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STUDY OF SUPEROXIDE- AND NO-DEPENDENT PROTECTIVE MECHANISMS OF N-ACETYLCYSTEINE AND LOSARTAN IN RAT'S AORTA AND LIVER UNDER STREPTOZOTICIN-INDUCED TYPE 1 DIABETES MELLITUS

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Мета. Дослідити супероксид- та NO-залежні механізми дії N-ацетилцистеїну (NAC) та лозартану (LOS) в аорті та печінці щурів із індукованим цукровим діабетом 1 типу (ЦДІ).

Методи. Щури із індукованим ЦДІ отримували NAC (1,5 г/кг), LOS (20 мг/кг) або їх комбінацію (NAC+LOS) протягом 4-х тижнів.

Результати. Швидкість генерування CP мітохондріями аорти нелікованих тварин із ЦДІ була достовірно вищою порівняно до групи контролю, в той час рівень NO знижувався. Продукція CP та NO значно підвищувалася у печінці тварин із ЦДІ. Підвищувалася екскреція з сечею маркера окисного пошкодження ДНК -8-оксогуаніну у щурів із ЦДІ. Всі досліджувані фармакологічні схеми сприяли достовірному зниженню швидкості генеруванні CP та NO мітохондріями аорти та печінки; рівень NO був значно нижчий у тканині печінки щурів порівняно із групою ЦДІ. Тільки NAC статистично значуще відновлював рівень NO в аорті діабетичних щурів. Введення NAC/ LOS або NAC+LOS сприяло збільшенню рівня 8-оксогуаніну порівняно із групою ЦДІ.

Висновки. Корекція NAC та LOS чи їх комбінації пов'язана із захистом клітин аорти та печінки у тварин із ЦДІ від токсичної дії CP, перешикоджаючи мітохондріальній дисфункції і послідуєчого руйнування ДНК, що свідчить про кардіопротекторні ефекти та загальне метаболічне покращення

Ключові слова: стрептозотозин, супероксид, аорта, печінка, щури, N-ацетилцистеїн, лозартан

1. Introduction

Diabetes mellitus (DM) is the most widespread endocrinological disease, and its complications, especially diabetic cardiomyopathy (DC) decreases quality of patients' lives and often results in fatal outcome. According to the WHO's data, 2.2 million deaths induced by cardiovascular complications in case of excessive hyperglycemia were registered at the beginning of 2012 [1, 2].

2. Formulation of the problem in a general way, the relevance of the theme and its connection with important scientific and practical issues

One of the basic mechanisms of DC development is hyperglycemia-induced oxidative stress (OS) that includes excessive formation of reactive oxygen species (ROS) – superoxide radicals (SRs) as well as antioxidant system defense depletion. SRs have significant implication in the intracellular signaling and mediate various cellular functions, including activation of transcription factors, kinases and ion channels. Furthermore, increased generation of SRs initiates activation of 5 basic signaling ways including in the pathogenesis of diabetic complications, such as: polyol pathway, increased formation of

advanced glycation end products (AGEs), elevated expression of the receptor for AGEs and its activating ligands; activation of protein kinase C (PKC) isoforms; hyperactivity of the hexamine pathway [3, 4].

Additionally, overproduction of SR initiate formation of the most aggressive hydroxyl radicals (HO[•]) and reactive nitrogen species (RNS) in reaction with NO in form of toxic peroxynitrite ONOO⁻, especially under hypoxia condition [5].

3. Analysis of recent studies and publications in which a solution of the problem and which draws on the author

It has been proved that hyperglycemia-induced accumulation of mitochondrial ROS is associated with disturbance of transmembrane potential that directly induces opening of the mitochondrial pore and results in mitochondrial dysfunction and DC development [6]. Therefore, the search for new pharmacological schemes in DC treatment which minimize oxidative damage of cells has become an actual problem.

Lately, the effectiveness of synthetic antioxidant N-acetylcysteine (NAC) for correction of cardiovascular

diseases in experiments *in vivo* has been actively discussed [7, 8]. The recent systemic review has also pointed out that NAC has cardioprotective potential in case of hyperglycemia-induced OS [9].

The European Society of Cardiology (ESC Guidelines, 2013) have recommended the renin-angiotensin-aldosterone system blockers, including angiotensin II (AT II) type 1 receptor blockers for patients with DM and decreased left ventricular ejection fraction [10]. In addition, cardioprotective properties of losartan (LOS) in patients with heart failure have been dynamically highlighted [11, 12].

4. Allocation of unsolved parts of the general problem, which is dedicated to the article

Moreover, one can find quite fragmental information about the possible SR- and NO-dependent mechanisms of cardioprotection of NAC as well as its multiple administration in correction of DC. However, the approach of complex action of NAC and LOS in case of this pathology hasn't been studied yet.

5. Formulation of goals (tasks) of the article

Therefore, the aim of this study was to investigate the efficacy and redox-dependent mechanisms of protection in case of various pharmacological schemes including NAC and LOS in aorta and liver of rats with experimental type 1 diabetes mellitus.

6. Statement of the basic material of the study (methods and objects) with the justification of the results

Wistar rats (200–250 g) were procured at our institution animal house facility in Bogomolets National Medical University (Kiev, Ukraine) and housed in an air-conditioned room at a temperature of 25 ± 2 °C and relative humidity from 45 % to 55 % under 12-h light: 12-h dark cycle. The animals had free access to food pellets except when starvation was required. Water was provided *ad libitum*. The experimental protocol was approved by Law of Ukraine N 3447-IV about "Animal protection from cruel treatment" and in accordance with the rules and guidelines of the Council Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes [13].

Type 1 Diabetes mellitus was induced by intraperitoneal injection of streptozotocin (STZ, Sigma, USA) at the dose of 50 mg/kg dissolved in 0.1 M citrate buffer pH 4.5 [14]. After 72 hours of STZ injection, the blood glucose level was measured with the using of a glucometer Accu-Chek Performa Nano (Roche Diagnostics, Germany). Only rats, which had blood glucose level higher than 15mmol/L were used for the study.

Animals were randomly divided into 5 groups: 1st - Control (n=6; intact rats); 2nd - DM1 (n=6; untreated diabetic control rats supplemented with 0.9 % normal saline *per os*); 3rd - NAC (n=6; diabetic rats treated with N-acetylcysteine at a dose 1.5g/kg *per os*); 4th - LOS (n=6; diabetic rats treated with losartan at a dose 20mg/kg *per os* as reference drug); 5th - NAC+LOS

(n=6; diabetic rats treated with combination of NAC and LOS *per os*). One week after induction of diabetes investigated pharmacological agents were administrated to diabetic treated groups for 4 weeks.

Molecular marker of oxidatively damaged DNA in urine - 8-oxoguanine (8-oxoG) was determined by analysis of ultraviolet specters of eluates after their solid-phase extraction on the column (Merck, Germany) spectrophotometrically. Sample aliquot of urine with $A_{280} = 50$ µg/ml were brought on the column which was previously washed with 5 ml of ethanol, 10 ml of distilled water and balanced with 10 ml 50 mM KH_2PO_4 (pH 7.5). Elution of 8-oxoG was provided by 15% methanol with 50 mM KH_2PO_4 (pH 5.5) [15].

The rate of superoxide generation by mitochondria of aorta cells was determined by electron paramagnetic resonance (EPR) method using a spin trap (2,2,6,6-tetramethyl-4-oxypiperidine) at room temperature in a special paramagnetic pure quartz cuvette. The level of NO in the aorta tissue was investigated by EPR using the Spin Traps technology (spin trap - diethyldithiocarbamates (Sigma, USA) at the temperature of 77 K) [16].

The data was expressed as mean \pm standard error of mean (SEM). One way analysis of variance (ANOVA) was applied to test the significance of difference between average parameters of different groups and multiple comparisons were determined by post hoc Dunnett's test. The statistical analysis was performed using IBM SPSS Statistics Base version 22.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

During 5-week experiment, the rate of SR generation by mitochondria of aorta in group of animals with type 1 diabetes mellitus was significantly higher than corresponding values of control group (0.53 ± 0.13 nm/ g tissue \cdot min vs 0.21 ± 0.05 nm/ g tissue \cdot min, $p < 0.01$). The level of NO generation in aorta diabetic animals was decreased compare to control rats (0.56 ± 0.08 nm/ g tissue \cdot min vs 0.80 ± 0.05 nm/ g tissue \cdot min, $p < 0.01$). The results are shown in Fig. 1–3.

Comparing changes of free radical rate generation by mitochondria of aorta after 4 weeks of pharmacological correction in diabetic rats, it was determined statistically significant decrease the rate of SR generation compare to type 1 diabetes mellitus group ($p < 0.01$; Fig. 2, a). Administration of NAC resulted in the 2-fold decrease the rate of SR generation (0.28 ± 0.08 nm/ g tissue \cdot min, $p < 0.01$). Treatment with LOS caused 1.8-fold decrease the rate of SR generation (0.32 ± 0.03 nm/ g tissue \cdot min, $p < 0.01$). The combination therapy with NAC and LOS resulted in the 2.2-fold decrease the rate of SR generation (0.25 ± 0.06 nm/ g tissue \cdot min, $p < 0.01$) compared to diabetic control group DM1 (0.53 ± 0.13 nm/ g tissue \cdot min). The results are shown in Fig. 1 (2) and Fig. 2, a.

As shown in Fig. 2, b, only NAC exposure caused significant increase NO level in aorta of diabetic rats (0.79 ± 0.09 nm/g tissue \cdot min vs 0.56 ± 0.08 nm/g tissue \cdot min, $p < 0.01$) compared to type 1 diabetes mellitus group; however, LOS and combination treatment showed results approximated to control (0.80 ± 0.05 nm/g tissue \cdot min).

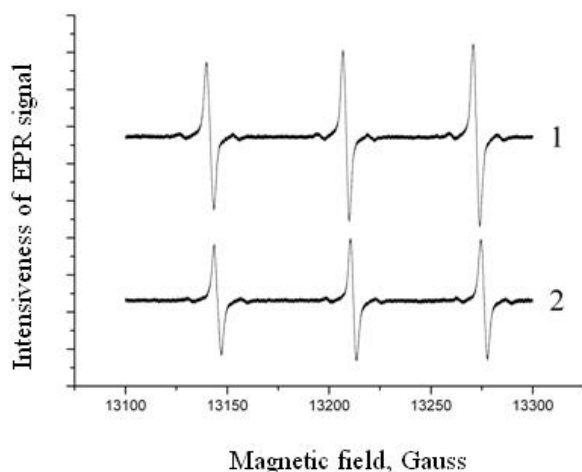


Fig. 1. Effects of N-acetylcysteine (NAC) and losartan (LOS) on the specters of electron paramagnetic resonance of superoxide radical in mitochondria of aorta cells. Untreated diabetic rats (1); diabetic rats treated with NAC (1.5g/kg *per os*) and LOS (20mg/kg *per os*) for 4 weeks (2)

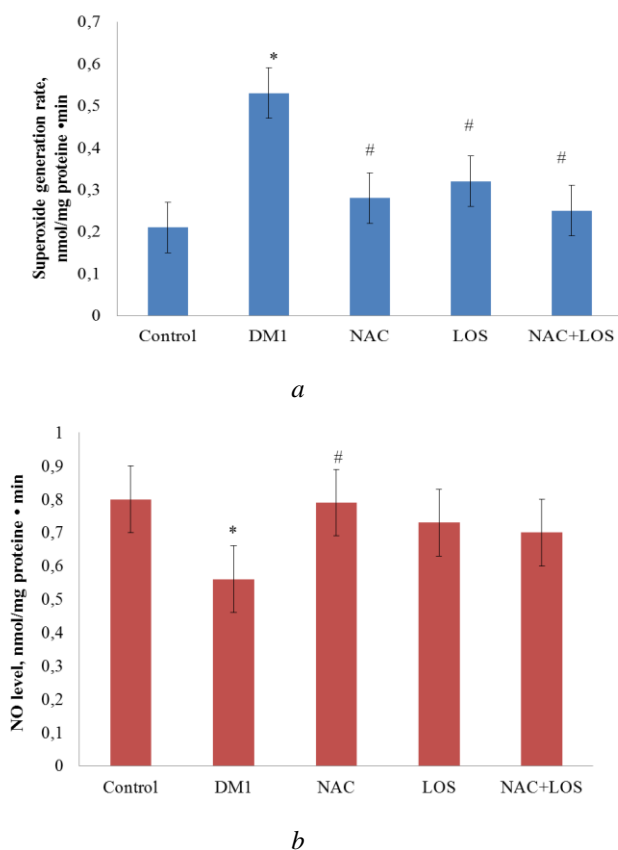


Fig. 2. Effects of N-acetylcysteine (NAC) and losartan (LOS) on the rate of superoxide radical generation and NO level in mitochondria of aorta: *a* – the rate of superoxide radical generation; *b* – NO level. Control (intact rats); DM1 (untreated diabetic control rats supplemented with 0.9% normal saline *per os*); NAC (diabetic rats treated with NAC 1.5g/kg *per os*); LOS (diabetic rats treated with LOS 20mg/kg *per os*); NAC+LOS (diabetic rats treated with combination of NAC and LOS *per os*). NAC / LOS or NAC+LOS were administered for 4 weeks. All values are expressed as mean \pm SEM. *n*=6 per group. *vs Control, *p*<0.01; #vs DM1, *p*<0.01

In the liver tissue of type 1 diabetes mellitus group were detected follow changes (Fig. 3): the rate of SR generation was increased by 2.8 times more than in control group (0.39 ± 0.07 nmol/g tissue •min vs 1.09 ± 0.14 nmol/g tissue •min, *p*<0.01) as well as twice increase of NO level (1.75 ± 0.16 nmol/g tissue •min vs 0.89 ± 0.07 nmol/g tissue •min, *p*<0.01).

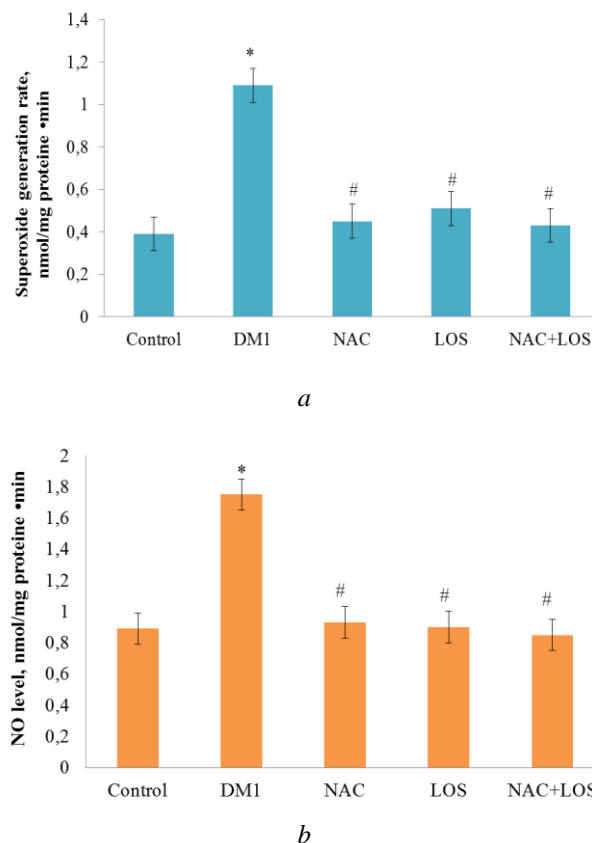


Fig. 3. Effects of N-acetylcysteine (NAC) and losartan (LOS) on the rate of superoxide radical generation and NO level in mitochondria of liver cells: *a* – the rate of superoxide radical generation; *b* – NO level. Control (intact rats); DM1 (untreated diabetic control rats supplemented with 0.9% normal saline *per os*); NAC (diabetic rats treated with NAC 1.5g/kg *per os*); LOS (diabetic rats treated with LOS 20mg/kg *per os*); NAC+LOS (diabetic rats treated with combination of NAC and LOS *per os*). NAC / LOS or NAC+LOS were administered for 4 weeks. All values are expressed as mean \pm SEM. *n*=6 per group. *vs Control, *p*<0.01; #vs DM1, *p*<0.01

In the groups of pharmacological correction were determined statistically significant changes against rate of SR generation and NO in mitochondria of liver cells which were relative to control group (*p*<0.01). As shown in Fig. 3, *a* NAC significantly reduced SR generation by 2.4 times less than in type 1 diabetes mellitus group (0.45 ± 0.08 nmol/g tissue •min vs 1.09 ± 0.14 nmol/g tissue •min; *p*<0.01). Exposure to LOS significantly reduced SR generation by 2.1 times less than in type 1 diabetes mellitus group (0.51 ± 0.08 nmol/g tissue •min vs 1.09 ± 0.14 nmol/g tissue •min; *p*<0.01). Treatment with combination of NAC and LOS showed significant 2.5-fold decrease of SR generation compared to diabetic control type 1 diabetes mellitus group (0.43 ± 0.05 nmol/g tissue •min vs 1.09 ± 0.14 nmol/g tissue •min; *p*<0.01).

As shown in Fig. 3, *b* the level of NO was decreased in all of researched groups of pharmacological correction (NAC, LOS and NAC+LOS) by 1.9–2 times less than in non-treated diabetic rats ($p < 0.01$).

The marker of oxidatively damaged DNA, 8-oxoG, significantly raised in urine of diabetic rats by 5 times more than in control group (0.87 ± 0.11 nm/day/g vs 0.18 ± 0.06 nm/day/g; $p < 0.01$). After pharmacological intervention with NAC / LOS or NAC+LOS the level of 8-oxoG was significantly increased in compare to diabetic control rats of type 1 diabetes mellitus ($p < 0.01$). Minimum parameters were obtained for diabetic rats treated with NAC+LOS (1.34 ± 0.17 nm/day/g vs 0.87 ± 0.11 nm/day/g; $p < 0.01$). Maximum one were obtained for LOS group (2.3 ± 0.26 nm/day/g) and NAC (1.90 ± 0.27 nm/day/g) which were by 2.6 and 2.2 times more than in type 1 diabetes mellitus group respectively ($p < 0.01$). The results are shown in Fig. 4.

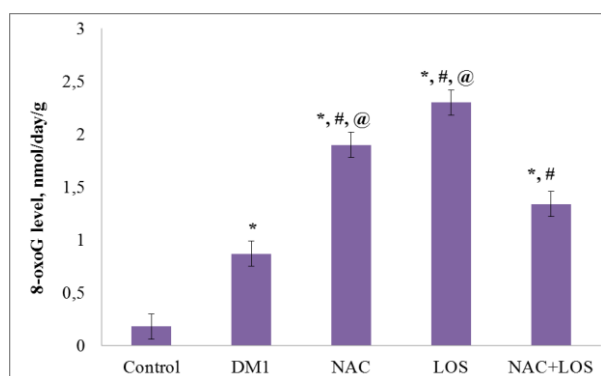


Fig. 4. Changes of urinary excretion of 8-oxoG in untreated diabetic rats and after pharmacological correction. Control (intact rats); DM1 (untreated diabetic control rats supplemented with 0.9% normal saline *per os*); NAC (diabetic rats treated with NAC 1.5g/kg *per os*); LOS (diabetic rats treated with LOS 20mg/kg *per os*); NAC+LOS (diabetic rats treated with combination of NAC and LOS *per os*). NAC/ LOS or NAC+LOS were administered for 4 weeks. All values are expressed as mean \pm SEM. $n=6$ per group. *vs Control, $p < 0.01$; #vs DM1, $p < 0.01$; @ vs NAC+LOS, $p < 0.01$

Consistent with the literature data the current study has shown that SRs are the major sources of cardiovascular complications of type 1 diabetes mellitus [3]. Mitochondrial damage is related to ROS formation (especially SRs) and plays an important role in the development of DC, which results in myocardial cell death, hypertrophy, fibrosis, abnormalities of calcium homeostasis, and endothelial dysfunction [4]. The rate of SR generation increases in mitochondria of cells which are sensitive to high glucose concentrations and its metabolites in animals with STZ-induced type 1 diabetes mellitus [4]. Hyperglycemia induces SR generation in the heart primarily by disruption of the activity of electron transport chain (ETC) complex enzymes [5]. Thus, in cells with high glucose concentration, pyruvate is oxidized in the Krebs cycle, enhancing the electron donor flux (NADH and FADH₂) into the ETC, blocking ETC inside the Complex III, causing reverse of electron flow to coenzyme Q, which donates electron on molecular oxygen, generating SR in such way [5, 17, 18].

Mitochondria provide organism with energy, but in case of excessive mitochondrial OS, autophagy is

activated and apoptosis is activated via mitochondrial DNA damage [19]. Consequently, the major cause of cardiac dysfunction has been considered ROS which initiate apoptosis of cardiomyocytes and endothelial cells via mitochondrial OS [20]. Moreover, ROS directly initiate fibrosis through proliferation of fibroblasts and collagen synthesis, contributing to DC development [21]. In the current study, our results showed 2.5-fold increase the rate of generation of SRs by mitochondria of aorta cells along with 1.4-fold reduce of NO level in STZ-induced diabetic rats compared to control group ($p < 0.01$). The regulation balance of ROS and eNOS/NO have been considered as an essential part in DC progression. The authors of the research [22] have shown increase the SR production accompanied both with decrease phosphorylation of eNOS and attenuation of NO level in myocardium tissue in case of STZ-induced type 1 diabetes mellitus of diabetic animals.

Obtained findings suggest that the early 4-week treatment with various pharmacological schemes of NAC, LOS and their combination have positive action against ROS formation in aorta cells, significantly decreasing the rate of SR generation by 1.8-2.2 times less than in diabetic control group of type 1 diabetes mellitus ($p < 0.01$).

M. Sleem et al. have explained the antioxidant (AO) properties of selective antagonist of AT II type 1 receptors LOS as well as its combination with L-carnitine by reducing the overproduction of SR in aorta, prevented diabetes-induced endothelial dysfunction and risk of cardiovascular complications [23]. Co-administration of losartan and vanillic acid reduced OS-induced ischemia-reperfusion in isolated myocardium [24]. Losartan treatment suppressed the excessive NO and lipid peroxidation production, decreasing generation of toxic peroxynitrite in diabetic rats' pancreas [25]. Due to the results of previous work, the quantum-chemical calculations have confirmed that LOS has property of quick scavenging of free radicals and donating of hydrogen proton with minimum energy consumption due to its hydroxymethyl side chain, which explain its AO activity [26].

NAC is well-known synthetic mucolytic agent with AO properties due to its SH-group and ability to synthesize reduced glutathione. Lately, the possibility of NAC usage in correction of cardiovascular complications in case of diabetes mellitus has been actively discussed [9]. In experimental works, NAC increased activity of aldehyde dehydrogenase 2 -a key cardioprotective enzyme susceptible to oxidative inactivation in diabetic heart [8]. Moreover, NAC attenuated diabetic myocardial ischemia reperfusion injury through inhibiting excessive autophagy [19]. The other study [21] indicated that NAC treatment in diabetes effectively protects from DC through inhibiting the ROS production and fibrosis. It was shown that antioxidant NAC attenuated myocardial dysfunction and myocardial I/R injury by improving Cav-3/eNOS signaling [22].

Moreover, the study of NAC combinations with other pharmacological agents in case of DC has been considered. NAC and allopurinol synergistically reduced myocardial ischemia reperfusion injury in diabetes [27] through mechanisms of up-regulating phosphatidylinositol 3-kinase/Akt and Janus kinase 2/signal transducer and

activator of transcription-3 (JAK2/STAT3) pathways [28]. Other combination with multiple antioxidants decreased the expression of ROS-generating enzymes xanthine oxidase, monoamine oxidase-A along with 5-Lipoxygenase mRNA and/or protein expression, significantly attenuated cardiac dysfunction in diabetic rats [29].

Regarding NO level restoration in the current study, the results showed that the most comparable to control animals was group of diabetic rats treated with NAC ($p < 0.01$), thereby revealing the endothelial protective effect in experimental animals. The authors of the study [22] have pointed out that NAC recovers myocardial function via restoring Cav-3/eNOS signaling and stimulation of NO levels.

The current study was also aimed to investigate redox balance in liver of diabetic animals. So, in mitochondria of hepatocytes of diabetic animals were detected increasing the rate of SR generation and NO by 2.8 and 2.0 times more than in control rats respectively. Such difference in the formation of NO pools in aorta and liver tissue can be associated with enhancing of oxidative carbonylation of mitochondrial proteins in liver cells [18]. It is well known that NO is synthesized by different isoforms of NOS. The authors of the study [18] have experimentally proved that concentration of NO differentially increased in various tissues in type 1 diabetes mellitus which was also supported by increased NOS protein level in the mitochondria from diabetic animals.

Regarding influence of cardioprotective agents on the process of OS in liver, were detected that all of the elucidated schemes were demonstrated positive action on the reducing NO and SR formation in mitochondria of hepatic cells in rats with STZ-induced type 1 diabetes mellitus. Administration of NAC and its combination (no worse than reference agent LOS) significantly lowered the rate of SR generation in liver tissue 2.4 and 2.5 times against group of type 1 diabetes mellitus respectively ($p < 0.01$). Similar results were obtained in others studies which confirmed hepatoprotective effect of NAC [30, 31].

It is commonly known that SR can damage mitochondrial DNA because of it sensitive target. Therefore, ROS can initiate damage of DNA chain in form of production 8-hydroxydeoxyguanosine (8-OHdG) and 8-oxoguanine (8-oxoG) which are sensitive markers of OS-induced damage of DNA excreted in urine [32]. In the current study was indicated 5-fold elevating of 8-oxoG in urine of diabetic rats compared to control group ($p < 0.01$). The displacing balance in overproduction of ROS and RNS leads to downregulation 8-oxoG-DNA glycosylase and increase accumulation of 8-OHdG and 8-oxoG in DNA, initiating mechanisms of biomolecules damage, dysregulation of cell cycle and apoptosis [33].

The action of NAC and LOS, which would explain their systemic protective influence via ROS, including regulation of 8-oxoG urinary excretion in case of DC hasn't been studied yet. Moreover, one can find quite

fragmental information: the authors of the research [34] pointed out that action of NAC in kidney cortical tissue of diabetic animals significantly inhibited ROS generation, Akt/protein kinase B, and tuberin phosphorylation and resulted in decreased 8-oxodG accumulation and upregulation of 8-oxoG-DNA glycosylase protein expression. *In vitro* studies NAC prevented the 8-OHdG accumulation and promoted upregulation of 8-oxoG-DNA glycosylase expression in cell culture of glial cells supposed to high glucose concentration [35]. Other experimental work was highlighted [36] that 3 week administration of LOS caused decreasing level of 8-oxoG in renal DNA in case of STZ-induced type 1 diabetes mellitus.

Interestingly, administration of all investigated pharmacological groups (NAC/ LOS/ NAC+LOS) caused increasing the marker of oxidative damage of DNA, 8-oxoG by 2.2 / 2.6/ 1.5 times more than group of type 1 diabetes mellitus respectively ($p < 0.01$). The enhanced urinary excretion of 8-oxoG in presence of pharmacological correction may be associated with NUDIX hydrolase activation, preventing modified DNA precursors being incorporated into the genome. Obtained results may be explained by the review findings [37]: the enzyme 8-oxo-2'-deoxyguanosine triphosphatase (8-oxodGTPase) NUDT1 is activated, hydrolysing 8-oxodGTP to 8-oxodGMP, further processing, perhaps by 5'(3')-nucleotidases, may give rise to 8-oxoG, which can be removed from the cell to ultimately appear in the urine [37]. The given results are hypothesized that drugs-antioxidants activate the defense mechanisms of organism, which are focused on removal of modified bases, thereby providing protective action against harmful influence of ROS.

7. Conclusions from the research and prospects of further development of this area

1. The redox-dependent mechanism of cardioprotection of NAC, LOS and their combination is associated with protection of aorta cells in diabetic animals against toxic action of superoxide radicals, preventing mitochondrial dysfunction and further DNA damage via activation of defense mechanisms (activation of NUDIX hydrolase, 8-oxodGTPase etc.), removal modified bases, preventing in such way development of OS-induced diabetic cardiomyopathy.

2. The enhancement of NO level in mitochondria of aorta cells in diabetic animals points out the endothelial protection effect of NAC, preventing progression of myocardial dysfunction.

3. The positive action of studied pharmacological schemes against damage of free radicals in liver cells emphasizes recovery of metabolic disturbance in rats with STZ-induced type 1 diabetes mellitus.

4. So, NAC or their combination with LOS can be used as cardioprotective agents in prevention and treatment of diabetic cardiomyopathy.

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