Section 2. Medical science

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EFFECT OF H_2S METABOLISM MODULATORS ON THE LEVEL OF GALECTIN-3 IN AORTA, HEART AND KIDNEYS OF RATS WITH STREPTOZOTOCIN-INDUCED DIABETES MELLITUS

Abstract. Diabetes mellitus (DM) is often comorbid with developing angiopathy, cardiomyopathy and nephropathy. Immune-inflammatory activation of the endothelium, cardiac and renal fibrosis following chronic hyperglycemia can be linked to impaired production of hydrogen sulphide (H_2S) and galectin-3. The causal relation between these factors remains disputable. Aim of the research was to evaluate the connection between changes in H_2S system and the level of galectin-3 in the blood and tissues (aorta, heart and kidneys) of rats with streptozotocin-induced diabetes mellitus. It is established that streptozotocin-induced diabetes mellitus is associated with the increase in galectin-3, the decrease in H_2S, the decrease in the activity and expression of cystathionine-γ-liase (CSE) in blood, aorta, heart and kidney. The decline in the activity of H_2S / CSE system is accompanied by a significant increase in galectin-3 in blood and tissues which can accelerate the development of diabetes-associated organ dysfunction and angiopathy.

Keywords: galectin-3, hydrogen sulfide, diabetes mellitus.

Introduction. Diabetes mellitus (DM) is a global medical and social problem due to its high prevalence, early development of complications, high disability and mortality. According to the World Health Organization, the prevalence of diabetes in different countries vary from 4% to 8% [1; 2]. Among

severe and common complications of diabetes there are diabetic angiopathy, cardiomyopathy and nephropathy. The various pathobiochemical disorders (glycosylation of proteins, oxidative stress, inflammation, mitochondrial dysfunction) are involved in the pathogenesis of heart, vascular and renal disease in diabetes, among which the hyperproduction of profibrogenic mediators takes an important place [3; 4]. The literature analysis showed that galectin-3 is one of the sensitive biochemical marker of the development of endothelial dysfunction, myocardial and renal fibrosis in patients with diabetes [5; 6; 7].

Recently, the attention of scientists has been focused on the study of a biologically active molecule – hydrogen sulfide (H_2S). This molecule possesses the properties of an antiplatelet agent, anticoagulant, vasodilator, regulator of insulin secretion and glucose metabolism in the liver [3; 8; 9]. H_2S is an important nephro- and cardioprotector due to its antioxidant and anti-inflammatory activity and ability to stabilize cell membranes [3; 8; 9; 10]. It was shown that the use of H_2S donors in a streptozotocin (STZ) diabetic model has a cardio- and renoprotective effect [3]. Disorders of hydrogen sulfide (H_2S) and galectin-3 production may be potential factors in immunoinflammatory activation of the endothelium, myocardial and renal fibrosis in the setting of chronic hyperglycemia, yet, the relationship between these factors remains debatable.

Aim of the research was to evaluate the connection between changes in H_2S system and the level of galectin-3 in the blood and tissues (aorta, heart and kidneys) of rats with streptozotocin-induced diabetes mellitus.

Materials and methods. The experiments were conducted on 40 white non-linear male rats, initial body mass within 200–250 g, which were obtained from the vivarium of the National Pirogov Memorial Medical University (Vinnytsya). DM was modelled in three animal groups (n = 10) by single intraperitoneal (IP) injection of streptozotocin (Sigma, USA) which was freshly dissolved in 0.1 M citrate buffer (pH 4.5) at 40 mg/kg of rat mass. Control rats received IP equivalent volumes of 0,1 M citrate buffer (0.1 ml /100 g). Substances were injected after the animals had been deprived food for 24 hours. Two animal groups (Group 3 and 4) from day 3 to day 28 after streptozotocin injection were injected modulators of the H_2S system IP once a day as freshly prepared water solution at 0.1 ml per 100 g of rat mass. A CSE inhibitor D, L-propargylglycine (PPG) (Sigma, USA) was injected at 50 mg/kg, and H_2S donor NaHS (Sigma, USA) – at 3 mg/kg. The doses, routes and duration of H_2S modulators' delivery were taken from the literature and did not cause animal mortality [11; 12; 13]. Rats of the first group (control) and the second group after STZ-diabetes initiation were injected IP once a day with 0.15 M NaCl (0.1 ml per 100 g rat mass). The pathology signs were polydipsia, polyuria, and body mass loss. The rats with glycaemia levels >15 mmol/l were selected for the study.

All stages of experiments were carried out according to general ethical principles approved by European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). Research protocols were approved by the Committee on Bioethics of National Pirogov Memorial Medical University (Vinnytsya).

Whole venous blood was obtained by decapitation and collected into sterile plastic Vacuette tubes (Greiner Bio-One, Austria) without anti-coagulant and with EDTA, when necessary. Plasma was obtained by centrifugation of the whole blood at 1500 g for 25 minutes at 18–22° C, plasma aliquots were collected into sterile plastic Eppendorf microtubes and stored at –20° C until further use.

To determine H_2S level in organs we used post-nuclear homogenates prepared in the following way: the myocardium and kidneys were washed with cold 1.15% KCl solution, cut up with scissors, homogenized in 0,01 M NaOH at the ratio of 1:5 (m/v) at 3000 rpm (teflon-glass). To 1 ml of homogenate we added 250 microliter 50% TCA, centrifuged for 15

minutes at 1200 g, then aliquots were collected into Eppendorf microtubes and the H_2S level was immediately determined in the supernatant.

For other biochemical studies, myocardium and kidney homogenates were prepared by the following procedure: tissue samples were homogenized in 0.25 M sucrose, 0.01 M Tris (pH 7.4) at the ratio of 1:5 (m/v) at 3000 rpm (teflon-glass), centrifuged for 30 min at 600 g at 4° C, then aliquots were collected into Eppendorf microtubes and stored at –20°C.

Levels of glucose in peripheral blood were measured using electronic glucometer Accu-Chek Active (Rouche Group, Germany). Levels of galectin-3 in blood plasma, postnuclear myocardium homogenates and kidney were determined by immunoassay using the Rat Galectin 3 (GAL-3) ELISA Kit (MyBiosource, Cat№ MBS2600708) according to the instruction. Standard solution concentrations were GAL-3 0.156; 0.312; 0.625; 1.25; 2.5; 5; 10 ng /ml. The detection was carried out using the STAT-FAX 303 analyser (USA) at 450 nm (differential filter 630 nm).

Levels of H_2S in aorta, myocardium and kidneys were determined spectrophotometrically by the reaction with N, N-dimethyl-para-phenylenediamine sulfate in the presence of $FeCl_3$ [14]. All manipulations were performed in hermetically sealed plastic tubes to prevent losses of H_2S. Sulfide ion content in the sample was calculated using a calibrated graph. The standards were water solutions of $Na_2S{\cdot}9H_2O$ in the range of 31.2–3120 μM. Optical density was measured at 670 nm in a cuvette with optical path of 1,0 cm using Apel PD-303 spectrophotometer (Japan).

Activity of the H_2S-synthesizing enzyme cystathionine-γ-liase (CSE, EC4.4.1.1) in the postnuclear supernatant of the homogenates of aorta, myocardium and kidneys was determined by the increase in sulfide anion as described here [15]. Activity of the CSE in the reaction of cysteine desulfuration was determined in incubation medium containing in final concentrations pyridoxal phosphate 1.34 mM, L-cysteine 6.0 mM, Tris-HCl buffer 0.08 M (pH 8.5). To 0.5 ml incubation medium were added postnuclear homogenates of organs (protein content 1–2 mg). The samples were incubated at 37° C for 60 min in sterile hermetically sealed plastic Eppendorf tubes (to prevent H_2S losses). The reaction was stopped by cooling the tubes on ice, then there was added 0.5 ml 1% zinc acetate solution to bind the produced H_2S. The control samples were treated similarly, for the exception that the investigated material was added to the medium only after incubation and cooling. The amount of H_2S was determined by the methylene blue production by a standard method [16]. To the samples were added 0.5 ml 20 mM N, N-dimethyl-p-phenylenediamine in 7.2 M HCl, 0.4 ml 30 mM $FeCl_3$ in 1.2 M HCl, incubated for 20 min at 18–22° C, then added 1 ml 20% TCA, centrifuged for 10 min at 3000 rpm. The optical density of the supernatant was measured at 670 nm in a cuvette with optical path of 1.0 cm using Apel PD-303 spectrophotometer (Japan). Sulfide anion content in the sample was calculated using a calibrated graph.

The expression of the CSE gene in aorta, myocardium and kidneys were determined by Real-Time PCR. Total RNA was isolated from tissues using AmpliSens RIBO-zol-B kit (AmpliSens, Russia). To obtain cDNA a reverse transcription kit (Sintol, Russia) was used, that included 2.5 x reaction mix, 15 U/ml primer of oligo$(dT)_{15}$, 50 U/μl reverse transcriptase MMLV-RT, 5 U/μl RNAase inhibitor and deionized water free from RNases. 1–2 μg total RNA was added to the mix.

The determination of the CSE gene expression was done in the presence of SYBR Green I dye, using detection amplifier DT-Light (DNK-Tekhnologia, Russ) in the reactionary mix: 10 x buffer for amplification with SYBR Green I dye; 25 mM $MgCl_2$; 2.5 mM deoxynucleoside triphosphates; specific primers to *CSE* gene (5'-GCTGAGAGCCTGGGAGGATA-3', 5'-TCACTGATCCCGAGGGTAGCT-3') and 5 U/μl SynTag DNA-polymerase. 5 μl DNA sample were added to the mix. The primers to the β-actin gene: 5'-ACCCGCGAGTACAACCTTCTT-3' and 5'-TATCGTCATCCATGGCGAACT-3' were used

as the referent gene. Amplification regime was: 94° C, 3 min, 40 cycles: – 94° C, 15 s; – 64° C, 40 s. For data analysis, we used the Ct method: the relative level of mRNA CSE / β-actin was estimated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{CSE} - Ct_{\beta\text{-actin}}$, and Ct_{CSE} is the threshold cycle of cDNA amplification of the target gene CSE; $Ct_{\beta\text{-actin}}$ – threshold cycle of cDNA amplification of the referent gene for β-actin.

Raw data were treated using universal statistical programs MS Excel, SPSS22 for Windows, STATISTICA 6,0 (license № AXXR910A374605FA). To evaluate between-group difference we used Student's parametric t-criterion, when the data were not normally distributed – Mann-Whitney U-test. The normality was evaluated using the Shapiro-Wilk test. The correlation was evaluated after Pearson. Statistical significance was assumed at $p<0.05$. Results are given as mean and standard deviation (M±SD).

Results. It was shown that at STZ-diabetes there is an increase in galectin-3 in blood, heart and kidneys by 3.5, 5 and 5.5 times, respectively ($p<0.05$), compared to control (Fig. 1). Injection of propargylglycine to STZ-diabetic rats caused further increase in galectin-3 in blood, heart and kidneys of rats by 55, 77.6 and 80%, respectively ($p < 0.05$), compared to untreated animals with DM. Whereas, introduction of NaHS decreased galectin-3 in blood, heart and kidneys of STS-diabetic rats: its level was lower by 22, 58.2 and 63.2%, respectively ($p < 0.05$) than in untreated animals with DM.

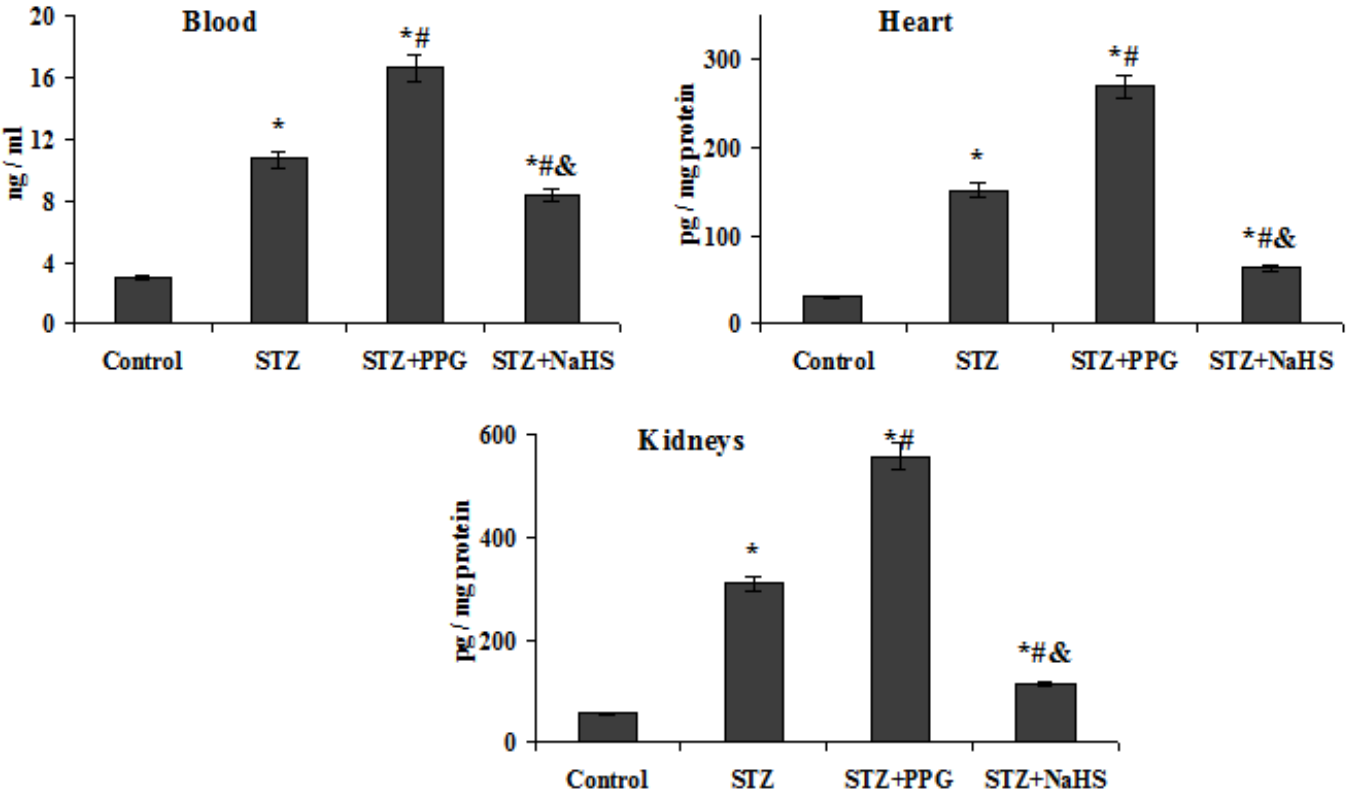

Figure 1. Effect of propargylglycine and NaHS on galectin-3 in blood, heart and kidneys of rats with streptozotocin-induced diabetes (n=10):
* – $p<0.05$ compared to control; # – $p<0.05$ compared to untreated animals with STZ-diabetes;
& – $p<0.05$ compared to animals with STZ-diabetes which were given propargylglycine

Experimental DM causes decrease in H_2S in aorta, heart and kidneys by 40; 36.7 and 38.6%, respectively ($p<0.05$) compared to the control rat group (Fig. 2). The treatment of DM-rats with propargylglycine worsen H_2S deficit in aorta, heart and kidneys – the level was lower by 34.8; 29.4 and

33%, respectively ($p<0.05$) compared to untreated animals. On the other hand, introduction of NaHS to rats was followed by increase in H_2S in aorta, heart and kidneys by 28.1, 23.5 and 26.5%, respectively ($p<0.05$) compared to DM-rats.

According to the correlation analysis, under STZ-diabetic condition in rats, H_2S content in aorta, myocardium and kidneys has statistically significant anti-correlation with the correspondent levels of galectin-3 ($r= -(0,76–0,82)$, $p < 0.05$).

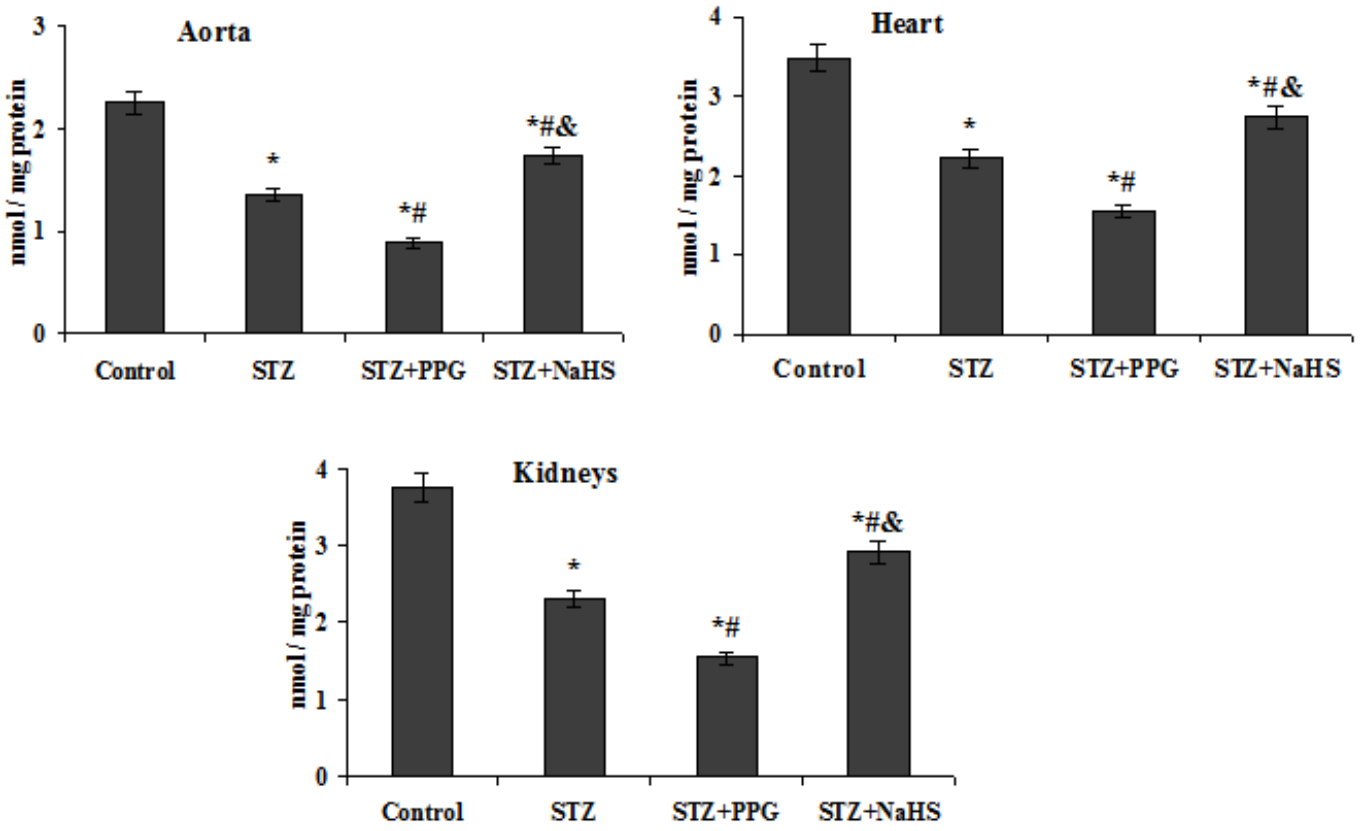

Figure 2. Effect of propargylglycine and NaHS on H_2S in aorta, heart and kidneys of rats with streptozotocin-induced diabetes (n=10):
– $p<0.05$ compared to the control group; # – $p<0.05$ compared to untreated animals with STZ-diabetes; & – $p<0.05$ compared to animals with STZ-diabetes treated with propargylglycine

DM causes decrease in the activity of H_2S synthesis by the mean of cysteine desulfation reaction catalyzed be CSE in aorta, heart and kidneys by 57.7; 55.1 and 56.6% respectively ($p<0.05$) compared to control (Fig. 3). Propargylglycine potentiated the DM's negative effect on the enzymatic synthesis of H_2S: in aorta, heart and kidneys of rats there was registered alleged reduction in CSE activity by 44.4; 35.9 and 42.4%, respectively ($p<0.05$) compared to untreated DM animals. Meanwhile injection of NaHS was followed by the opposite changes; in aorta, heart and kidneys the CSE activity was 2.1, 2 and 2.2-times higher ($p<0.05$) than in untreated animals.

In animals with modelled DM expression of the CSE gene was lower in aorta, heart and kidneys by 72, 60.5 and 70.6% ($p<0.05$), respectively, compared to control (Fig. 4). H_2S metabolism modulators had opposite effects on the parameter. Propargylglycine in DM animals caused yet further depression of this gene. At these conditions the mRNA of CSE/β-actin in aorta, heart and kidneys was lower by 44, 29.4 and 38.5%, respectively ($p<0.05$), compared

to untreated animals with STZ-diabetes. The introduction of NaHS, on the other hand, induced the CSE gene expression: the mRNA of CSE / β-actin in aorta, heart and kidneys of rats was higher by 49, 35.3 and 42.8%, respectively ($p<0.05$), compared to untreated animals.

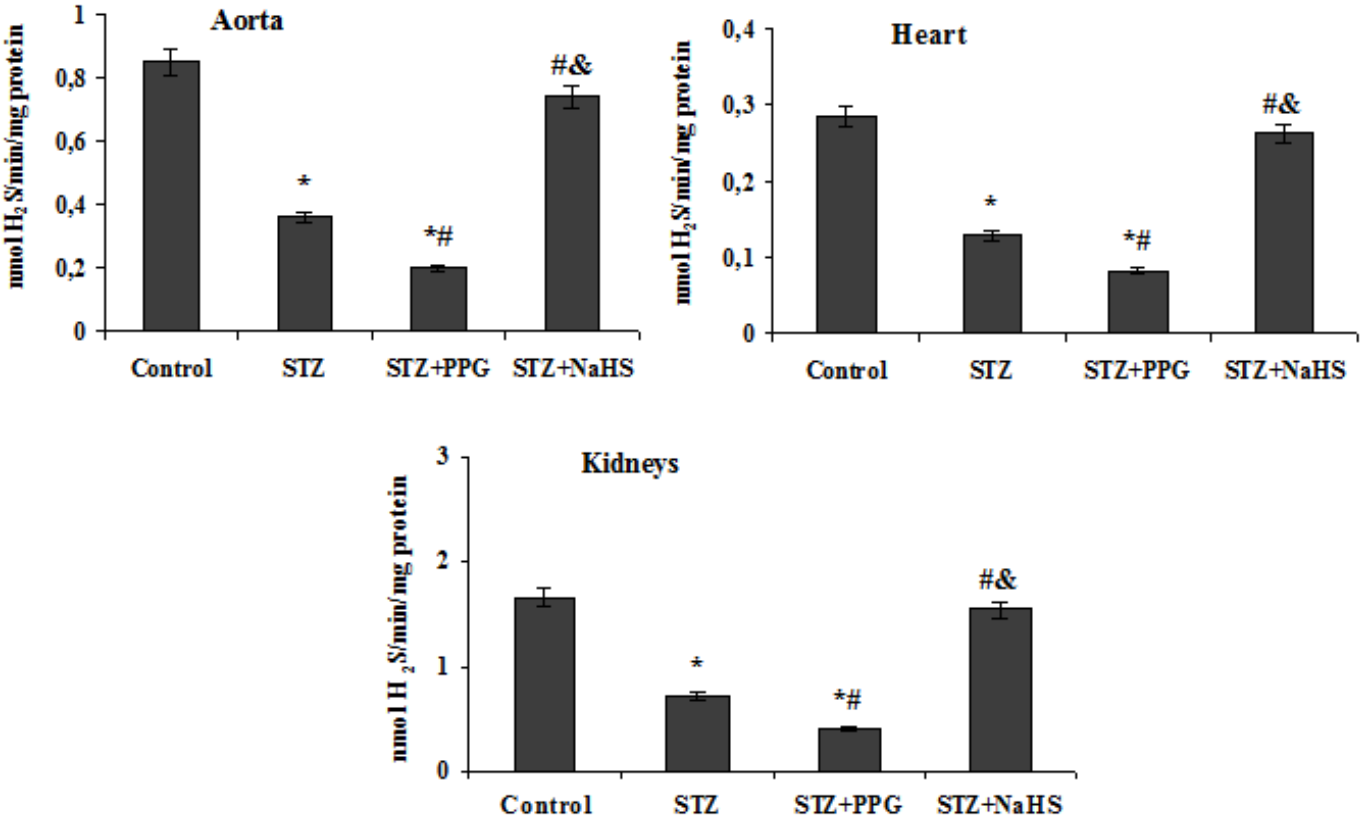

Figure 3. Effect of propargylglycine and NaHS on CSE activity in aorta, heart and kidneys in rats with STZ-induced diabetes (n=10): * – $p<0.05$ compared to the control group; # – $p<0.05$ compared to untreated animals with STZ-diabetes; & – $p<0.05$ compared to animals with STZ-diabetes which were treated with propargylglycine

Discussion. According to the obtained data, diabetes is accompanied by a decrease in the activity and expression of CSE, an increase in utilization of exogenous H_2S and a decrease in H_2S content in the aorta, heart and kidneys of rats. The development of H_2S deficiency in the organs of diabetic rats is closely correlated with the level of hyperglycemia. The obtained results are confirmed by the literature: incubation of endothelial cells of mice in a medium with a high glucose concentration was accompanied by a decrease in H_2S [17].

Disorders of H_2S metabolism in diabetes are among the pathogenetic factors in the development of endothelial dysfunction, cardio- and nephropathy [3; 9]. According to the literature, the negative impact of low concentration of H_2S on the cardiovascular system and kidneys in diabetes is realized through various mechanisms, including the activation of fibrogenesis. Nowadays it is known that the profibrogenic effect of H_2S deficiency is realized through the induction of inflammation, oxidative stress, hyperexpression of transforming growth factor TGF-β1, myogen-activated protein kinase MARK, matrix metalloproteinase MMP-9 [3; 8; 9; 10].

According to our studies, in diabetes the disorders of H_2S metabolism closely correlate with an increase in galectin-3, another important profibrogenic mediator. The use of propargylglycine,

an inhibitor of H_2S synthesis, in diabetes deepens H_2S deficiency in tissues and increases galectin-3, while H_2S donor (NaHS) – alleviates these changes.

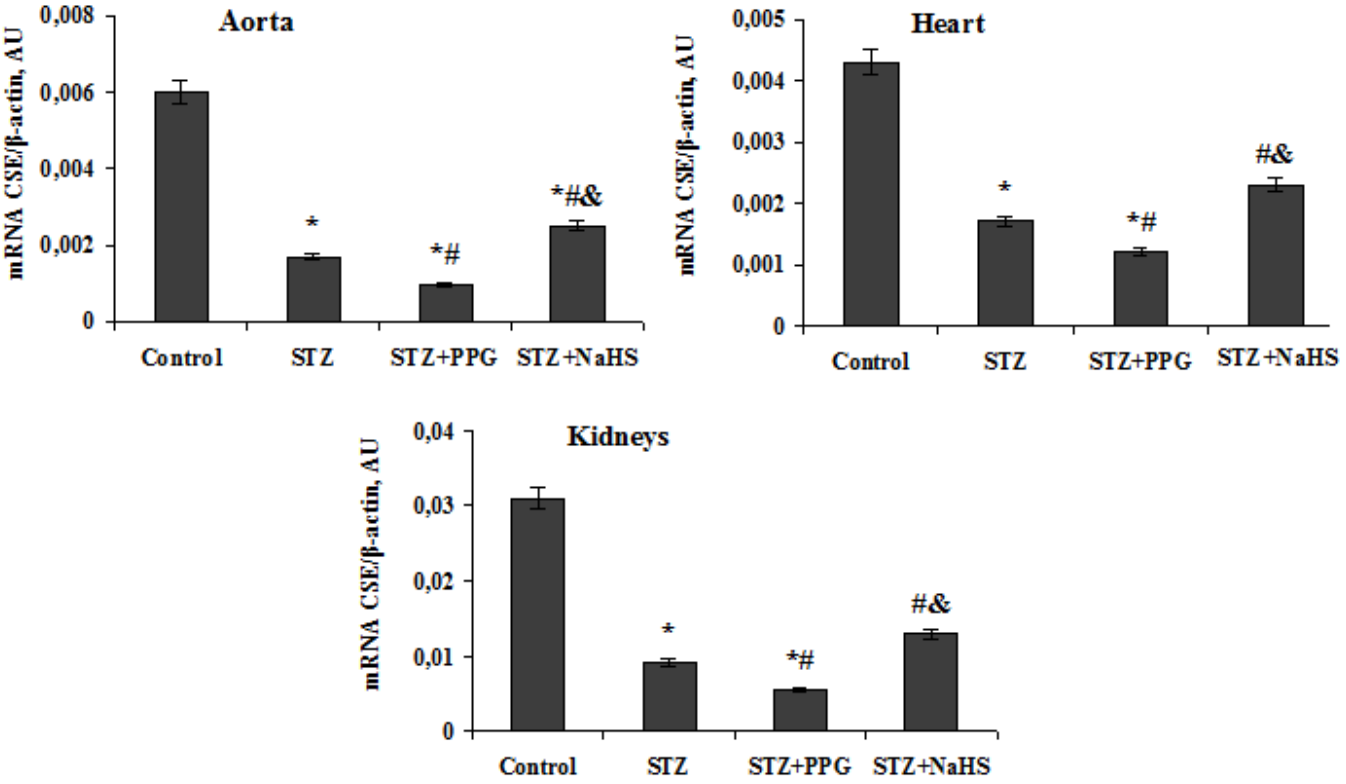

Figure 4. Effect of propargylglycine and NaHS on the expression of the CSE gene in aorta, heart and kidneys of rats with streptozotocin-induced diabetes (n=10):
* – $p<0.05$ compared to control group; # – $p<0.05$ compared to untreated animals with STZ-diabetes; & – $p<0.05$ compared to animals with STZ-diabetes that have received propargylglycine

However, how do the modulators of H_2S effect galectin-3 system, through which biological mechanisms? To answer these questions, it requires further research. Another promising area is the development of new drugs with a stimulating effect on the H_2S system in tissues in order to provide effective correction of the endothelial dysfunction and fibrogenesis in animal organs in diabetes.

Conclusions. Diabetes mellitus, induced by streptozotocin, is associated with an increase in galectin-3 in the blood, aorta, heart and kidneys, a decrease in H_2S, a decrease in the activity and expression of CSE. Inhibition of activity of the H_2S / CSE system is accompanied by a significant increase in galectin-3 in the blood and tissues, which can accelerate the development of diabetes-associated organ dysfunction and angiopathy.

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