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ROLE OF NITRIC OXIDE-SYNTHASE AND CYCLOOXYGENASE/LIPOOXYGENASE SYSTEMS IN DEVELOPMENT OF EXPERIMENTAL ULCERATIVE COLITIS

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Development of ulcerative colitis was accompanied by the activation of iNOS/COX-2/5-LOX and increased contents of nitric oxide (NO), prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4). The following information was assessed: morphological changes, activity of nitric oxide-synthase, content of nitric oxide, and indexes of lipoperoxidation processes in the mucous membrane of the large intestine (MMLI). Colitis was induced in rats by intrarectal administration of 1 ml of 4% acetic acid. Aminoguanidine - selective inducible nitric oxide-synthase (iNOS) blocker, celecoxib – cyclooxygenase-2 (COX-2) inhibitor, indomethacin - non-selective COX inhibitor and AA-861 – 5-lipooxygenase (5-LOX) blocker were administered in 1 ml volumes *per os 1* hour before and 24 hours after the intrarectal application of acetic acid. It was noticed that blockage of iNOS by aminoguanidine caused enhancement of cytoprotective mechanisms, reduction of iNOS activity and oxidative stress, and an increase in blood L-arginine level as compared to their respective indexes in colitis. Combined blockage of iNOS and COX-2 displayed additive character of their effect on the processes of lipoperoxidation and activity of iNOS. Combined blockage of iNOS, COX-2 and 5-LOX had a manifested cytoprotective effect under condition of ulcerative colitis and was accompanied by a sharp decline in NOS activity and oxidative stress. If each of these systems, iNOS/NO, COX-2/PGE_2 and 5-LOX/LTB_4 are simultaneously activated due to inflammation, they contribute to the destructive damage of the MMLI, development of oxidative stress, and affect components of the antioxidant protection system. The obtained results substatiate the relevance of treatment of the inflammatory processes with the use of madication capable of combined blockage of iNOS, COX-2, and 5-LOX.

Key words: *5-lipooxygenase, cyclooxygenase-2, inducible nitric oxid-synthase, lipoperoxidation, ulcerative colitis*

INTRODUCTION

Ulcerative colitis is one of the most common diseases of the large intestine in humans whose development is affected by different factors including, genetic disorders of the regulatory immune system, stress, side-effects of pharmacological preparations, irregular diet, dysfunction of mucous barrier, and dysbiosis (1, 2).

The crux of the study of pathochemical mechanisms on the development of ulcerative colitis are roles of the main proinflammatory cellular systems – nitric oxide (NO) inducible nitric oxide-synthase (iNOS)/NO, cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) and 5-lipooxygenase (5-LOX)/leukotriene B_4 (LTB_4) in this pathology. This is explained by the key parts played by these cellular systems in the genesis of inflammatory processes of the large intestine. In patients with ulcerative colitis were noticed: increased expression of iNOS and COX-2, activation of lipoperoxidation processes, and higher content of nitrosylated thyrosin in the mucous membrane of the large intestine (MMLI) (3).

Development of ulcerative colitis is characterized by increased mucosal infiltration by neutrophils and monocytes which, having become activated, release proinflammatory cytokines – tumor necrosis factor α (TNF-α), interferon-γ – (IFN-γ), interleukin β (IL-β) and free oxygen radicals, induce increased expression of iNOS COX-2, nuclear transcription factor NF-κB, protein p66, and enhancement of the lipoperoxidation processes. These changes cause destructive affections of the MMLI, *e.g.* impairment of mucous barrier, swelling, ulcers, erosions, and hemorrhages (4, 5). The destructive changes the intestinal mucosa is subjected to due to ulcerative colitis are associated with increased numbers of active forms of oxygen, enhanced synthesis of NO, expression of iNOS by epithelial cells, macrophages, and neutrophil infiltraton into the damaged mucous membrane. Concomitantly, the activity of myeloperoxidase, the marker of inflammation, was enhanced (2).

Nitric oxide under physiological conditions is synthesized from L-arginine with the involvement of constitutive NO-synthases – eNOS and nNOS. In the mucous membrane of the large intestine (MMLI), eNOS is identified in the endothelial and epithelial cells, smooth muscles, thrombocytes and T-cells while nNOS is mainly localized in the central and peripheral nerves, but also is detected in myocytes, epithelial cells, mast cells, and neutrophils. Ulcerative colitis is indicative of increased expression of NO-synthases, predominantly iNOS. iNOS is identified in the cells of the proper plate and submucous membrane of the large intestine. Expression of eNOS mostly occurs in the endothelial cells and to a lesser extent, in the epithelial cells (6).

Another important system involved in the development of ulcerative colitis is presented by prostaglandins produced by

COX-1 and COX-2. COX-1 of mRNA is expressed in the large intestine under physiological conditions. At the same time, COX-2 is also expressed under the normal conditions in the interstitial tissue, epithelial cells, and neurons of submucous and intermuscular plexuses. Prostaglandins synthesized by COX-1, provide for the processes of water and electrolyte transport, vasodilatation, proliferation, and intercellular integration. Considerable amounts of prostaglandins synthesized by COX-2 are involved in the inflammatory process. Concomitantly, with the activation of COX-2, prostaglandines enhance the activity of 5-lipooxygenase (5-LOX) and the release of inflammatory leukotriens – LTB_4, LTD_4, LTC_4 (7).

Determination of the role of NO-synthase system in its interrelationship with the activity of COX-2, 5-lipooxygenase, and processes of lipoperoxidation in the pathogenesis of ulcerative colitis remains largely unresolved and open to dispute (3, 7-9).

MATERIALS AND METHODS

Animals

The structure of this study and the experimental procedures performed on the animals were approved by the Ethical Committee of Lviv National Medical University. Sixty female albino rats weighing 200-250 g were used. The animals were housed for 12-24 hour under light/dark cycles at a constant temperature 21-22°C and fed on a standard rat chow and water ad libitum. All rats were fasted 24 hours prior to the experimental procedure.

The investigation reported on comprised the following series of experiments: 1 – intact animals were used as controls; 2 – ulcerogenic lesions in rats were induced by 4% acetic acid as previously described (n=10) (27); 3 –inducible NO-synthase inhibitor, aminoguanidine (Sigma), was introduced in a dose of 20 mg/kg at the background of colitis (n=10); 4 – COX-2 blocker, celecoxib (Arterium, Ukraine) was introduced in a dose of 10 mg/kg at the background of colitis (n=10); 5 – non-selective COX inhibitor, indomethacin was introduced in a dose of 10 mg/kg at the background of colitis (n=8); 6 – of 5-lipooxygenase blocker, AA-861 (Wako, Japan) was introduced in a dose of 50 mg/kg at the background of colitis (n=8); 7 – aminoguanidine and celecoxib were introduced at the background of the acetic acid action (n=10); 8 – aminoguanidine, celecoxib and AA-861 were introduced at the background of the acetic acid actions (n=10).

Induction of colitis and treatment protocols

Colitis was induced in rats by intrarectal administration of 1 ml of 4% acetic acid (group 2). After anesthesia, a soft 6F pediatric catheter was introduced 8 cm into the anus and acetic acid was carefully injected. After 30 second 1 ml of phosphate buffer pH 7,4 was injected for neutralization of acetic acid. Control rats (group 1) were treated in a similar way but instead of 4% acetic acid, 1 ml of 0.9% saline was infused. Aminoguanidine (20 mg/kg) (group 3), celecoxib (10 mg/kg) (group 4), indomethacin (10 mg/kg) (group 5), AA-861 (50 mg/kg) (group 6), aminoguanidine and celecoxib (group 7), aminoguanidine, celecoxib and AA-861 (group 8) were administered in 1 ml volumes per os 1 hour before and 24 hours after the intrarectal application of acetic acid. Under general anesthesia, rats were sacrificed by decapitation and 10 cm of the distal colon were excised, freed from adherent adipose tissue and opened longitudinally. After washing the mucosa with saline, mucosal injury (macroscopically) was assessed using the grading scale of Morris *et al*. (1989). No damage (score 0); localized hyperemia but no ulcers (score 1); linear ulcer with no significant inflammation (score 2); linear ulcer with inflammation at one site (score 3); two or more sites of ulceration and inflammation (score 4); two or more sites of ulceration and inflammation or one major site of inflammation and ulceration extending >1cm along the length of the colon (score 5).

For histological investigation additional samples were fixed in 10% formalin, embedded in paraffin and sections were prepared and stained with hematoxylin-eosin.

After that colonic tissue samples were homogenized in saline (or phosphate buffer pH 6.0) 1:4, centrifuged at 5000 rpm and supernatant was used to determine of biochemical parameters.

Lipid peroxidation determination (measurement of MDA levels)

Lipid peroxidation level was expressed as MDA concentration in homogenates of MMLI. It was measured according to the procedure of Timirbulatow *et al*. (10). The reaction mixture contained 0.1 ml sample, 10 mmol phosphate buffer in 125 mmol potassium chloride and volume was finally adjusted to 8.0 ml. Next, 0.5 ml of 1 mmol potassium permanganate was added and incubated for 10 min at 24°C. Then, 0.5 ml of 10 mmol $FeSO_4$, 1 ml of 20% trichloracetic acid, 0.5 ml of 0.1 N HCl and 1 ml of 0.7% solution of thiobarbituric acid in water were added. The mixture was heated for 20 min in a boiling water bath. After cooling of the samples, they were centrifuged at 4000 rpm for 10 min and than the complexes formed by thiobarbituric acid reactant substances were extracted with n-butanol. Color intensity was measured at 532nm using a spectrophotometer. Plasma MDA level was expressed as μmol/g.

Assessment of antioxidant status in colonic tissues

Catalase (CAT) activity

CAT was determined by measuring of the decrease in hydrogen peroxide concentration at 410 nm by the Korolyuk method (11). Assay medium consisted of 1 ml Tris HCl buffer solution 0.05 mmol, pH 7.8, 0.1 ml sample (homogenates from colon tissue) and 2 ml of 0.03% H_2O_2. The reaction was stopped after 10 min by 1 ml of 4% ammonium molybdate. Samples were measured at 410 nm by a spectrophotometer. Colon mucosal catalase activity was expressed in μmol H_2O_2/min. mg of protein.

Activity of superoxide dismutase (SOD)

Determined by the reaction of reduction of nitrotetrazoliume blue to nitroformazan (12). Incubation assay medium contained 0.1 ml of homogenate (1:10), 0.9 ml of distilled water, 0.5 ml of absolute ethylic alcohol, 0.25 ml of chloroform and 0.3 g KH_2PO_4. The mixture was shaken vigorously. After centrifugation at 5000 rpm for 30 min, 0.1 ml of NADPH and 0.05 ml of the incubation solution (37 mg EDTA, 330 mg p-iodonitrotetrazolium violet, 55 mg phenazinmethosulfate) were added to a supernatant and incubated for 10 min at 20°C. The absorbance was immediately read in a Stat fax at 540 nm. SOD activity was expressed in μmol/min•mg of protein.

Determination of nitric oxide-system in colonic tissues

Measurement of nitrate in colon tissue

The content of nitrogen oxide in homogenate was determined as nitrites by the method of Green L., David A., (13). 0.3 ml of homogenate 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. 1 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 The absorbance was read in a Stat fax at 550 nm. NO concentration was expressed as μmol/g.

Measurement of nitric oxide-synthases

NO-synthases activity was measured by the method described in detail (Sumbajev V. (14)). 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 method; b – volume of tissue homogenates samples.

Colon mucosa NOS activity was expressed in nmol NADPH/min•mg protein. Activity of iNOS was measured by similar method, but the reaction mixture contained a buffer without $CaCl_2$. eNOS=NOS-iNOS.

Measurement of L-arginine in plasma samples

Level of L-arginine in plasma samples was measured according to the procedure of Alejnikova (15). 0.5 ml of 5% trichloracetic acid was added to 0.5 ml of rat's plasma samples and the precipitate was eliminated by centrifugation at 3000 rpm for 10-15 min. 1 ml of 5% NaOH, 0.05 ml of 0.02% alcohol solution of α-naphtolle, 0.05 ml hypobromidi reagent, 0.2 ml 10% water solution of urea were added to 0.5 ml of obtained supernatant. Incubated 20 min and measured at 500 nm using a spectrophotometer against water. Plasma L-arginine level was expressed as μg/ml.

Experimental results were analyzed by ANOVA and t-tests for multiple comparisons between groups. The data was finally expressed as mean ±standard deviation. P value less than 0.05 was considered statistically significant.

RESULTS

Injection of 4% solution of acetic acid induced structure-hemorrhagic lesions, manifested by ulcerative colitis, erosions, hemorrhages (*Fig. 1*) with a total area of 77.2±25.1 mm^2 (*Fig. 2*). Character of the damage of the MMLI had a score of 4.6±0.69 (*Fig. 2*). Histological investigations revealed disorders of the mucous barrier of the MMLI, destructive changes of its components, presence of edema, deep erosions, infiltration of polymorphonuclear leukocytes and lymphocytes (*Fig. 3*).

Ulcerative colitis in the MMLI was associated with enhancement of the activity of NO-synthases: activity of general NOS enhanced by 145% (from 1.09±0.09 to 2.68±0.58 μmol/min•g), activity of eNOS – by 21%, activity of iNOS – 6.9-fold (P<0.01). In colonic mucosa content of NO increased by 64% and concomitantly, content of L-arginine in blood decreased by 51% (from 37.8±12.8 to 18.53 ±4.98 mg/ml) (*Table 2*).

Affected with ulcerative colitis MMLI was subjected to the following changes: enhanced activity of lipoperoxidation processes manifested by a steep rise of MDA content – by 116% (from 255.5±24.3 to 556.3±15.7 μmol/g) (P<0.01), SOD activity enhanced by 71% (P<0.01), catalase activity by 54% (P<0.01).

Therefore, development of ulcerative colitis induced by per rectum introduction of acetic acid was accompanied by the characteristic destructive changes in the MMLI *i.e*., enhanced lipoperoxydation processes, increased content of nitric oxide, and considerable activation of iNOS. Due to activation of NO-synthases, concentration of L-arginine in the plasma of blood decreased and content of NO in the MMLI increased.

Due to injection of aminoguanidine, the area of lesions of the colonic mucosa decreased and separate erosions and hemorrhages were localized in the rugae (*Fig. 1, 2*). Histological data showed increased area of the mucosal barrier devoid of damage, although punctuate hemorrhages and sites with erosions were still detectable (*Fig. 3*). Aminoguanidine produced a decrease of iNOS activity by a considerable 48% (p<0.05), but failed to induce any significant change in the activity of eNOS. As a result, NO content in the colonic mucosa decreased by 50% (p<0.05) and concentration of L-arginine in the blood plasma increased (p<0.05). iNOS blockage with aminoguanidine resulted in the decrease of MDA by 28% (p<0.01), 2.3 - fold reduction of SOD activity (p<0.01), and 20% reduction of catalase activity (p<0.05).

Therefore, iNOS blockage with aminoguanidine led to in the enhancement of cytoprotective processes. Under these conditions, destructive changes of the MMLI became less manifested, activity of iNOS and eNOS diminished by 46% and 34%, respectively, and liperoxidation processes reduced. Decline of the activity of NO-synthases was accompanied by a drop in nitric oxide content in the MMLI (by 50%) and increase of L-arginine concentration in the blood plasma.

COX-2 inhibition with celecoxib led to decrease of area of lesions of the MMLI, hereby activity of iNOS and eNOS diminished by 46% and 40%, respectively, NO content decreased by 16%. L-arginine concentration in the blood plasma was increased. The content of MDA and SOD activity decreased by 20% and 46% respectively. Therefore, as the COX-2 inhibition led to the increase of cytoprotective processes, it suggests that prostaglandins, produced by COX-2, are associated with inflammation and have many proinflammatory activities that could contribute to symptoms of ulcerative colitis.

Non-selective COX inhibition with indomethacin resulted in significantly worse colitis as compared with acetic acid colitis alone (*Fig. 1*). The total area of lesions of colonic mucosa was 96±16.9 mm^2 (*Fig. 2*) character of a score of 4.9±0.42 (*Fig. 2*). Nevertheless indexes of NO-synthase system and oxidative stress were on the same height with those in ulcerative colitis.

Administration of 5-LOX inhibitor AA-861 at the background of colitis had no effect on the severity of lesions (*Fig. 1*). Activity of NO-synthase had a tendency to decrease, whereas iNOS activity was declined by 44% (p<0.05) as compared to their activity in colitis. Contents of NO and MDA also showed a tendency to decrease. Hence, under condition of LOX-5 inhibition the intensity of lipoperoxidation processes remained high as well as NOS activity. This suggests that the product of 5-LOX reaction – LTB_4 didn't play a central role in pathogenesis of experimental ulcerative colitis.

Combined blockage of iNOS with aminoguanidine and COX-2 with celecoxib resulted in a decrease of both macroscopic and microscopic destructive changes of the MMLI. Separate punctuate hemorrhages and erosions were observed on a macroscopic assessment of MMLI (*Fig. 1*). Histological investigations revealed that crypts preserved their integrity although separate damages areas of the mucous membrane were visible (*Fig. 3*). It has been noticed that iNOS activity and nitric oxide content tended to reduce and eNOS activity remained unaltered in contrast to the action exerted by aminoguanidine at

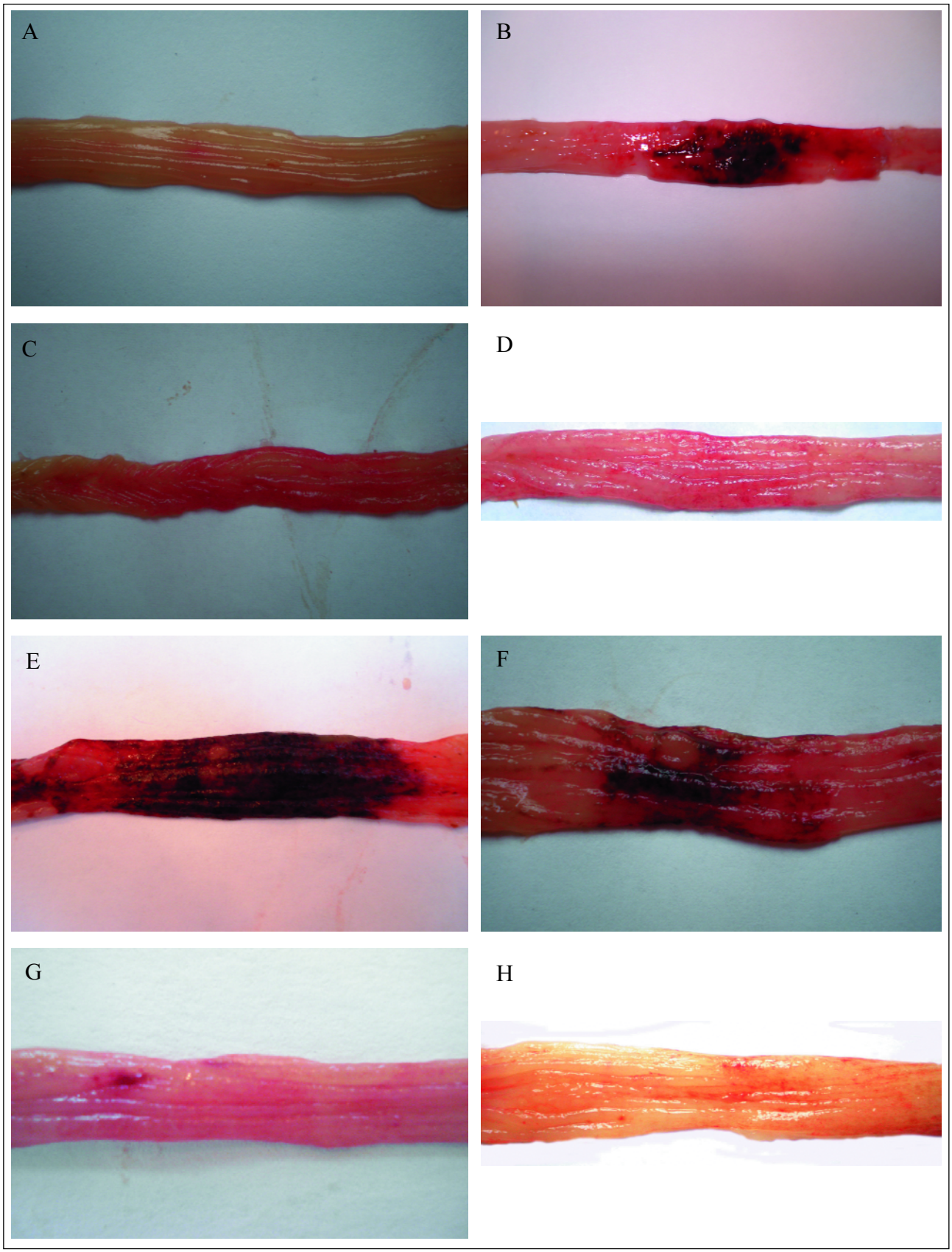

Fig. 1. Macroscopic status of the surface of mucous membrane of large intestine: A – control; B – under conditions of colitis evoked by 4% acetic acid introduced per rectum; C – resulting from *per os* introduction of iNOs-synthase blocker, aminoguanidine, at the background of colitis; D – resulting from per os introduction of COX-2 inhibitor, celecoxib, at the background of colitis; E – resulting from per os introduction of non-selective COX inhibitor indomethacin; F – resulting from *per os* introduction of 5-lipooxigenase blocker, AA-861; G – induced by aminoguanidine and celecoxib introduced *per os* at the background of the acetic acid action; H – caused by the action of aminoguanidine, celecoxib, and blocker of 5-lipooxigenase AA-861 introduced *per os* at the background of the acetic acid action.

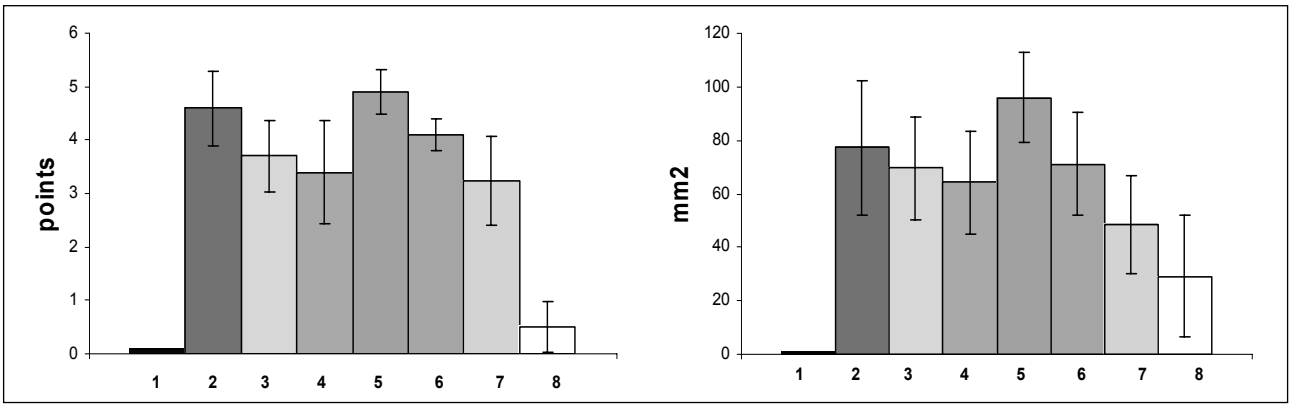

Fig. 2. Character (in score – 1) and area (in mm²) of the structure-hemorrhagic damage of the MMLI: 1 – control; 2 – under conditions of colitis evoked by 4% acetic acid introduced per rectum; 3 – resulting from *per os* introduction of iNO-synthase blocker, aminoguanidine, at the background of colitis; 4 – resulting from *per os* introduction of COX-2 inhibitor, celecoxib, at the background of colitis; 5 – resulting from *per os* introduction of non-selective COX inhibitor indomethacin; 6 – resulting from *per os* introduction of 5-lipooxigenase blocker, AA-861; 7 – induced by aminoguanidine and celecoxib introduced *per os* at the background of the acetic acid action; 8 – caused by the action of aminoguanidine, celecoxib, and blocker of 5-lipooxygenase AA-861 introduced *per os* at the background of the acetic acid action.

Table 1. Activity of NO-synthases, content of NO in MMLI and L-arginine in blood under conditions of independent and combined blockage of iNOS, COX-2 and 5-LOX at the background of ulcerative colitis.

	NO, μmol/g	iNOS, nmol/min·g	eNOS, nmol/min·g	NOS, nmol/min·g	L-arinine, μg/ml
Control group	1.37±0.14	0.239±0.07	0.851±0.095	1.09±0.092	37.8±12.8
4% acetic acid	2.25±0.43	1.65±0.5	1.03±0.32	2.68±0.58	18.6±4.7
4% acetic acid +aminoguanidine	1.13±0.63*	0.851±0.11*	0.817±0,26	1.58±0.37*	35.7± 11.7*
4% acetic acid +celecoxib	1.89±0.27	0.898±0.03*	0.623±0,101	1.52±0.12*	22.4±3.81*
4% acetic acid +indomethacin	1.96±0.11	1.64±0.37	0.49±0.27	2.13±0.52	20.1±6.7
4% acetic acid +AA-861	2.12±0.28	0.91±0.13	0.73±0.15	1.64±0.39	23.1±8.4
4% acetic acid +aminoguanidine +celecoxib	1.08±0.21	0.735±0.15	0.827±0.19	1.56±0.24	33.1±9.97
4% acetic acid +aminoguanidine +celecoxib +AA-861	1.5±0.36	0.444±0.148	0.565±0.116	1.01±0.162	24.5±3.16

Note: * P<0.05, ** P<0.01 *vs*. the indexes in colitis.

Table 2. Changes of the indexes of lipoperoxidation and activity of antioxidant protection enzymes (SOD, catalase) under conditions of independent and combined blockage of iNOS, COX-2 and 5-LOX at the background of ulcerative colitis.

	MDA(μmol/g)	SOD (μmol/min•mg of protein)	Catalase (μmol/min•mg of protein)
Control group	255.5±24.3	18.8±5.5	2.42±0.3
4% acetic acid	556.3±15.7	32.0±9.5	3.72±0.54
4% acetic acid + aminoguanidine	401.3±54.9*	14.2±7.6**	2.98±0.44
4% acetic acid + celecoxib	444.0±58.4*	17.2±1.8*	3.18±0.98*
4% acetic acid + indomethacin	543.4±61.2	26.7±3.1	3.54±0.49
4% acetic acid + AA-861	543.6±25.7	23.4±2.4	3.34±0.31
4% acetic acid + aminoguanidine +celecoxib	339.5±45.5	17.4±1.95	2.93±0.54
4% acetic acid + aminoguanidine +celecoxib +AA-861	292.0±5.65	17.1±3.2	2.53±0.32

Note: * P<0.05, ** P<0.01 *vs*. the indexes in colitis.

the background of colitis. Content of MDA decreased by 39% (p<0.01) as compared to the indexes resulting from iNOS blockage at the background of colitis. Activity of SOD displayed a sharp decline – by 79% (p<0.01) *versus* the indexes of SOD activity in colitis and was by 53% (p<0.05) lower than the activity due to concomitant effect of amine guanidine at the

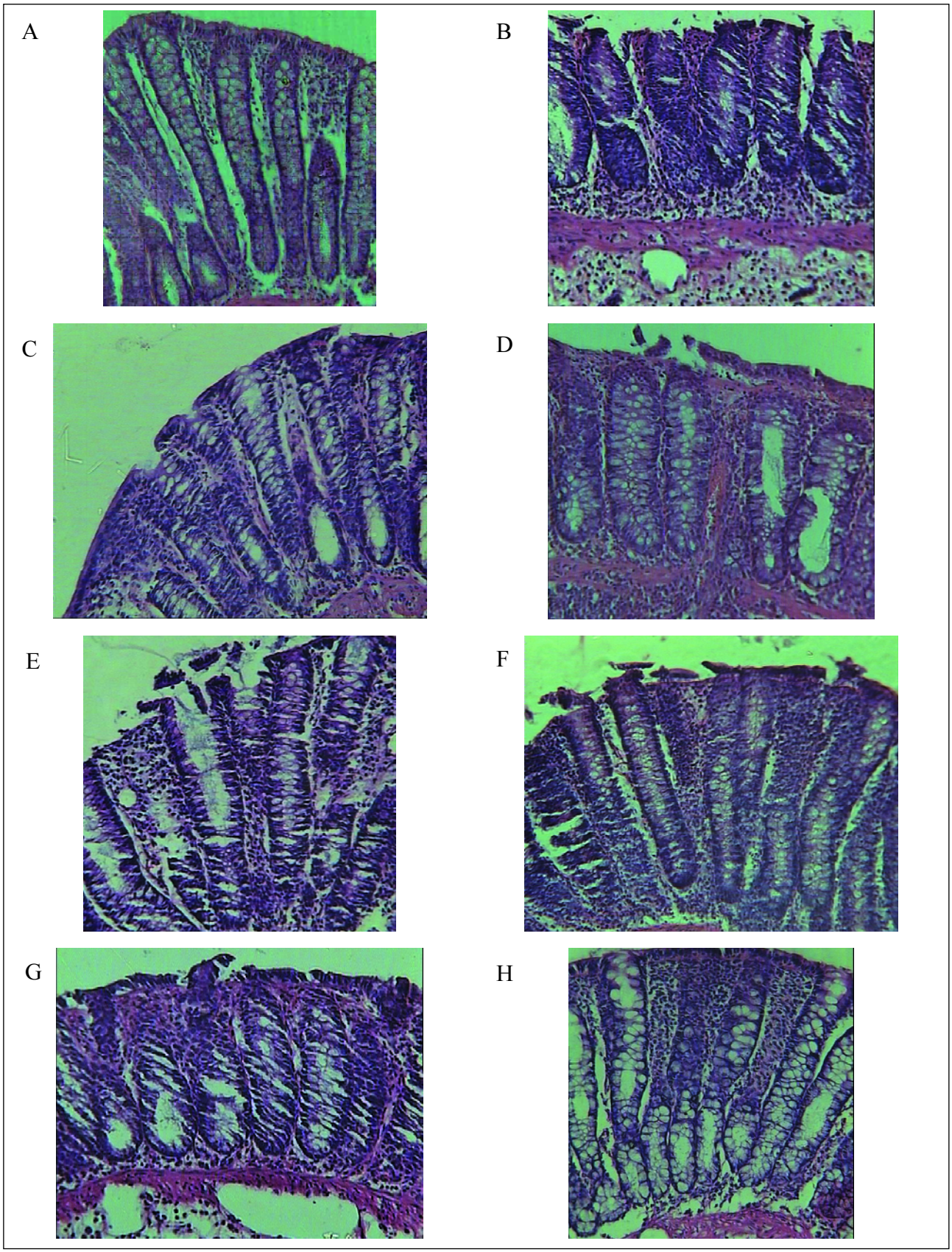

Fig. 3. Histological status of the MMLI caused by: A – control; B – under conditions of colitis modeled with 4% acetic acid introduced per rectum; C – resulting from per os introduction of iNOS-synthase blocker, aminoguanidine, at the background of colitis; D – resulting from *per os* introduction of COX-2 inhibitor, celecoxib, at the background of colitis; E – resulting from *per os* introduction of non-selective COX inhibitor, indomethacin; F – resulting from *per os* introduction of 5-lipooxygenase blocker, AA-861; G – induced by aminoguanidine and celecoxib introduced *per os* at the background of the acetic acid action; H – caused by the action of aminoguanidine, celecoxib, and blocker of 5-lipooxygenase AA-861 introduced *per os* at the background of the acetic acid action. Magnification of 1:300.

background of colitis. Content of L-arginine in blood was high. Hence, under conditions of combined blockage of iNOS and COX-2 enhancement of cytoprotective processes and reduction of the destructive damage of the MMLI were noticed. Activity of iNOS and eNOS tended to diminish and the content of nitric oxide decreased, which resulted in high L-arginine content in the blood plasma.

Combined blockage of iNOS, COX-2 and 5-LOX displayed significant cytoprotective effect. Microscopy detected separate hemorrhages with the absence of considerable destructive changes of the MMLI. Histological investigations showed that the mucous barrier as well as the structure of crypts and epithelial cells were preserved, and edema was only mild (*Fig. 3*). Activity of iNOS and eNOS declined by 73% ($p<0.05$) and 45% ($p<0.05$), respectively, and total activity of NO-synthases by 62% ($p<0.05$) as compared to their activity in colitis. Content of nitric oxide manifested a concomitant decrease by 34% ($p<0.05$). It has to be mentioned that due to the blockage of COX-2 at the background of aminoguanidine, iNOS activity tended to attenuate and reduced by 14%. Yet under combined blockage of COX-2/5-LOX activity of iNOS was reduced by 48% ($p<0.05$) as compared to the indexes of iNOS activity under the impact of aminoguanidine at the background of colitis. Intensity of lipoperoxidation processes were determined 14% lower than under the effect of aminoguanidine and celecoxib. For this reason, combined blockage of NO synthesis by iNOS and synthesis of proinflammatory prostaglandins COX-2 and leukotriens 5-LOX has a significant cytoprotective effect accompanied by reduction of oxidative stress and activity of NO-synthases.

DISCUSSION

Nitric oxide under physiological conditions is synthesized from L-arginine by the constitutive forms of NOS (eNOS and nNOS) and plays a significant role in providing for the processes of modulation of nervous impulses, regulation of motility and secretion, as well as cytoprotection of the large intestine (8, 10). Activity of iNOS in the large intestine of intact rats was either undetectable or very low. Expression of iNOS in the norm mainly occurs in histocytes, neutrophils, smooth muscles and occasionally in epithelial cells (8, 17, 18).

In ulcerative colitis, cytokines (interleukin-1β, interferon-γ) and lipopolysaccharides induce expression of iNOS leading to a steep rise of nitric oxide synthesis. As a result of interaction between nitric oxide and superoxide radical, cytotoxic peroxinitrite ($OONO^-$) is synthesized, capable of splitting into secondary radicals – OH^- and NO_2^-. These free radicals can induce activation of lipoperoxidation processes, oxidation of sulfhydryl groups of proteins, changes of valence of metal ions and an increase of nitrosamines content that cause functional disorders in the cellular membranes and intracellular proteins (19).

Components of NO-synthase system, namely eNOS/NO, are known to provide for the cytoprotective mechanisms whereas action of iNOS/NO is opposite*, i.e*. it causes development of inflammatory processes and ulcerogenesis. Proinflammatory effect of iNOS/NO is manifested in different models of colitis: *Salmonella typhinarum* lipopolysacccharides, 2,4,6-trinitrobenzene sulfuric acid (TNBA), dextran sodium sulfate, ischemia-reperfusion. Ulcerogenic effect of iNOS/NO is manifested, in particular, by the activation of lipoperoxidation processes and production of nitrogen compounds which cause cell damage and even apoptosis (4, 8).

Results obtained in the research on the non-selective blocker of NO-synthases, L-NAME, in ulcerative colitis have been so far disputable. On the one hand, due to injection of L-NAME, oxidative stress levels were reduced along with area of lesions (18). On the other hand, L-NAME manifested its inhibitory effect on the secretion of mucus and fluid, enhanced motility, bacterial translocation and aggravated ulcerative lesions of the MMLI (20). The protective effect of L-NAME was less pronounced as compared with the protective effect of the selective blocker of iNOS – 1400 W (21).

Contrary results have been noticed due to injection of iNOS blocker, aminoguanidine (in the dose of 20 mg/kg) in ulcerative colitis modeled by means of dextran sodium sulfate salt. According to the data reported by Rumi and co-workers (18), effect of aminoguanidine induced a considerable decrease in the area of lesions with a maximum effect being achieved on the 4^{th} to the 6^{th} days. Other investigations stated that as a result of iNOS blockage with aminoguanidine, the degree of damage to the MMLI and inflammatory processes intensified and were accompanied by enhanced activity of myeloperoxidase in the mucous membrane, enhanced activity of lactate dehydrogenase in blood, and decreased concentrations of NO and 6-keto-prostaglandin 1α (22).

In our investigations it was established that under iNOS blockage with aminoguanidine, the area of structure-hemorrhagic damage decreased, lipoperoxidation processes and activity of antioxidant protection enzymes were reduced, as with the activity of iNOS and NO. This was accompanied by a increase of L-arginine concentration in blood. Activity of eNOS was determined less considerable. The aforementioned provides evidence that iNOS/NO plays a significant part in the pathochemical mechanisms of damaging processes in the MMLI under conditions of ulcerative colitis. Due to the blockage of iNOS/NO activity, cytoprotective processes were enhanced and oxidative stress levels were reduced.

Development of ulcerative colitis was accompanied by the activation of not only NO-synthase system but also by the increased expression of COX-2 (23). The role of COX-1 and COX-2 in the development of ulcerative lesions so far remains disputable. It should be pointed out that independent blockage of COX-1 or COX-2 failed to cause any manifested destructive damage to the small intestine whereas combined blockage of COX-1 and COX-2 resulted in development of ulcerative disorders. Injection of nonselective blockers of COX-1 and COX-2, the nonsteroidal anti-inflammatory drugs (NSAIDs) – indomethacin, naproxen, and diclofenac caused destructive changes in the small intestine. For this reason, the content of PGE_2 considerably decreased which is evidence that deficiency of prostaglandins is the key factor whose impact results in damage to the small intestine induced by the effect of NSAIDs (2, 24). The decrease of prostaglandins synthesis in the site of inflammation considerably correlated with the reduction of COX-1 activity, but not COX-2 (25). It was ascertained that COX-1, but not COX-2, plays a role in maintaining the mucosal integriti (20). Other reports showed that COX-2 is the main form responsible for PGE_2 production at the site of inflammation (25).

Several reports previously reported that inhibition of COX-2 aggravated experimentally-induced colonic lesion, that caused considerable inhibition of biosynthesis of prostaglandins and increased area of lesions in colon (27, 28). It should be suggested that COX-2 activation during inflammation caused production of anti-inflammatory prostaglandins PGD_2, PGF_2) (29).

Previously, it was shown that under blockage of COX-2 with celecoxib, damage to the MMLI was reduced as well as neutrophilic infiltration and the level of IL-1β. Celecoxib caused a substantial reduction of the degree of colonic injury, rose the myeloperoxidase activity in mucosa and increased the tissue level of MDA (5, 24). Combined blockage of iNOS and COX-2, as determined in our investigations, strengthened their effects and tended to reduce iNOS activity. The content of nitric oxide

decreased and lipoperoxiation processes and activity of SOD in the MMLI diminished (8).

Recent investigations have established close interrelationship existing between iNOS and COX-2. Nitric oxide synthesized by iNOS and peroxinitrite possesses a property of inhibiting COX-2 activity and, consequently, the synthesis of PGE_2. However, PGE_2 is capable of affecting the biosynthesis of nitric oxide. Inhibition of NO synthesis, in turn, reduces secretion of PGE_2 (25). This provides substantial evidence to new approaches for the treatment of ulcerative colitis and for development of drugs capable of combined blockage on iNOS and COX-2.

The interrelation between NO and PG-generating systems happens on different levels. NO can directly stimulate the expression of COX isoforms as well as prostaglandins biosynthesis. Yet, arachidonic acid and its metabolites have an influence on NOS activity. NOS expression closely correlates with receptors of prostaglandins. NO increases COX-1 activity directly, that cause PGE_2 production to increase by 7 times (30). COX-2 is activated by NO even more then COX-1. COX activation by NO takes place by means of its action upon the heme of the prosthetic group or, probably, by superoxide, produsd during COX action. Another mechanism is related with the influence on nitrizothiols and peroxynitrite. Endotoxins induce both iNOS and COX-2 activities which cause a considerable rise NO and PGE_2 concentrations. iNOS inhibition decrease excretion of NO as well as PGE_2. COX blockage leads to reduction of NOS activity (25).

Enhanced activation of lipooxygenases and increased content of leukotriens are characteristic of ulcerative colitis (7). A steep rise in the content of proinflammatory leukotriens – C_4, D_4, and B_4, released predominantly by macrophages, monocytes, eosinophils, and mast cells, is observed in experimental ulcerative colitis in the MMLI (31, 32). Under inflammatory conditions, leukotriens (D_4 and B_4) are the cause of increased expression and accumulation of membranous COX-2, beta-catenin, Bc1-2 and enhanced production of PGE_2 (28). Although in a number of research works a higher production of leukotriens in colitis has been reported (32-35), but it was not always accompanied by an increased production of PGE_2 (9). LeDuc *et al*. reported that intestinal release of both PGE_2 and LTB_4 into the colonic instillate was low in control rats, but they increased markedly in rats with ulcerative colitis. Treatment of colitis rats with indomethacin, alone or with AA-861, significantly reduced these evaluated PGE_2 levels. When AA-861 alone or with indomethacin was administrated a significant reduction in LTB_4 was observed. When the drugs were used in combination, both PGE_2 and LTB_4 values were below the levels seen with each drug alone (35).

L-NAME injected to intact (control) animals failed to exert any considerable influence on the content of leukotriens B_4 and C_4 in the MMLI. However, the effect of L-NAME at the background of ulcerogenic colitis led to reduction of leukotriene B_4 level and is accompanied by decrease in the destructive changes of the MMLI. Protective action of L-NAME was accompanied by a significant reduction of NOS-activity and generation of nitric oxide. Cytoprotective action of L-NAME is considered to be related to the reduction of mesenteric blood flow and diminished permeability of the capillaries (19).

Our investigations have determined that combined blockage of inducible NO-synthase, cyclooxygenase-2, and 5-lipooxygenase contributes to enhancement of cytoprotective mechanisms in the MMLI under conditions of ulcerative colitis modeled by means of acetic acid. Additionally, there is a noticeable reduction in the total activity of NO-synthase and in the activity of iNOS and eNOS. However, the dominating role in the development of inflammatory processes is played by NO and iNOS due to considerable activation of iNOS and excessive production of peroxinitrite. Injection of aminoguanidine resulted in attenuation iNOS activity by 48%, because of the combined blockage of iNOS and COX-2 which reduced activity of iNOS by 55%. Furthermore, the combined blockage of iNOS, COX-2 and 5-LOX caused a sharp decline of iNOS activity (by 73%) that was accompanied by a considerable decrease in the content of TBA products. Proceeding from the aforementioned, it can be stated that each of the systems, namely, NO/iNOS, PGE_2/COX-2, or leukotriens/5-LOX, being activated under inflammatory conditions, play their particular role in contributing to destructive changes in the MMLI, development of oxidative stress, and affect components of the antioxidant protection system.

Obtained results have proved that involvement of NO/iNOS, PGE_2/COX-2 and leukotriens/LOX in the mechanisms of development of ulcerative colitis provide substantiation for new approaches to the treatment of ulcerative colitis and for the development of pharmaceutical preparations possessing properties of combined blockage of iNOS, COX-2, and 5-LOX.

Under conditions of ulcerative colitis modeled by means of acetic acid, destructive damage of the MMLI, activation of NO-synthases, predominantly iNOS, accompanied by increase of NO content, enhancement of lipoperoxidation processes in the MMLI and a concomitant decrease of L-arginine content in blood were observed. iNOS blockage with aminoguanidine resulted in reduction of the activity of NO-synthases (eNOS – by 34%, iNOS – by 46%), decrease of NO content, reduction of lipoperoxidation processes, SOD and catalase activity in the MMLI, and increase of L-arginine concentration in blood. Non-selective COX inhibition with indomethacin led to the development of higer destructive changes in the MMLI, however indexes of oxidative stress and NOS system were at the level of colitis. 5-LOX inhibition with AA-861 revealed no significant effect on structure-hemorrhagic lesions in MMLI caused by the administration of 4% acetic acid. Concomitantly, iNOS activity was reduced, MDA content displayed a tendency to the decrease. Combined iNOS and COX-2 blockage displayed an additive effect in the mechanisms of regulation of the cytoprotective processes, activity of NO-synthases, and lipoperoxidation processes: activity of iNOS diminished, content of NO and MDA decreased, SOD activity in the MMLI reduced. Combined blockage of iNOS, COX-2, and 5-LOX manifested pronounced cytoprotective effect under conditions of modeled ulcerative colitis that was accompanied by a sharp decline in the activity of NO-synthases and indexes of oxidative stress. The obtained results substatiate the relevance of treatment of the inflammatory processes with the use of preparations capable of combined blockage of iNOS, COX-2, and 5-LOX.

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