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Original article

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EFFECTS OF NITROSATIVE STRESS AND REACTIVE OXYGEN-SCAVENGING SYSTEMS IN ESOPHAGEAL PHYSIOPATHY UNDER STREPTOZOTOCIN-INDUCED EXPERIMENTAL HYPERGLYCEMIA

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Experimental and clinical gastrointestinal data reported that nitrosative stress development involved in impaired barrier function, altered motility and a lowered threshold to noxious stimuli, but its pathogenetic role in diabetic esophagopathy remains unexplored. We tested the hypothesis that an imbalance in nonenzymatic glycation and glycooxidation, enhanced peroxynitrite formation, may play an important role in development esophageal mucosa (EM) lesions during streptozotocin-induced experimental hyperglycemia (EHG). To understand the biological significance of EM resistance *in vivo* used a glycomic approach to identification of lectin receptors glycosylation pattern. Were enrolled rat groups without/with EHG & modification of NO/NOS activity by L-arginine (L-arg) and indomethacin pre-treatment. Survival rate, destruction occurrence *ratio,* the size of EM lesions, and the number of EM lesions was investigated. To access the oligosaccharide residues the peroxidaseconjugated lectin (HPA, SNA, WGA, PNA)-diaminobenzidine procedure was performed to EM sections. EHG was monitored daily by glucometer. Content of NO (NO_n) was determinated by Griess reagent and reactive oxygen-scavenging systems (ROSS) activity - generally accepted biochemical methods. In EHG and L-arg pretreatment group reduced NO_n and EM injury with markedly rise ROSS activity significantly *vs* to control; in the group with indomethacin pretreatment existed different ROSS activity. Presence of heterogeneous glycosylation pattern in different layers of EM was shown. In EHG staining with PNA and SNA were strongly positive. NS and ROSS play a critical role in esophagoprotection induced by EHG, because both involved increases in iNOS expression. These results indicate the usefulness of glycomic approach as multifunctional substrate of early evaluation of NS in esophageal physiopathy.

Key words: *esophageal mucosa, streptozotocin-induced hyperglycemia, nitrosative stress, reactive oxygen-scavenging systems, lectin histochemistry*

INTRODUCTION

Nitrosative stress (NS), the process of the excess reactive nitrogen oxide (NO) species (RNOS) formation such as peroxynitrite (ONOO-) and nitroxyl (NO-), which are derived from NO metabolism and not counter-balanced by endogenous reactive oxygen-scavenging systems is a feature of numerous pathological conditions (1, 2). It leads to misbalance between different pathways of arginine metabolism: NO-synthase (NOS, oxidative) pathway is activated, whereas arginase (nonoxidative) one is depressed (3). Particularly, the cytotoxicity of RNOS causes cellular damage by oxidizing proteins, membrane lipids and DNA leading to the disorders with microcirculation, anticoagulation, leukocyte adhesion, smooth muscle proliferation and the antioxidative capacity (4).

Diabetes mellitus (DM) – one of the modern global growing problem and the data from WHO on this time counted over 180 million people, that have different forms of DM and its prevalence will increased twice at forecast to 2030 year (5). Complications involving the gastrointestinal (GI) tract such as gastro-esophageal reflux disease (GERD), peptic ulcer disease, irritable bowel syndrome, non-alcoholic fatty liver disease, gallstones or some other malady, are frequent in patients with DM but not commonly recognized in clinical practice (4, 6). In addition, it has been shown that the GI symptoms do not correlate with the duration of the disease, metabolic control and other chronic complications as neuropathy (7, 8, 9). Recent epidemiological studies have been suggested about correlation between cancerogenesis and DM (10, 11, 12). Importantly, GERD is closely associated with the development of Barrett's oesophagus, premalignant condition and previous studies indicate on the key role of pro- and antioxidant disbalance in esophageal cancerogenesis (13). Cancer-related mortality of esophageal adenocarcinoma are highest than other and a median survival time of <1 year. However, the physiopathy of esophageal damage during DM is not well understood. Hyperglycemia and protein glycation, increased inflammation, a prothrombotic state and endothelial dysfunction have all been implicated as possible mechanisms for such complications (14). A linking element between many of these phenomena could possibly be, among other factors, increased production of reactive oxygen-scavenging systems (ROSS) (15). Identifying the pathogenesis of this increased risk provides a basis for secondary intervention to reduce GI morbidity and mortality in diabetic patients.

Glycoconjugates creates a regular esophageal epithelial barrier in the the paracellular and intracellular pathways and play a crucial role in structural esophageal defence against injury. Our previous studies have shown the physiological importance of NO/NOS, prostaglandin (PG)/cyclooxygenasa (COX) signalling pathways and epithelial barrier glycoconjugates in the esophageal integrity at normal conditions and during experimental acid-pepsin and bile acid-trypsin induced esophageal damage (16, 17). Since the NS is one of the most important factor in the development of esophageal mucosa lesions we

hypothesized that differences in activity of NO/NOS and PG/COX systems during DM will modified signs of nitrosative stress due to activity of ROSS, as important player enzyme gluthatione peroxidase (GPx) and might contribute to modification of synthesis of glycoproteins, proteoglicans, glycolipids and their expression and localization in the esophageal epithelium.

This study follows from the previously published papers and investigates the relationship between esophageal integrity markers and nitrosative stress (18, 19), to determine whether nitrosative stress is independently associated with esophageal damage during experimental uncontrolled hyperglycemia (EHG), and hence may play a role in the pathogenesis of GERD during DM. Therefore, the aims of the present study were: 1) to investigate the morpho-functional characteristic of esophageal lesions and accompanied changes in esophageal mucosal glycoconjugates in rat diabetic model of streptozotocin (STZ)-induced EHG; 2) to study the effect of pre-treatment of L-arginine (L-Arg) and indomethacin (Indo) on esophageal lesions induced by EHG and accompanied changes in malondialdehyde (MDA), major lipid peroxidation product, and nitrate/nitrite (NO_2^- and NO_3^-) amounts (NOn) and ROSS; 3) to examine the involvement of PG/COX, NO/NOS systems in resistance of esophageal epithelial barrier during EHG.

MATERIAL AND METHODS

Male Wistar rats, weighing 180-220 g and fasted for 24 h before the study though having free access to water, were used in our studies. These experimental procedures were approved by were approved by the University Ethical Committee for Animal Research. All trials were followed Lviv National Medical University Guide for Care and Use of Laboratory Animals that run in accordance to the statements of European Union regarding handling of experimental animals.

Induction of rat diabetic model by streptozotocin (STZ) – mediated hyperglycemia

The animals were rendered diabetic by a single injection into the tail vein of STZ (45mg·kg-^{1} bw, Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in saline. The diabetes was assessed by blood glycose measurements from the tail vein and determined with glycometr (Achtung TD-4207, Germany) every 3 days. The animals were considered with EHG if the blood glucose level was 20mM. Two weeks elapsed between the induction of diabetes and the animals were killed on 14 and 28 days of experiments. The non-diabetic control animals received a sham injection of vehicle solution.

Experimental protocols in vivo

For determination role of NO/NOS and PG/COX signalling influence to mechanisms in esophagoprotection studies were carried out on the following experimental groups (seven rats in each group): [1] control animals; [2-7] animals with EHG and treated with saline [2-3]; N^{ω}-nitro-L-arginine, (L-arg) donor of eNOS activity [4-5] at the doses 300 mg/kg bw, intraperitoneally; indomethacin (Indo), nonselective inhibitor of COX-1 and COX-2 activity at the doses 5 mg/kg bw *per os* [6-7] during 14 and 24 days, respectively.

Macroscopic and microscopic structural examination

Non-diabetic control and diabetic animals were euthanised under anaestesia and the esophaguses removed. Survival rate for rats with EHG was investigated. For the analysis macroscopic esophageal changes esophaguses were weighed and destruction occurrence ratio were ranged for score 0: normal shimmering mucosa; 1- hyperemic or edematous mucosa; 2 - erosions, and the size of EM lesions. The number of EM lesions were evaluated by the microscopic examination segments of the lower third samples of esophagus. In order to analyze the histological characteristics of esophageal tissue of the haematoxylin and eosin specimens were used histological activity index (HAI). HAI is based on the degree of light microscopic lesions (*Fig. 2*) and by according system of the epithelial loss: 0 - none, 1- splitting and erosion, 2 - ulceration, 3 - large ulcer and necrosis; for leukocyte infiltration: 0- none, 1- mild, 2- moderate, 3- severe; regenerative epithelial changes: 0-none, 1- basal hyperplasia, 2- mitosis, ballon cells, akantosis, 3 - parakeratosis.

Lectin labeling

For microscopic analysis segments of the lower third of esophagus were used for the routine histological examination and the lectin histochemistry methods. The lectin set included using peanut agglutinin (PNA, specific to βDGal→3DGalNAcDGal), *Helix pomatia* agglutinin (ÍĐŔ, specific to DGalαNAc), snail agglutinin (SNA), wheat germ agglutinin (WGA, specific DGlcNeuNAc) conjugated to peroxidase (purchased from "Lectinotest Lab", Ukraine). Lectin label was visualized with diaminobenzidine (DAB) in PBS as described elsewhere (20, 21). All incubation procedures were conducted at room temperature. 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) and carried out by semi-quantitative optical analysis, taking account the intensity, indicated as absent (–), weak (+), moderate (++) or intense (+++).

Determination of total nitrate and nitrite concentration

Nitrate/nitrite (NO_2^- and NO_3^-) amounts (NOn) in esophageal mucosa were determined using Griess reagent (22). Sample proteins were sedimented by 30% ZnSO4. After the centrifuging the supernatant was incubated with metal cadmium for 12 hours thereby reducing nitrate to nitrite. Then Griess reagent was added, and total NOn was measured at 550 nm spectrophotometrically (SOLAR, Model PV 125 1C) and expressed in terms of microM.

Determination of lipid peroxidation (LPO)

Malondialdehyde (MDA) was resolute by the method of Timirbulatov R. *et al*. (23). In briefy 0,1M standard phosphate solution (SPS) in pH 7,4 was added to 0, 1 mM $KMnO_4$ and 10 mM $FeSO_4$ to the homogenate and incubated for 10 minutes at room temperature, followed by boiling with 20% acetic acid and 0,6% thiobarbituric acid for 60 minutes in a water bath. On cooling, butanol pyridine was added and centrifuged for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of microM/mg.

Determination of glutathione peroxidase (GPx) level

GPx activity was determined as described by Moin V.M. [24]. Briefly, activity of GPx depended from oxidation rate of glutation in the presence of protein precipitation. Formation color reaction was result of response of SH-group with 5,5-ditiobis(2-nitrobensoic) acid (DTNNA) with development

colored product – tionitrophenolic anion. The level of this product was directly proportional to level of SH-groups that react with DTNNA. The level of reduced form of glutathione (GSH) before and after incubation was measured by spectrophotometer. Results were expressed as microM GSH/mg×h.

Statistical analysis

Statistical analysis was performed with program package STATISTICA for Windows 5.5 (Stat Soft, USA). The results of evaluations according the semiquantitative scale are expressed as means ± SEM. For comparison of data used paired Newman-Keuls's test with a level of significance at P < 0,05. The data obtained from the experiments were expressed as mean ± SE. Differences in the data of experiments were analyzed statistically using ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

The animal survival rate (n) in the all experimental groups (non-diabetic control and diabetic rats) represented in *Fig.1*. The diabetic animals from EHG 1 and EHG 2 groups (14 and 24 days of noncontrolled hyperglycemia) had lower body weight (172,14±16,14; 172,14±16,14 g, respectively) than the non-diabetic control animals (216,14±8,17 g, p<0.05) but higher esophageal weight (1,74±0,08 g on 14 days EHG and 2,03±0,08 g on 24 days EHG) than the non-diabetic control animals (1,27±0,06 g, p<0.05). Changes of body and esophagus weight for rats with EHG and pretreatment L-Arg and Ind represented in *Tabl. 1*. No pathological signs on esophaguses were detected by macroscopical examination in control

Table 1. Data of baseline conditions, histology activity index (HAI) and content of plasma levels of NOx, MDA content and activity of GPx in non-hyperglycemic control animals and uncontrolled streptozotocin-induced hyperglycemic animals after 14 (EHG1) and 24 (EHG2) days treatment of saline (EHG 1, EHG 2); L-Arginine (EHG + L-arg 1, EHG + L-Arg 2) and indomethacin (EHG + Indo 1, EHG + Indo 2). Values are means ± SEM.

Index	Experimental group						
	Control (n=7)	EHG 1 + saline (n=7)	EHG 2 + saline (n=6)	EHG + L-Arg 1 (n=6)	EHG + L-Arg 2 (n=5)	EHG + Indo 1 (n=5)	EHG + Indo 2 (n=5)
Body weight , g	216,14±8,17	172,14±16,14*	172,67±5,61*	169,33±4,22*	159,00±3,32*	176,60±4,85*	167,00±5,39*
Esophagus weight, g	1,27±0,06	1,74±0,08*$^{\S\#\beta}$	2,03±0,08*$^{\beta}$	2,32±0,08*$^{\dagger\alpha}$	2,26±0,09*$^{\dagger\alpha}$	1,86±0,06*$^{\S\#\beta}$	2,38±0,15*$^{\dagger\ddagger\alpha}$
HAI	0,00±0,00	1,43±0,20*$^{\ddagger\S\alpha\beta}$	2,50±0,22*$^{\dagger\S\#\beta}$	0,50±0,22$^{\dagger\ddagger\#\alpha\beta}$	1,20±0,20*$^{\ddagger\S\alpha\beta}$	2,80±0,20*$^{\dagger\S\#}$	3,20±0,20*$^{\dagger\ddagger\S\#}$
MDA, microM/mg	55,57±1,17	88,00±1,43*$^{\ddagger\#\alpha\beta}$	139,67±2,65*$^{\dagger\S\#\alpha\beta}$	88,33±2,60*$^{\ddagger\#\alpha\beta}$	120,80±3,12*$^{\dagger\ddagger\S\alpha\beta}$	147,20±2,87*$^{\dagger\ddagger\S\#\beta}$	188,80±2,94*$^{\dagger\ddagger\S\#\alpha}$
GP_x , microM GSH/mg×h	3,65±0,16	2,73±0,07*$^{\ddagger\alpha}$	2,20±0,11*$^{\dagger\S\#\alpha\beta}$	2,91±0,12*$^{\ddagger\alpha}$	2,83±0,11*$^{\ddagger\alpha}$	3,86±0,15$^{\dagger\ddagger\S\#\beta}$	2,83±0,14*$^{\dagger\ddagger\alpha}$
NO_x mkM	17,86±0,63	20,43±0,72$^{\ddagger\S\#\alpha\beta}$	24,00±0,82*$^{\dagger\S\#\alpha\beta}$	34,67±0,99*$^{\dagger\ddagger\alpha\beta}$	37,00±1,55*$^{\dagger\ddagger\alpha\beta}$	42,60±0,93*$^{\dagger\ddagger\S\#\alpha\beta}$	56,20±0,86*$^{\dagger\ddagger\S\#\alpha}$

* p<0.01 *vs* control
† p<0.05 *vs* EHG 1
‡ p<0.05 *vs* EHG 2
§ p<0.05 *vs* EHG + L-Arg 1
$^{\#}$ p<0.05 *vs* EHG + L-Arg 2
$^{\alpha}$ p<0.05 *vs* EHG + Indo 1
$^{\beta}$ p<0.05 *vs* EHG + Indo 2

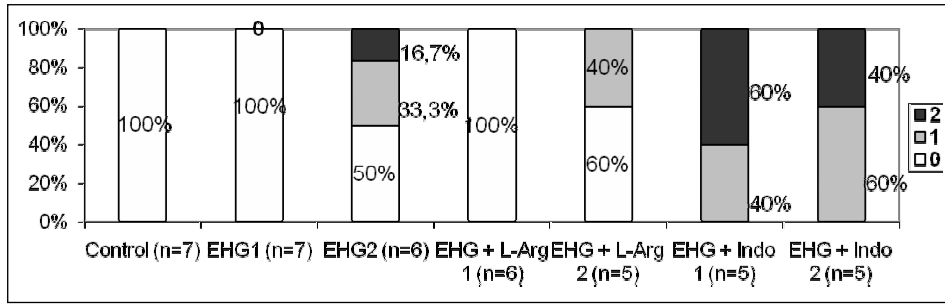

Fig.1. The animal survival rate (n) and esophageal destruction occurrence ratio in the all experimental groups (non-diabetic control and diabetic rats); score 0 - normal shimmering mucosa; 1- hyperemic or edematous mucosa; 2- erosions; values are means ± SEM.

group and EHG 1, but the changes of macroscopic morphology (esophaguses were swollen and enlarged) in other experimental groups were ranged between 1-2 score and represented by esophageal destruction occurrence *ratio* (*Fig.1*). Administration of SZT caused changes in esophageal epithelial barrier. At light microscopic level, prominent subepithelial and moderate intraepithelial edema was accomponied with intracelllular splitting and different degree destructive lesions, diffuse inflammatory leukocyte infiltration (*Fig.2*). Differences of expression morphological features of esophageal tissue in rat with EHG treated with saline, L-arg, Indo shown by HAI in *Tabl.1*. We first determined the effect of the various treatments on LPO product levels – MDA. In vehicle treated control rats esophageal MDA synthesis reached 55,57±1,17 microM/mg. EHG per 14 days affected significally increase MDA content to 60% and after 24 days in 1,5 times more. Pre-treatment of L-arg per 14 days was without effect but after 24 days it attenuated the reduction in MDA synthesis on 15% from rats of EHG 2 group. In group EHG Indo 1 (pre-treatment with Indo per 14 days) caused a strong increased MDA levels in EHG rat, approximately three times those in control vehicle treated rats. Treatment at prolonged times to 24 days (group EHG Indo 2) led to a marked increased MDA content, it reached 188,80±2,94 microM/mg.

Esophageal mucosa GPx activity in rats from control group reached 3,65±0,16 microM GSH/mg×h, EHG decreased it in all animal groups, except rats with Indo pretreatment per 14 days (increased in 6% without statistical authenticity). Significant increases of mucosal NOn values were measured in rats exposed to EHG with saline, L-arg and Indo pretreatment when compared with result from control group. However, the Indo treatment aggravated significantly EHG-induced esophageal lesions and this was accompanied by a rise of NO synthesis in comparison to the results from groups EHG L-arg 1 and EHG L-arg 2 that observed by the microscopic examination.

To determine whether modification of glycoconjugates expression of esophageal epithelial barrier is responsive to non controlled EHG were performed

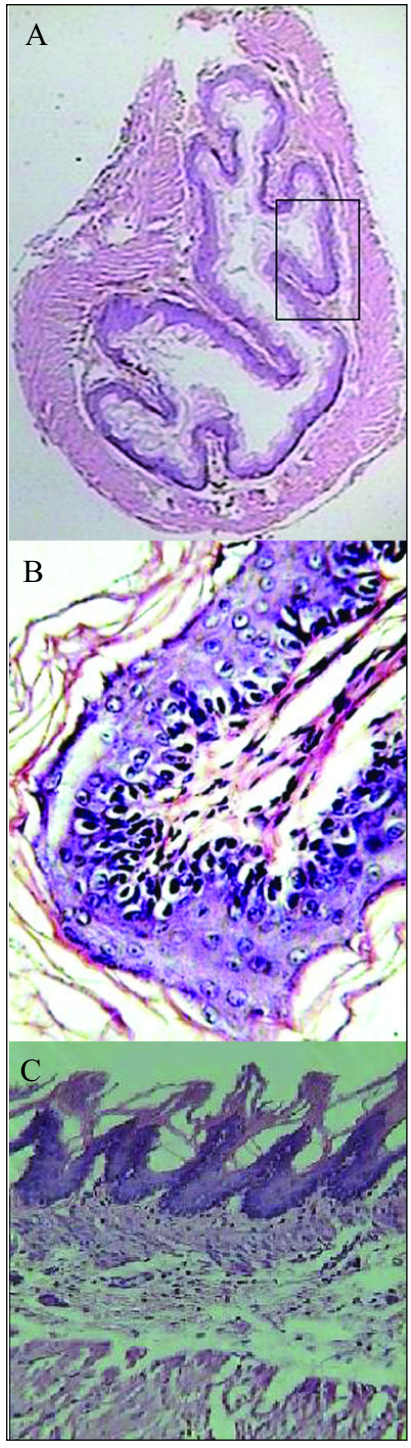

Fig. 2. Histological section of esophagus from rats with streptozotocin-induced non-controled hyperglycemia per 24 days. Hematoxylin-esosin stain, magnification x 40 (A); x 80 (B); x120 (C).

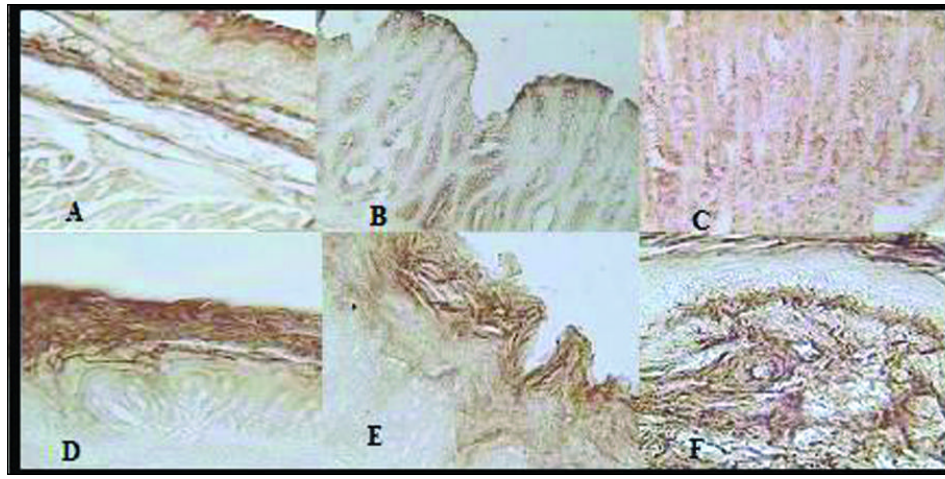

Fig. 3. Modification of expression of PNA (A, B), HPA (C, F), SNA (D) and WGA (E) lectin receptors in esophageal mucosa in experimental non controlled hyperglycemia without (A, D) and with L- arginine (B, E) and indomethacin-treated (C, F) rats; magnification x 300.

lectin histochemistry analysis (*Fig.3*). PNA and WGA were expressed at low levels in stratum spinosum of esophagus, whereas their expression was strongly in superficial epithelial layer at 24 days of EHG in comparison to that recorded in vehicle-pretreated rats (*Fig. 3 A,D*). Relative to expression in EHG controls, SNA and WGA expression in EHG with L-arg pretreatment was increased in superficial esophageal layer but more less (*Fig. 3 B,E*). By contrast, in HPA labeling were exhibited frequent and strong to intense staining in the highly proliferative epithelium (*Fig.3. C,E*). In addition, PNA and HPA overexpression in esophageal epithelia displayed during EHG with Indo pretreatment in the hyperplastic cell layers but not in parakeratotic regions of the outer layers.

DISCUSSION

Data from *in vitro* and *in vivo* investigations revealed nitrosative stress as a basis of development of many pathological conditions from acute to chronic diseases (25, 26, 27). Molecular biology tools suggest a strong link between implication of increased formation of peroxynitrite, superoxide anion and nitrotyrosine in high glucose level and their membranodestuctive damaging effect on cellular integrity, wide-ranging in the epithelium, endothelium and perineurium (28, 29, 30, 31). Excess of synthesis NO and activity iNOS influences on vascular tone, causes release of vasoactive neurohormons, changes adhesion of leukocytes (modificate expression of VCAM-1, ICAM-1, E-selectin and stability of mRNK of macrophage chemotactic factor, MCF) (11, 14, 32, 33). Although hyperglycemia has been proven to cause gastrointestinal and

hepatobilliary dysfunction in patients with DM (4, 34, 35), the physiopathology mechanisms for this effect on esophageal epithealial barrier are poorly understood. In present study increased amount of NOn production in esophageal mucosa during of EHG was demonstrated and proposed to be responsible for the breakdown of the esophageal epithelial barrier in diabetic animals. Nitrosative stress mediated remodeling of epithelial layer and initiated modification in carbohydrate moieties of esophageal glycoconjugates. To clarify the possible mechanism of esophagoprotection at EHG by which esophageal lesions were attenuated, we examined changes in esophageal LPO levels and activity of potent antioxidant enzyme GPx. We have linked the metabolism of glucose associated with increased production of MDA, end product of LPO, and decreased activity of GPx in esophageal tissue suggesting their pathogenetic role in the development of non-erosive changes in esophagus wall (36). Accumulating literature data suggest a strong link between disbalance in pro- and antioxidative system during DM (37, 38). Our observations evaluated the implication and interplay of NO/NOS and PG/ COX signaling pathways in esophagoprotection during EHG in the experiments with L-arg and Indo pretreatment. Esophageal NO levels were rise in animals with EHG treated by L-arg and Indo, but expression of destructive morphological outcomes is attenuated associated in diabetic rats with L-agr administration. In addition increased peroxynitrite-mediated PNA, HPA, SNA and WGA expression versus control were demonstrated and proposed to be responsible for the modification of preepithelial and epithelial mucosal layers due to defensive reactions against RNOS in diabetic animals (39, 40).

In summary, obtained results suggested that esophageal resistance during EHG when excess of production RNOS initiate nitrosative stress depends of upon a balance of pro- and antioxidative activity, paracrine regulation such as NO/NOS and PG/COX signaling pathways and pre-epithelial and epithelial cellular homeostasis. Glycomic approach for identification glycoconjugates of epithelial barrier are multifunctional substrate of early evaluation of mucosal integrity in esophageal physiopathy.

Conflict of interest statement: None declared.

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