 – none; 1 – edema, 2 – submucosal vascular dilation, 3 – perivascular hemorrhage) combined with leukocyte intraepithelial infiltration (0 – none, 1 – mild, 2 – moderate, 3 – severe).

Lectin histochemistry for oesophageal lesions

Sections of the lower third of oesophagus were evaluated to fucose (Fuc) and mannose (Man)-containing expression in EOB for lectin histochemistry analysis. The set included lectins which can bind L-Fuc-rich (specific to Fuc α1-2 Gal β1-4Glc> α-L-Fuc) glycoconjugates: the ovary of perch agglutinin from *Persa fluviatalis L.* (PFA), the bark agglutinin from the shrub golden rain *Laburnum anagyroides* (LABA), Man-rich glycoconjugates (specific to Man (α-1-3) Man > α-D-Man): snowdrop agglutinin from ground part *Galanthus nivalis* (GNA), pea seed agglutinin from *Pisum sativum L.* (PSL), *Lens culinaris* agglutinin from seeds *Lens culinaris L.* (LCA), Jack-bean agglutinin from seed *Canavalia ensiformis*, Concanavalin A (Con A) purchased from "Lectinotest Lab" (Ukraine). The intensity of the conjugation reaction was assessed by semi-quantitative optical analysis, being considered as absent (0), weak (1), moderate (2) or intense (3) by score esteemed. Images of histological slices were investigated using a digital video camera connected to a microscope (MBI-15-2, LOMO, Russia) and were processed using the AVerMedia FZC Capture image analysis program (AVerMedia Technologies, Inc., USA).

Determination of nitric oxide- nitric oxide-synthase system activity in oesophageal tissue

The content of NO in homogenate was determined as nitrites (NO_2^-) by the method of Green *et al*. (53). 0.3 ml of oesophageal homogenate sample was deproteinized by adding 0, 25 ml of 75 mmol/l $ZnSO_4$ solution, stirring and centrifuging at 10,000 rpm for at least 3 min at room temperature, after which 0.35 ml of 55 mmol/l NaOH was added. The solution was stirred and centrifuged at 10,000 rpm for 3 min and the supernatant was collected. One ml of reagent, prepared by mixing 50 mg of N-naphthylethylendiamine dissolved in 250 ml distilled water and 5 g sulfanilic acid, dissolved in 500 ml of 3 M HCl, was added to 1 ml of supernatant in proportion 1:1 Absorbance was read in a Stat fax at 550 nm. NO concentration was expressed as μmol/g.

Measurement of nitric oxide-synthases

NOS activity was measured by the method described in detail by Sumbajev and Jasinskaja (54). The reaction mixture contained 2.6 ml of 0.1 M Tris-HCl buffer, pH 7.4 with 10 mmol/l $CaCl_2$, 0.3 ml of water solution of arginine, 0.1 mmol of NADPH and 0.3 ml $MgCl_2$. The reaction was initiated by introducing the homogenate. The in NADPH absorbance was followed at 340 nm during 3 min every 30 seconds at 37°C. Activity of the NOS was: $X = \Delta E \bullet P/(6.22 \bullet a \bullet b)$; ΔE - change of absorbance per 1 min; P - volume of reaction mixture; 6.22 - optical coefficient for waves 340 nm; a - concentration (content) of protein measured by Lowri means; b - volume of tissue homogenates samples. OEM NOS activity was expressed in nmol NADPH/min•mg protein. Activity of inducible NOS (iNOS) was calculated by similar method, but the reaction mixture contained a buffer without $CaCl_2$ and esteemed by endothelial NOS (eNOS)=NOS-iNOS.

Serum cytokines levels

Blood was collected from the abdominal aorta and centrifuged at 3,000 rpm for 15 min at 4°C. Serum was used for interleukines determinations. Interleukin-1β and interleukin-8 levels in the rat serum were assessed in duplicate using next ELISA kits: GRO/CINC-1 (rat) ELISA kit («Enzo Life Sciences», United Kingdom) and IL-1β (rat), ELISA kit («Enzo Life Sciences», United Kingdom). All of them were performed in accordance with the manufacturer's instructions.

Statistical analysis

Results are presented as the mean ± standard deviation (S.D.). Data were analyzed by Statistica software incorporating an aposteriori test with the comparison of middle index after Newman-Keuls criteria for variance homogeneity. Differences with $p<0.05$ were considered as statistically significant.

RESULTS

In the first series of the study it was demonstrated that the animal body weight from experimental groups with PHG increased by 5–8% versus control. The blood glucose baseline of normal and experimental animals was 5.8±0.5 mmol/L. In rats from the control group OEM did not show any macroscopical or microscopical alterations. Macroscopic evaluation of PHG impact on OEM revealed mostly 1 score changes (mucosal edema with focal superficial erosions) in the distal part of oesophagus only in the rats with WRS versus other groups.

The severity of microscopic changes in rat OEM during PHG without and with WIS and COX pre-treatment and L-Try and Mel effects established by vascular index (VI) was used with score system of oesophageal subepithelial vascular changes, including edema, submucosal vascular dilation, and perivascular haemorrhage, combined with intensity of leukocyte intraepithelial infiltration are shown in *Fig. 2A* and *2B*, respectively.

Fig. 2 presents the levels of NO and NOS activities in PHG-induced oesophageal injury when rats were pretreated by vehicle or COX without/with L-Try or Mel and underwent WIS-induced injury. The basal level of NOS was 7.38±0.64 nmol /min•mg protein, it should be noted that activity of iNOS was 4.70±0.63 nmol /min•mg protein, while eNOS 2.68±0.20 nmol /min•mg protein. *Fig. 2A* depicts the activities of total NOS, and activity of iNOS and eNOS, respectively, and NEOL *via* vascular index in oesophagus of normal, PHG and WIS-inducted and L-Try/COX treated rats during PHG and WIS. The common tendency was an obviously significantly decreased activity of NOS in rats pre-treated by L-Try (P<0,05) than those without L-Try. The administration of COX to rats with PH decreased iNOS

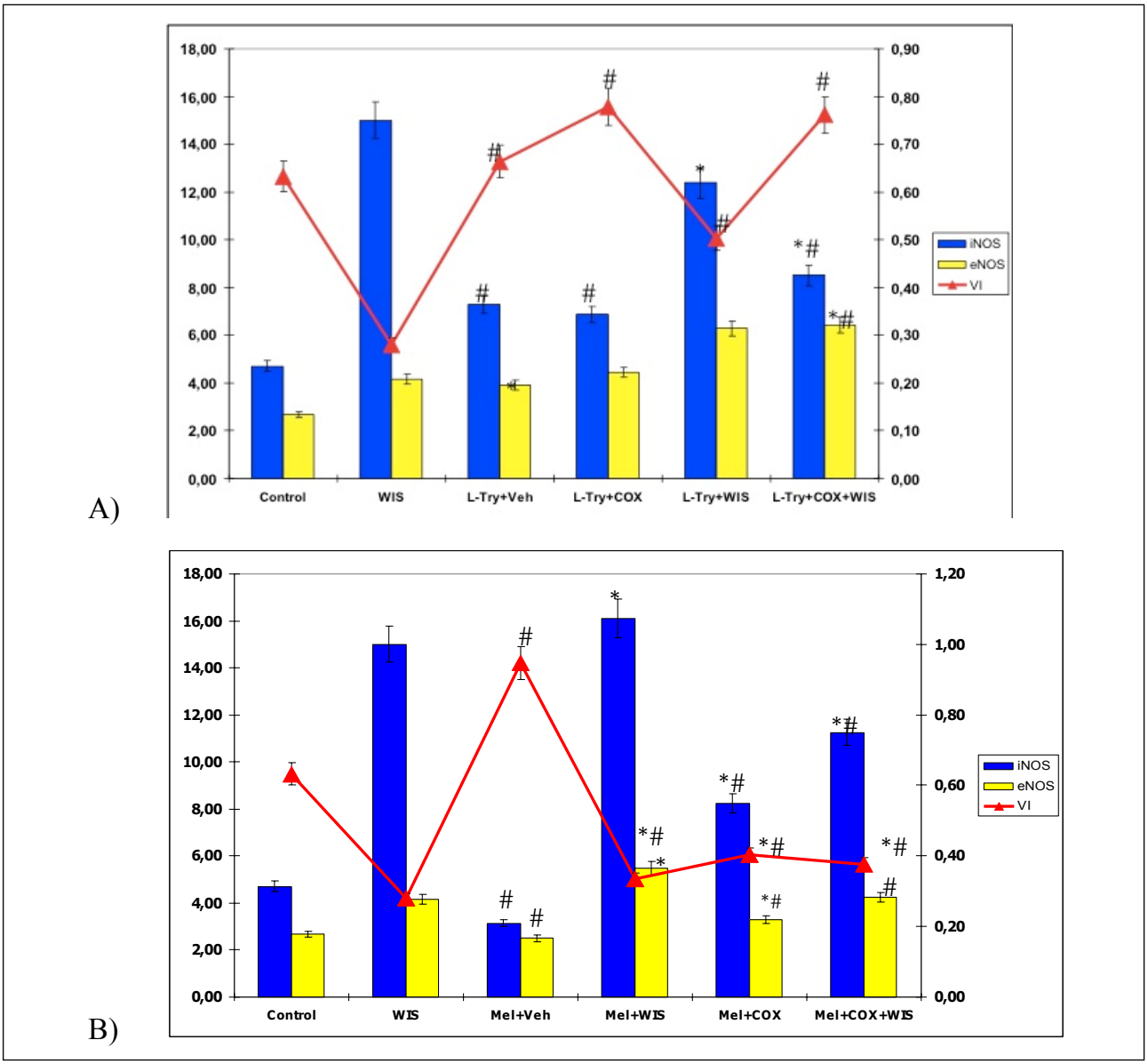

Fig. 2. Effect of L-tryptophan (*A*) and melatonin (*B*) on oesophageal lesions presented *via* vascular index (VI) and iNOS and eNOS contents induced by postprandial hyperglycemia and restraint water immersion stress (WIS) in rats with vehicle (Veh) and indometacin (COX) pretreatment. Data show for mean values for 6–7 animals each. L-Try, L-tryptophan; Mel, melatonin. Significant differences from control * p<0.05; # p <0.01.

activity two-fold ($p<0.05$). The results of co-treatment of L-Try and COX decreased activity of iNOS and significally stimulatated of eNOS activity in comparison to the data obtained in rats with WIS and without drug pre-treatment (*Fig. 2A*). Application of Mel enhanced activities of eNOS, which increased by 35% versus data obtained in rats without Mel pre-treatment, but this effect was abolished by administration of COX (*Fig. 2B*). We could conclude that identified PHG-related initial vascular injury is very important in the genesis of oesophageal mucosal injury and prevention. The activity of iNOS was significally inhibited by prior administration of L-Try and Mel while during WIS neither L-Try or Mel had any effect. During co-administration drugs with COX these results of significantly reduced iNOS acitivity with slighly decreased eNOS activitity were parallel with and without WIS.

Using high magnification in histological analysis revealed nonspecific markers of PHG-related injury in the epithelial layer of OEM: epithelial splitting, desquamation, elongation of papillae, increased mitoses in the epitheliocytes, spongiosis and balloon-cell change (swelling) of keratinocytes. The main characteristic of subepithelial injury in OEM during PHG were vascular congestion in papillae and dilated vascular channels at the tips of the papillae. Furthermore, WIS-related lesions of OEM during PHG characterized by signs of irregular hyperemia, stasis, perivascular diapedesis with microthroms and subepithelial excessive oedema. L-Try treated animals had significantly less NEOL than those receiving COX (*Fig. 2A*). The severity of these oesophageal lesions decreased with Mel pre-treatment verus L-Try pre-treatment.

Lectin histochemistry of OEM of control rats revealed a more specific and accurate picture of the structure-functional organization of OEM and marked heterogeneity of Fuc-specific (PFA, LABA) and Man-specific (LCA, PSL, GNA, ConA) binding was considered as a norm. During labeling in the lower part of oesophagus three layers of the esophageal epithelium were clearly seen: stratum corneum (SC), stratum spinosum (SS) and the basal cell layer (BC) which were used into interpretation of lectin space orientation and ligand expression. Modifications of expression Fuc- and Man-specific glycoconjugates in NEOL formation are represented on *Fig. 3*. PHG induced higher expression of Fucα1-2Gal β1-4Glc>α-L-Fuc-specific glycans labeling by PFA in the pre-epithelial and epithelial parts of OEM then in subepithelial structurs in the rats with stress-associated eosphagitis. Moreover, Fuc-containing PFA expressing increased in some cells in SS, as well as in dendritic cells, components of ENS, basal membrane in fibroblastic cells of lamina propria of OEB. In addition, in the subepithelial part of OEB expression of PFA lectin receptors were in the collagen fibers. In the muscular layer fucosylated PFA and LABA binding were weak, therefore in the external layer of OEB there was moderate expression of Fuc-specific glycoconjugates in the fibrils of connective tissues. Excessive expression of fucose residues was evaluated in the internal elastic membrane and the external layer in vessels in oesophageal microcirculation in OEB. L-Try-treated rats had enhanced expression of PFA and LABA in SC and SS in OEB against the background of a detected decrease in oesophageal lesions appearance. De-fucosylation of nerve endings in OEB marked by PFA were in COX-treated rats; therefore administration of L-Try reversed these changes contributed to the main mechanism of maintenance of gastric mucosal integrity.

These results revealed a more specific and accurate picture of the structure-functional organization of oesophageal mucosa during PHG injury. The results showed the higher expression of Fuc-specific labelling by PFA, LABA in the pre-epithelial and epithelial parts of OEM, as well as in subepithelial structures were in the rats with stress-associated oesophagitis. Moreover, Fuc-containing PFA expressing increased in some cells in SS, as well as in dendritic cells, enteric nervous system components, BC, and in fibroblastic cells of lamina propria of basal membrane in OEM. Our data suggested that the neural-glial-epithelial unit in OEM is involved in alteration barrier function of oesophagus and inflammatory responses. In addition, in the subepithelial part of OEM expression of PFA lectin receptors were in the collagen fibres. In the muscular layer fucosylated PFA and LABA binding were weak, therefore in the external layer of OEM there was moderate expression of Fuc-specific glycoconjugates in the fibrils of connective tissues. Additionally, a combination of PHG and WIS elicited excessive expression of Fuc-containing glycoconjugates in the internal elastic membrane and external layer in vessels in subepithelial oesophageal microcirculation in OEM. Man-specific glycopolymers were intensively expressed in pre-epithelial and epithelial layers and subepithelial vascular walls of OEM during PHG, as well as in a combination of PHG and WIS injury with the administration of COX. An L-Try-treated rat had an enhanced expression PFA and LABA in SC and SS in OEB against the background of decreasing oesophageal lesions appearance. De-fucosylation of nerve endings in OEB marked by PFA were in COX-treated rats, therefore administration of L-Try reversed these changes contributed to the main mechanism of maintenance of OEM integrity.

In the second series of the study the effect of mESNR on oral and oesophageal mucosal integrity, as well as the effect of pretreatment with eCMS was assessed in comparison with vehicle on the OEM injury and oral mucosal damage. The structural and functional analysis of treatment of symptoms of oesophagitis and gingivitis included research of the state of the SC, presented as masses of keratin; the stratified squamous epithelium, the basal layer, and the submucosal layer together with the microcirculatory network with delineation of the level of damage, inflammation, hyperplasia (as a sign of healing) in the OEM and the oral mucosa (*Fig. 1A* and *1B*). In the animals that had R injected once only, the mucosal folds were mildly visible or absent in separate sections, and the papillary pattern was almost absent. The epithelium was multilayered, with signs of keratinization. The submucosal base of OEM showed signs of subepithelial oedema and the lumen of the oesophageal microvasculature was sharply narrowed. In rats that had R administered twice, the detachment of the SC of the epithelial lamina was observed, with desquamation in some places. The manifestations of proliferation were the characteristic signs of nonerosive oesophagitis and gingivitis after the administration of R over three days. Papillary structures were clearly visible. Proliferation of the epithelium of the lamina propria, local detachments of the epithelium from the basal membrane, and destructive changes to the epithelial plate were observed. Application of R, A and L-NAME caused obvious oral (*Fig. 1A*), and oesophageal mucosal lesions: edema of subepithelial layer, submucosal vascular dilation, moderate leukocyte intraepithelial infiltration, basal hyperplasia, keratosis phenomena (*Fig. 1B*). These results confirmed the importance of ESNR and luminal NO in the pathogenic mechanism of NERD and the increased risk of concomitant disruption of oral mucosa. We examined the time-response relationship experimentally induced mESNR from the point view of inflammation by blocking cholinergic, histaminergic control mechanisms. The significant rise of IL-1β was observed after two days of treatment with a slight decrease on the third day A (*Fig. 4A* and *4B*, respectively).

As shown in *Fig. 4B*, rats with mESNR regulation by A blocking had a significant increase in the production of IL-1β by 32.8% over two days, while over three days a similar pretreatment level of GRO/CINC-1 increased by 13.9% in comparison with control. In rats with dual blocking cholinergic

and histaminergic mechanisms of ESNR by combination of R with A a significant increase in the level of GRO/CINC-1 (by 137.1%) was registered after two days of treatment versus control (*Fig*. *4B*). In the group of animals with mWSNR with combined blocking of cholinergic, histaminergic and NOS regulation (R, A and L-NAME) significantly accelerated production of IL-1β (by 14.3%) appeared after three days as well as an accelerated production of GRO/CINC-1 (by 191.4%) in comparison with control results (*Fig*. *4D*). The results of the NO serum content showed an increasing tendency with a maximum rise after three days of blocking of histaminergic and both cholinergic and histaminergic and a combination of triple blocking cholinergic, histaminergic and NO/NOS pathways *via* L-NAME administration. The pre-treatment of eCMS showed an anti-inflammatory effect *via* a significantly decreased level of IL-1β in rats with blocked cholinergic mechanism after one-day treatment. The combination of cholinergic and histaminic pathways blocking, as well as at triple blocking of cholinergic, histaminic and NO/NOS revealed decreased level of GRO/CINC-1 versus to vehicle treatment.

DISCUSSION

Modern interpretation of oesophageal disorders includes a chain of events related to the frequency of TLESRs, abnormal oesophageal and gastric peristaltics, a decrease in defensive properties of the oesophageal epithelial barrier function due to impairment of complex tissue (pre-epithelial, epithelial and sub-epithelial) resistance and visceral hypersensitivity to each other

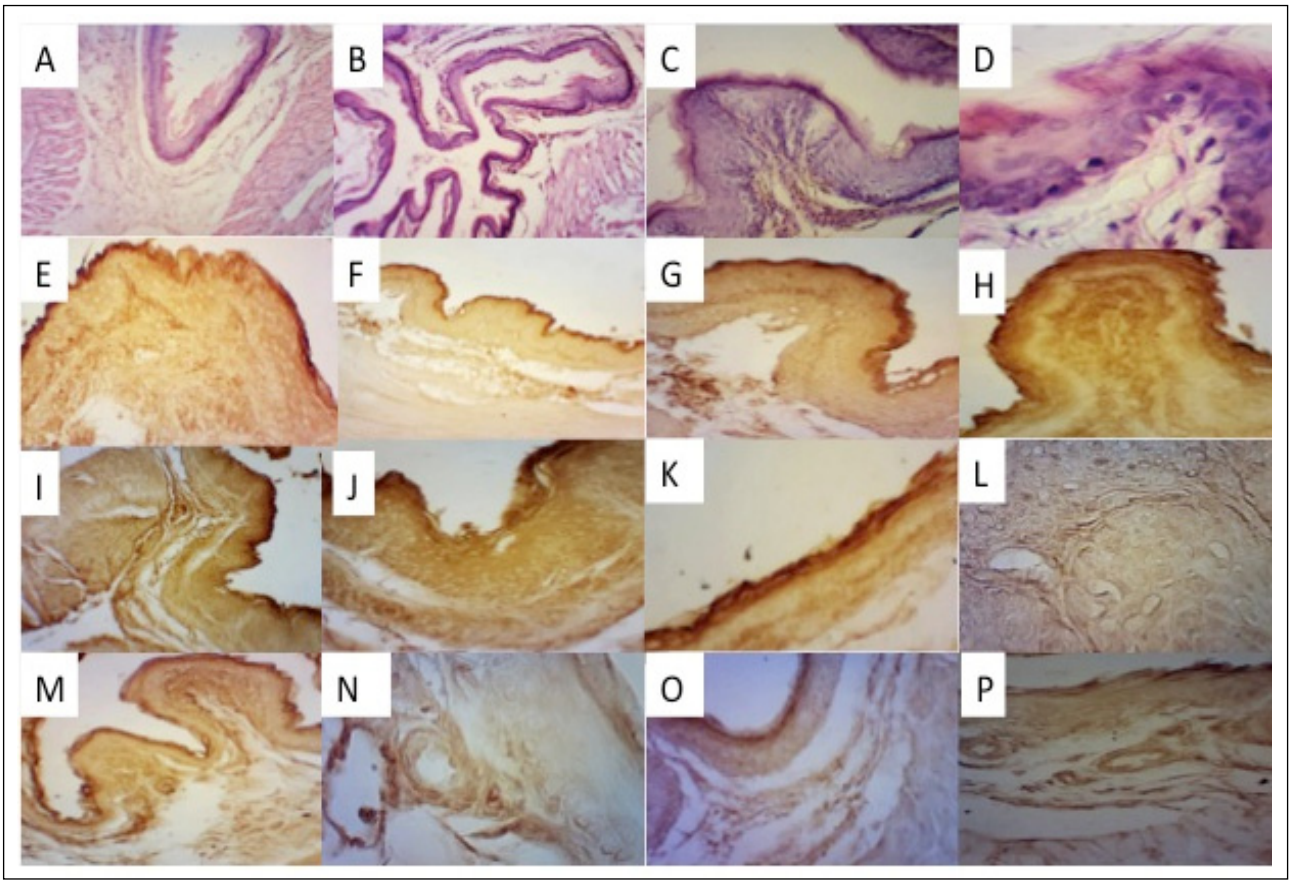

Fig. *3*. Microscopic and lectinhistochemical findings of rat oesophageal mucosa (OEM) lesions. (*A*) – transmitted light micrograph of esophagus with postprandial hyperglycemia (PHG) with findings of non-erosive oesophageal lesions (NEOL), HE×180; (*B*) – OEM after combined PHG and WIS-induced injury with findings of intensity of elongation of papillae, particular losing keratin or destruction of epithelial layer, prominent subepithelial edema, disorganization of connective tissue, HE×180; (*C*) – typical nonerosive esophagitis after WIS plus indomethacin (COX) pretreatment with irregular hyperemia, stasis, perivascular diapedesis with microthroms and subepithelial excessive edema, HE×300. (*D*) – effect of L-Try on OEM with findings of increased mitoses in the epithelium, spongiosis and balloon-cell change (swelling) of keratinocytes HE×600. (*E*) – PFA staining were graded ‘strong’ in the pre-epithelial and subepithelial layers in OEM in rat with PHG and WIS plus COX pre-treatment, ×300; (*F*) – LABA staining were graded ‘intense’ in stratum corneal (SC) and ‘weak’ in other parts of OEM in rat with PHG and WIS, ×300; (*G*) – PFA staining were graded ‘intense’ at OEM with PHG, ×300; (*H*) – GNA staining were graded ‘intense’ at oesophageal mucosa in rats with L-Try, ×300; (*I*) and (*J*) – LCA staining were graded ‘intense’ at pre-epithelial and epithelial layers of OEM after combined PHG with WIS plus COX pretreatment, ×120 and ×300, respectively; (*K*) – PFA staining were ‘intense’ at epithelial layer OEM of effect of L-Try, ×600; (*L*) – ConA staining were graded ‘weak’ in OEM of effect of Mel pre-treatment, ×600; (*M*) – and (*P*) – LABA staining were ‘intense’ and ‘weak’ at OEM and vascular walls in the stroma during PHG and WIS without and with Mel pretreatment, respectively, ×300; (*N*) – LCA staining were graded ‘intense’ in vascular walls, connective tissue in OEM with combined PHG with WIS plus COX pretreatment, ×300; (*O*) – ConA staining were ‘strong’ in SC and subepithelial vascular walls in OEM with combined PHG with WIS plus COX pretreatment, ×300.

(55-57), but their genesis in relation to CDL remains poorly understood. Despite progress in investigation of early molecular and biochemical changes in the oesophageal lesions (3, 58, 59), it remains essentially unknown how risk factors of CDL influence oesophageal ulcerogenesis. Moreover, the traditional drug therapy of GERD has resulted in incomplete efficacy (60); in long-term use it is risky in view of unexpected effects, as altered microbial profile with domination gram-negative flora which elicits inflammation, increased TLESRs, hypokinetic gastric dysmotility *via* cyclooxygenase-2 activity (61), inhibition

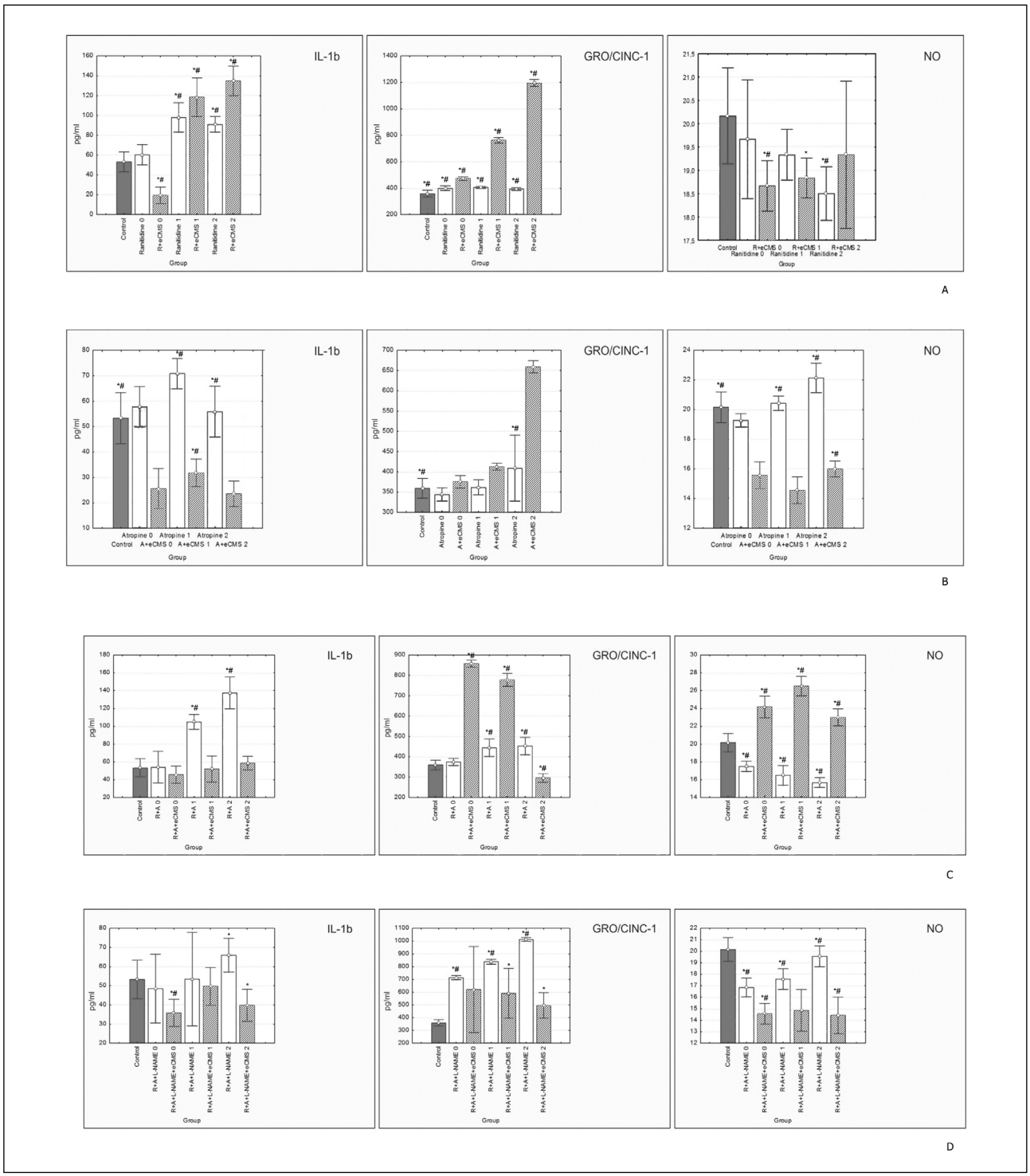

Fig. 4. Effect of extract of *Cucurbita Maxima sweet* seed (eCMS) applied in dose 0.5 ml/200 g/day *per os* on serum IL-1β, GRO/CINC-1 and NO contents *via* experimental imbalance of ESNR regulation by blocking of cholinergic (*A*), histaminergic (*B*) regulation (R - ranitidine, 100 mg/kg i.g., A- atropine, 3 mg/kg i.g.), by combined blocking of cholinergic and histaminergic (*C*), and cholinergic, histaminergic and nitroergic (*D*) regulation (R - ranitidine, 100 mg/kg i.g., A- atropine, 3 mg/kg i.g., L-NAME, 10 mg/kg i.g.); 0 – first day, 1 – second day, 2 – third day of drug apply. Data show for mean values for 6–7 animals each. Significant differences from control * $p<0.05$; # $p<0.01$.

of activity of dimethylarginine dimethylaminohydrolase, an endogenous enzyme of asymmetrical dimethylarginine, modest inhibitor of endothelial NOS which increase the risk of vascular inflammation and thrombosis (62) and induction selective apoptosis for cancer (2-4). Moreover, recent study had shown the key role of the esophageal microcirculation and inhibition of proinflammatory cytokines IL-1β, TNF and IL-6 induced by acidic refluxate in early stage of GERD and preventive effect of NO-releasing anti-infammatory drug (63).

Therefore, we hypothesized that those dietary risk factors of CDL, as PHG and an abnormal functioning of ESNR may be involved in the early stages of oesophageal ulcerogenesis and possibly reflect early pathophysiological changes, as pre-erosive injury and RO. However, there has been no experimental study of PHG effect on oesophageal barrier function in scientific literature (64, 65). After carefully assessing previous research into the role of ESRN in foregut cytoprotection, we also did not find data on using ESNR activity in experimental rodent modeling gastric reflux taking into account that an over-releasing NO have a potential to cause changes in oesophageal motility and can trigger its harmful effect on OEM damage.

The present rat model of NERD related to PHG demonstrated that this model produced easily oesophageal subepithelial vascular injury which was confirmed by relatively constant lesions characterised by increased vascular permability, inflammation and rised iNOS and dropped eNOS activities. Our data supported by other results which confirmed the implication of NO/NOS system for endothelial injury (66-68). As expected, COX did not inhibit lesion formation and rather aggaravated it (61, 69). This model having properties to confirm that long-term PHG is a risk factor for initiation decreasing integrity of OEM and induction of RO. The exogenous and endogenous Mel derivated from L-Try prevents OEM subepithealial injury induced by PHG.

There are several novel methods of visualization of cellular and tissue processes involved in OEM and gastric mucosa impairment, as powerful, confocal laser endomicroscopic imaging system with EGF-R and survivin labeled specific antibodies which allows non-invasive, *in vivo*, real time evaluation of state of receptor activation and its response to physiological and pharmacological stimuli (70). Our attention attracts assessment of structural changes OEM glycoconjugates, powerful method of dynamic morphology and physiology.

Mammalian glycoconjugates of gastro-intestinal mucosa possess multiple functions in intercellular signalling. Tight proteins occluding, claudin-1, -2 and zonula occludens-1, 2, as well as dilated intercellular spaces, play an important role not only in experimental animals but also in patients with GERD, as well as reflux-negative individuals (71, 72). Many recent basic and clinical studies have reported that lectins, due to their properties, are of great interest in practical applications. Our structural and conformational aspects of changes in Man- and Fuc-containing glycoconjugates expression are important through functional consequences, indicating that labeling by PFA is highly effective for epithelial-glial-vascular activity while LABA is a sensitive tool for cell injury, leukocytes recruitment, necrosis and also signs for apoptosis in the pre-epithelial and epithelial layers in OEB during PHG (73). Previously we developed animal models of oesophageal non-erosive lesions, *e.g.*, induced by stress-associated changes and streptozotocin, a compound that has preferential toxicity toward pancreatic β cells. Using modeling for experimental DM, we acquired new data which expanded insight into the genesis of DM oesophageal lesions *via* a shift in redox system over-production of ROS by nitroxidative stress-induced by disbalance in NO/NO-synthase (NOS) activity and changes of microcirculation and subepithelial endothelial cells of oesophagus and up-regulation of cellular glycopolymers with sialic acid in EOM (36, 37, 58, 59). We also demonstrated the central role of inflammation and endothelial dysfunction in oesophageal barrier impairment and starting point of its abnormal *restitutio ad integrum* (35, 38). It was confirmed by previous own data and results from other scientific groups obtained by animal model of oesophageal erosive injury induced by non-invasive approach *via* establishing structure-activity correlation that sialic acid-containing specific glycoconjugates are implicated in oesophageal ulcerogenesis and may be key biomarkers in ulcerogenic degenerative and regenerative processes regulated recognition processes, immune defence in barrier function (35-37, 61, 71-73). This implies that glycoconjugates may be early biomarkers of pre-ulcerogenic lesions of OEM. Our present observations reported that Man- and Fuc- specific glycopolymers expressed by lectins are promising biomarkers to diagnosis subepithelial microcirculation injury of OEM during non-erosive esophagitis related to CDL regarding the differences observed in their expression. In these investigations, variants of glycoconjugates expression in the animal model of NERD can be able to pave the way for advanced and deeper understanding of mechanisms of oesophageal defense because they almost depended on appropriate glycosylation.

In the series of mENSR which is the natural protection system in foregut, the series of experiments included blocking gastric acid and saliva production was done by histamine H-2-receptor antagonist ranitidine (R), antagonist of the muscarinic acetylcholine receptors, atropine (A), combined and combined with non-selective blocker NOS L-NAME which were used for determination of influence NO/NOS mechanism on oral and oesophageal lesions (74-76).

Many studies have demonstrated that tocopherol from plant-originated substances was capable of reducing inflammatory response, cellular differentiation, and control redox system (77), therefore under condition of ESNR disbalance where the amount of NO modulate ulcerogenic potential. Our results showed that eCMS induced oral and oesophageal cytoprotective and anti-inflammatory effects (78, 79). The results obtained, namely the high-level reparations, were due to the antioxidant and membrane-stabilizing activity of the eCMS, thanks to the high carotene content. This indicated that ESNR has the ability to maintain the mucosal integrity in foregut; however histamine and NO synthesis exert biphasic biological effect when in physiological dose are essential for natural functioning, but in high or low application seems to be a reason for cell and tissue damage.

Conclusions

The results of the present study revealed that experimental modelling CDL *via* induction of PHG, stress and ESNR disbalance caused NEOL in the rat, which is similar to human NERD. Furthermore, the vascular element and pro-inflammatory hypoxia are key in the early phases of pathogenesis of esophageal lesions. Esophageal glycoconjugates are good biomarkers of esophageal epithelial and subepithelial lesions. These models may be useful for the study of stress-associated oesophageal injury and healing and evaluation for drug therapy. Beneficial effects of L-tryptophan, being a source of endogenous melatonin and exogenous melatonin, on the development of esophageal lesions and healing were demonstrated. Plant-originated eCMS represented a uniform mucosal protective effect on oral and oesophageal integrity under different experimental circumstances of alteration of entero-salivary nitrities recirculation. Modification of activity gactric acid elecits changes in enterosalivary nitrites

recirculation suggesting on its important functional cytoprotective role in the foregut. The dysfunction of ESNR leads to low-grade inflammation of the foregut, inducing erosive lesions in oral and oesophageal mucosa. The pre-treatment of eCSE prevented mESNR-induced foregut lesions via anti-inflammatory, cytoprotective, and repair activities.

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Author's address: O. Zayachkivska, MD, PhD, Physiology Department, Lviv National Medical University, Lviv, Ukraine, 79010.
E-mail: ozayachkivska@gmail.com