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THE INDICES OF NITRIC OXIDE SYSTEM IN RATS WITH CARRAGEENAN-INDUCED ENTEROCOLITIS COMBINED WITH DIABETES MELLITUS

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Abstract

Background and aims: *Diabetes mellitus (DM) represents a considerable public health issue, being one of the major causes of morbidity and mortality in the modern societies. Chronic hyperglycemia is accompanied by significant physiological, biochemical, and histological changes, e.g. development of oxidative stress that affects the motor activity of the intestine. This study aimed to evaluate the indices of nitric oxide (NO) system in blood serum and colon tissue supernatant of rats with carrageenan-induced enterocolitis combined with streptozotocin-induced diabetes.* ***Material and methods:*** *The total NOS activity was determined by monitoring the rate of conversion of L-arginine into citrulline. The total quantity of NO metabolites was assessed by evaluating their amount, which included nitrite ions that were initially present in the sample (NO_2^-) and nitrate ions reducted to nitrites (NO_3^-).* ***Results:*** *We found a significant increase in total NOS activity in colon tissue of all experimental groups vs. control animals (54.8, 30.6 and 79.2 % respectively). The total content of NO metabolites in colon tissue of all experimental groups also significantly increased (2.8, 1.9 and 3.4 times respectively) compared to the control animals.* ***Conclusions:*** *We observed activation of nitroxydergic process in blood serum and colon tissue of rats with carrageenan-induced enterocolitis. Nitroxydergic processes markedly intensified in rats with carrageenan-induced enterocolitis combined with diabetes mellitus.*

key words: *nitric oxide; NO-synthase; enterocolitis; diabetes.*

Background and aims

Diabetes mellitus (DM) is a multifactorial metabolic disorder, characterized by hyperglycemia caused by insulin deficiency or insulin resistance [1]. DM is a significant public health issue, being one of the major contributors to morbidity and mortality in the modern societies [2]. The number of DM patients in the developed countries of the world is 2-6% of the total population. The latest International Diabetes Federation figures indicate that 415 million people (or 1 in 11 persons) have diabetes; this figure is expected to increase to 642 million or almost 10% of the general population by 2040 [3].

The prevalence of diabetes varies in different populations depending on the factors

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such as genetic and cultural background, ranging, for instance, from 10% in the Japanese to 40% in Pima Indians [4]. Some factors linked to the prevalence of diabetes in populations across the world are the region, level of economic development of the country, gender and age. Notably, about 75% of DM patients live in low and medium income countries [5].

Chronic hyperglycemia causes significant physiological, biochemical, and histological in the affected organisms [6]. In physiological conditions, constitutive forms of NO synthases (cNOS), endothelial (eNOS) and neuronal (nNOS) expressed in the mucous and muscular shell of the digestive organs, are calcium-dependent and ensure biosynthesis of insignificant quantities of nitric oxide, which regulates secretion, motility, absorption, blood flow, supports structure and function of the mucosal barrier, and the process of intercellular integration [7]. DM increases the activity of inducible NO-synthase (iNOS), which is localized in the endothelial, epithelial, immune cells and smooth myocytes of the intestine [7], while it decreases the expression of nNOS in NO-ergic neurons [9]. Hyperglycemia induces the development of oxidative stress and changes activity of antioxidant protection enzymes, affecting motor activity of the intestine [10].

This study tracked changes to the indices of nitric oxide system in blood serum and colon tissue supernatant of rats with carrageenan-induced enterocolitis combined with diabetes mellitus.

Material and methods

The experimental animals, white nonlinear mature male rats (n=48) were kept on a standard diet at the animal facility of I. Horbachevsky Ternopil State Medical University. Animal treatment and all experimental procedures were done in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [11].

Four control and experimental groups were set up: control group (n=12); animals with diabetes mellitus (n=12); animals with carrageenan-induced enterocolitis (n=12); and animals with diabetes mellitus and carrageenan-induced enterocolitis (n=12).

Diabetes mellitus (DM) was induced by a single intraperitoneal administration of streptozotocin (Sigma Aldrich, USA, at a dose of 60 mg/kg body weight) [12]. Immediately prior to the administration, streptozotocin was dissolved in 0.1 M citrate buffer (*p*H 4.5). The control group received a corresponding amount of citrate buffer. Animals with glucose level of at least 10.8 mmol/L in 2 weeks after streptozotocin administration were included in the DM experimental groups.

Carrageenan-induced enterocolitis (CEC) was modeled by giving the animals free access to 1.0% solution of carrageenan in drinking water for 1 month [13]. We used blood serum and colon homogenate of the rats to study the markers. Concentration of glucose in blood serum was determined using a glucometer (MediSense UK Ltd., Great Britain).

NOS activity assay in the supernatant of colon homogenate was performed by monitoring the rate of conversion of L-arginine into citrulline [14]. Total protein was measured by Lowry assay [15].

The total concentration of NO^{2-} + NO^{3-} was estimated by adding the quantity of nitrite ions that were previously present in the sample (NO^{2-}) to the amount of nitrate ions reduced to nitrites (NO^{3-}) [16].

The data were processed using the software package Statistica 6.1 for Windows. Intergroup comparisons were performed using Mann–Whitney U test. The median (Me) and

interquartile range (IQR [Q25-Q75]) were used. Differences with p-value of ≤0.05 were accepted as statistically significant.

Results

Total NOS activity in the rat colon tissue supernatant of was significantly greater in all experimental groups as compared to the control group (54.8, 30.6 and 79.2% respectively), being the largest in the rats with CEC combined with DM (Table 1).

Table 1. The indices of nitric oxide system in blood serum and colon homogenate supernatant of the control and experimental groups, Me [Q25-Q75]

Group of animals	Control (n=12)	DM (n=12)	CEC (n=12)	DM+CEC (n=12)
Blood serum				
NO_2^-+NO_3^-, μmol/L	38.2 [32.8; 46.1]	81.7* [76.1;84.4]	54.7* [50.8; 58.2]	101.2* [95.3; 104.1] p_1<0.05 p_2<0.002
Supernatant of colon homogenate				
NO_2^-+NO_3^-, μmol/L	15.9 [12.3; 17.8]	44.5* [40.3;48.9]	30.0* [25.8; 36.4]	54.7* [50.5; 57.3] p_1<0.05 p_2<0.01
Total NOS, pmol of L-citrulline/min per 1 mg of protein	47.1 [39.8; 52.2]	72.9* [65.7; 76.7]	61.5* [55.1; 67.3]	84.4* [76.7; 86.2] p_1<0.05 p_2<0.01

Notes: * - the difference between the control and the experimental group is statistically significant (p≤0.05-0.001); p_1 – the reliability coefficient between groups 2 and 4; p_2 – the reliability coefficient between groups 3 and 4.

The total concentration of NO_2^-+ NO_3^- in blood serum of animals with DM was significantly (2.1 times) higher, with CEC 43.2 % higher and with CEC combined with DM 2.6 times higher compared to the control group. Comparison of the total NO_2^-+ NO_3^- concentration in blood serum and colon tissue supernatant was of particular interest. It is evident that disrupted NO production had directionality towards the oxidative burst. Thus, the total concentration of NO_2^-+ NO_3^- in colon tissue supernatant of rats with diabetes mellitus was significantly, 2.8 times, higher, rats with carrageenan-induced enterocolitis 1.9 times higher and rats with carrageenan-induced enterocolitis combined with diabetes mellitus 3.4 times compared to the control group.

Discussion

Nitric oxide (NO) is a messenger molecule involved in a number of physiological pathways in different cells and tissues. It is formed by enzymatic oxidation of L-arginine by cytochrome P450-like hemoproteins and NO-synthases (NOS) [14]. Three isoforms of this enzyme are described: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) or macrophagal [17]. Pathophysiology studies revealed that NO can act as an inhibitory neurotransmitter messenger in human intestine [18]. NO plays a significant role in diverse processes such as nervous impulses modulation, regulation of motility and secretion, and cytoprotection of the large intestine [19].

In the mucous membrane of the large intestine, eNOS is primarily detected in the

endothelial and epithelial cells, smooth muscles, thrombocytes and T-cells. While nNOS is mainly localized in the central and peripheral nerves, it is also found in myocytes, epitheliacytes, mastocytes, and neutrophilic white blood cells [19].

NO is involved in several mechanisms that regulate functional state of smooth muscles of the intestine. NO is a lipophilic molecule, and thus easily diffuses through cell membranes into the neighboring cells (for instance, from endothelial cells to myocytes). There, cyclic guanosine monophosphate decreases free calcium concentration and activates myosin light chain kinase [20]. There are three main mechanisms of NO signaling. The first one involves activation of soluble guanylate cyclase (sGC) through binding to its heme group (thus forming a Fe^{2+}-nitrosyl complex); this leads to the formation of cGMP, which in turn stimulates protein kinase G. The second mechanism is S-nitrosylation: active forms of nitrogen reversibly attach to nitrosilate thiol groups of target protein cysteins. The final mechanism involves formation of peroxynitrite (ONOO-) with subsequent nitration of the tyrosine and tryptophan residues in proteins, leading to the engagement of mitogen-activated protein kinases, protein kinase C isoforms, transcription factor NF-κB, etc., in the process of signaling [21,22].

Normally, endogenous NO inhibits the motility of digestive organs [23]. Increasing the activity of iNOS leads to significant reduction in motor activity [24], whereas inhibition of NO-ergic blockage of the smooth muscle in the large intestine causes its intensification [8].

Chronic enterocolitis causes harmful changes in the intestinal mucosa associated with the increased number of reactive oxygen species, increased NO synthesis, iNOS expression in the epithelial cells, infiltration of macrophages and neutrophils into the damaged mucous membrane.

Hridneva (2003) notes that chronic enterocolitis impairs endothelial functions. This is manifested in the activation of free radical oxidation processes and\or decrease in the activity of the antioxidant system, which explains the excessive production of ROS [25].

Our previous studies also have shown that the number of ROS in rats with DM increased 3.0 times, in rats with CEC by 2.0 times, and in rats with combined pathology 3.4 times compared to the control values [1].

Our studies show that under streptozotocin-induced hyperglycemia, the total activity of NOS and total concentration of NO_2^-+ NO_3^- increased potentially causing decreased smooth muscle tone and disrupting the motor and evacuation functions of the large intestine.

Hyperglycemia accompanied by muscle relaxation, and with rising of iNOS activity in the muscular membrane, increases NO concentration. It should be noted, that some nitrogen oxide synthesized by iNOS interacts with the superoxide radical, which leads to the formation of ONOO-. This causes endothelial dysfunction, nitrosylation of cytoplasmic proteins, and activates the processes of lipoperoxidation. Increased activity of iNOS during muscles relaxation is also associated with increased formation of H_2O_2 and the impact of pro-inflammatory cytokines that activate expression of mRNA iNOS [10]. Therefore, TNF-α is very important in the development of both DM and CEC. The mechanisms by which TNF-α initiates and enhances inflammation in the intestine are very complex and have so far been poorly understood. A direct negative effect of TNF-α is associated with damage to enterocytes, which leads to increased epithelium permeability. In addition to direct effects on the integrity of the intestinal epithelial barrier, TNF

is a powerful inducer of matrix metalloproteinases in the stromal cells of the intestine; it induces production of keratinocyte growth factor, leading to hyperplasia of crypts and causing increased expression of major histocompatibility complex class II antigens [26].

In case of diabetes mellitus, the production of ROS and peroxynitrite, which cause the development of oxidative and nitrosative stress, is increasing. This may be due to auto-oxidation of glucose, enhanced glycosylation of cellular proteins, activation of the polyol pathway, and increased formation of superoxide radical in the respiratory chain of mitochondria [10]. Moreover, in diabetes mellitus, advanced glycation end products have damaging effect on cell and tissue DNA, accumulating in ganglia, villi and membranes of enterocytes of the intestine [27].

Study limitation

This study was carried out on a sample of small size. It is therefore essential to validate our findings with a larger sample size to determine the features of nitroxydergic processes more completely.

Conclusions

We determined that, in rats with modeled carrageenan-induced enterocolitis, nitroxydergic process was activated through significant increase in total concentration of NO_2^-+ NO_3^- and total NO synthases activity in blood serum and colon homogenate supernatant. A pronounced intensification of nitroxydergic processes was observed in rats with carrageenan-induced enterocolitis combined with diabetes mellitus.

Conflict of interest statements. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. **Marushchak M, Lisnianska N, Krynytska I, Chornomydz I.** The mechanisms of apoptosis initiation in rats with chronic enterocolitis combined with streptozotocin-induced diabetes. *Georgian Med News* 9(270):125-130, 2017.

2. **Guja C, Miulescu RD.** Heart failure in type 2 diabetes – the "forgotten" complication. *Rom J Diabetes Nutr Metab Dis* 25(2): 123-130, 2018.

3. **Demikhova N, Chernatska O, Mazur T et al.** Markers of cardiovascular complications in patients with type 2 diabetes mellitus and arterial hypertension. *Bangladesh Journal of Medical Science* 17(2): 319-322, 2018.

4. **Riyaz MSU, Rather MK, Koul PA.** Diabetes in Immigrant Tibetan Muslims in Kashmir. *North India J Immigrant Minority Health* 5(1): 18–30, 2017.

5. **Hwang CK, Han PV, Zabetian A, Ali MK, Narayan KMV.** Rural diabetes prevalence quintuples over twenty-five years in low- and middle-income countries: a systematic review and meta-analysis. Diabetes Res Clin Pract 96(3): 271–285, 2012.

6. **Guzyk MM, Dyakun KO, Yanytska LV et al.** Inhibitors of Poly(ADP-Ribose)Polymerase-1 as Agents Providing Correction of Brain Dysfunctions Induced by Experimental Diabetes. *Neurophysiology* 49(3): 183-193, 2017.

7. **Sklyarov AY, Detsyk OI.** The changes of the activity of NO-synthases and oxidative processes under conditions of 5-HT receptors activation in the stomach and large intestine in streptozotocin-induced diabetes mellitus. *Fiziol Zh* 58(5): 22-27, 2012

8. **Onori L, Aggio A, D'Alo S et al.** Role nitric oxide in the impairment of circular muscle contractility of distended, uninflamed mid-colon in TNBS-induced acute distal colitis in rats // *Word. J. Gastrointestinal* 11(36): 5677–5684, 2005.

9. Zandecki M, Vanden Berghe P, Depoortere I et al. Characterization of myenteric neuropathy in the jejunum of spontaneously diabetic BB–rats. *Neurogastroenterol Motil* 20(7): 818–828, 2008.

10. van den Oever I, Raterman HG, Nurmohamed MT, Simsek S. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators Inflamm* 2010:792393, 2010

11. Council of Europe Strasbourg. European convention for the protection of vertebrate animals used for experimental and other scientific purposes 123: 52, 1986

12. Ordodi VL, Paunescu V, Ionac M, et al. Indomethacin inhibits thymic involution in mice with streptozotocin-induced diabetes. *Artificial organs* 32(1): 66–70, 2008.

13. Moyana TN, Lalonde JM. Carrageenan-induced intestinal injury in the rat-a model for inflammatory bowel disease. *Ann Clin Lab Sci* 20(6): 420–426, 1990.

14. Fafula RV, Iefremova UP, Onufrovych OK, et al. Alterations in arginase-NO-synthase system of spermatozoa in human subjects with different fertility potential. *J Med Biochem* 37(2): 134–40, 2018.

15. Lowry OH, Rosebrough NG, Farr AL, Randall RC. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1): 265–75, 1951.

16. Kozar VV, Kudrya MY, Ustenko NV, Nikishina LE, Kravchenko SV. Determination of the concentration of metabolites of nitric oxide in serum. *Laboratory diagnostics* 3(53): 14–16, 2010

17. Strutynska NA, Kotsiuruba AV, Budko AY, Mys LA, Sagach VF. Mitochondrial dysfunction in the aging heart is accompanied by constitutive NO-synthases uncoupling on the background of oxidative and nitrosative stress. *Fiziol Zh* 62(2): 3–11, 2016.

18. Tomita R, Tanjoh K. Role of Nitric Oxide in the Colon of Patients with Ulcerative Colitis. *World J. Surg* 22: 88–92, 1998.

19. Sklyarov AY, Panasyuk NB, Fomenko IS. Role of nitric oxide-synthase and cyclooxygenase/lipo-oxygenase systems in development of experimental ulcerative colitis. *J Physiol Pharmacol* 62(1): 65-73, 2011.

20. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 33(7): 829–37, 2012.

21. Danylovych GV, Bohach TV, Danylovych YuV. The biosynthesis of nitric oxide from L-arginine. Nitric oxide formation features and its functional role in mitochondria. *Ukr Biochem J* 90(1): 3–24, 2018.

22. Krynytska I, Marushchak M. The indices of nitrogen (II) oxide system in case of experimental hepatopulmonary syndrome. *Ukr Biochem J* 90(5), 92-98 2018.

23. Meile T, Glatzle J, Habermann FM, Kreis ME, Zittel TT. Nitric oxide synthase inhibition results in immediate postoperative recovery of gastric, small intestinal and colonic motility in awake rats. *Int J Colorectal Dis* 2: 121–129, 2006.

24. Qu ZD, Thacker M, Baqyanszki P, Bagyánszki M, Epstein ML, Furness JB. Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res* 334(2): 147–161, 2008.

25. Hridneva SV. The role of nitric oxide and lipoperoxidation processes in the development of chronic non-ulcerative colitis. *Modern gastroenterology* 2: 43–46, 2003 (in Russian).

26. Mueller C. Tumor necrosis factor in mouse models of chronic intestinal inflammation. *Immunology* 105(1): 1–8, 2002.

27. Babu K, Babu S, Walker J, Walker AB, Pappachan JM. Gastrointestinal complications of diabetes mellitus. *World J Diabetes* 4(3): 51–63, 2013.