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HYDROGEN SULFIDE SYSTEM ATTENUATES INJURY BY HYPERGLYCEMIA AND STRESS: THE ROLE OF MESENTERIC ADIPOCYTES IN AGED ANIMALS

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Introduction. The mesentery is one of the recently described separate organs, whose functions and ability to protect itself from injuries are still unclear. Adipocytes are a part of the mesentery (MAC); however, little is known about their age-related changes, responses at times of damage induced by hyperglycemia, stress, and their interaction. Hydrogen Sulfide (H_2S) system is a key endogenous cytoprotective system that operates by catalytic activities of Cystathionine- γ -lyase (CSE), Cystathionine- β -synthase (CBS), and Sulfite Oxidase (SUOX) but their role in adipocyte cytoprotection is still unknown.

Aim. To evaluate the CBS, CSE, SUOX activities in the mesenterium and ultrastructural changes of MAC in aged rats fed with HSD, under conditions of stress, their combination, and modification of H_2S synthesis: by injecting NaHS and hybrid nonsteroid anti-inflammatory drug, which is rich in H_2S -acetylsalicylic acid (H_2S -ASA).

Material and Methods. The research was conducted on old rats, divided into the control group (animals were on the standard diet and received 1.0 ml of 0.9% NaCl solution per os); accordingly, the remaining animals with HSD in per os groups received 2) 1.0 ml of 0.9% NaCl solution; 3) NaHS at a dose of 100 mcM/kg/day, 9 days; 4) 1.0 ml of 0.9% NaCl solution, 9 days, and stress induction (Takagi, K, 1964); 5) NaHS at a dose of 100 mcM/kg, 9 days, and stress induction; 6) ASA in the dose of 10 mg/kg/day, 9 days, NaHS at a dose of 100 mcM/kg, and stress induction; 7) ASA, 10 mg/kg/day, 9 days, and stress induction; 8) H_2S -aspirin (H_2S -ASA, ATB-340), 17.5 mg/kg/day, 9 days, and stress induction. Subcellular modifications of adipocytes were researched using the transmission electronic microscopy (TEM). CSE, CBS, SUOX enzyme activity was assessed using the standard biochemical methods.

Results. In groups of animals with HSD and NaHS, no structural destructive changes were identified on the endothelial and subendothelial level; uneven chromatin condensation was observed in the nucleus of endothelial cells. The impact of hybrid H_2S -aspirin had a protective effect and led to the "loss" of mesenteric adipocytes. Administration of the hybrid aspirin led to an increase by 15% of the general H_2S /CSE i H_2S /CBS activity as compared to the effect of the regular aspirin ($p < 0,05$). Using the aspirin along with NaHS led to the 67% increase in H_2S /CSE i H_2S /CBS activity in rats with HSD as compared to the group receiving normal saline. SUOX activity constituted 5.27 nM/min•mg in the control group, 4.05 nM/min•mg in rats with HSD; in the groups of HSD with NaHS and stress, the obtained values were approximately equal to control values

Conclusions. This study confirms the important role of CSE/ H_2S pathway in the life of mesenteric adipocytes and its cytoprotective effect. The high activity of the expression of CSE and

SUOX pathways in case of H₂S synthesis in mesenteric adipocytes and disorders caused by age and hyperglycemia may be related to the development of non-specific inflammation and potentially used to treat obesity. H₂S-aspirin may become an effective drug for prevention of age-related inflammation and may have a protective effect in case of hyperglycemia.

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Key words: Gasotransmitters, Hydrogen Sulfide, Cystathionine- β -synthases, Cystathionine- γ -lyase, Sulfite Oxidase, mesenterium, fat tissue, cellular cytoprotection, inflammation, glucose homeostasis, age-related changes

СІРКОВОДНЕВА СИСТЕМА ЗМЕНШУЄ УШКОДЖЕННЯ АДИПОЦИТІВ БРИЖІ У СТАРИХ ТВАРИН, ІНДУКОВАНІ ГІПЕРГЛІКЕМІЄЮ ТА СТРЕСОМ

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Вступ. Брижа – новий нещодавно відкритий орган людського тіла. Однак функціональне значення брижі як органу травної системи та її роль у захисних механізмах організму залишаються нез'ясованими. Мезантеріальні адипоцити (МА) є частиною брижі, проте мало відомо про їхні вікові зміни, реактивні відповіді за індукції гіперглікемії, стресу та їхнього поєднаного впливу. Фізіологічні ефекти сірководню (H₂S) є ключовими для ендогенної системи цитопротекції, яка функціонує за допомогою каталітичної активності цистатионін- γ -ліази (CSE), цистатионін- β -синтази (CBS) і сульфит-оксидази (SUOX), але їхня роль у цитопротекції адипоцитів ще не з'ясована.

Мета. Дослідити активність цистатионін- β -синтази (CBS), цистатионін- γ -ліази (CSE), сульфит-оксидази (SUOX) брижі та дослідження ультраструктурних змін МА у старих щурів за умов висококалорійної високовуглеводної дієти (HSD), стресу, їхнього поєднання і модифікації синтезу сірководню (H₂S): введення NaHS та сполуки гібридного нестероїдного протизапального препарату, збагаченої H₂S - H₂S-аспірину (H₂S-ASA).

Матеріали та методи. Дослідження проводили на старих щурах, виділяли контрольну групу (тварини перебували на стандартній дієті й отримували 1,0 мл 0,9% NaCl р-ну per os), решта тварин з HSD отримували, відповідно, у групах per os: 2) 1,0 мл 0,9% NaCl р-ну; 3) NaHS в дозі 100 мкмоль/кг/добу, 9 днів; 4) 1,0 мл 0,9% NaCl р-ну 9 днів і індукцію стресу (Takagi, K, 1964); 5) NaHS в дозі 100 мкмоль/кг 9 днів і індукцію стресу; 6) АСК в дозі 10 мг/кг/добу 9 днів, NaHS в дозі 100 мкмоль/кг та індукцію стресу; 7) АСК, 10 мг/кг/добу, 9 днів та індукцію стресу; 8) H₂S-аспірин (H₂S-ASA, АТВ-340), 17,5 мг/кг/добу, 9 днів та індукцію стресу. Субклітинні зміни мезантеріальних адипоцитів досліджували за допомогою трансмісивної електронної мікроскопії (TEM). Активність ензимів CSE, CBS, SUOX оцінювали стандартними біохімічними методами.

Результати. У групі тварин з HSD та NaHS структурних деструктивних змін на ендотеліальному та субендотеліальному рівні не було виявлено, у ядрі ендотеліальних клітин спостерігалася нерівномірна конденсація хроматину. Вплив гібридного H_2S -аспірину виявився захисною дією і «схудненням» мезантеріальних адипоцитів. Застосування гібридного аспірину виявило збільшення на 15% загальної активності H_2S/CSE і H_2S/CBS на противагу від дії звичайного аспірину ($p < 0,05$). Використання аспірину одночасно з NaHS виявило збільшення на 67% активності H_2S/CSE і H_2S/CBS у щурів HSD при порівнюванні до групи, що отримувала фізрозчин. Активність SUOX становила 5,27 нмоль/хв•мг у контрольній групі, у щурів на HSD - 4,05 нмоль/хв•мг; у групі на HSD з NaHS і дією стресу отримані значення приблизно дорівнювали контрольним.

Висновки. Дослідження свідчить про важливу роль CSE/ H_2S шляху у життєдіяльності мезантеріальних адипоцитів і його цитопротекторну дію. Висока активність експресії шляхів CSE та SUOX при синтезі H_2S у мезантеріальних адипоцитах, при порушеннях зумовлених віком і гіперглікемією може бути пов'язана з розвитком неспецифічного запалення, мати потенційне використання з терапевтичною метою для лікування ожиріння. H_2S -аспірин може стати ефективним засобом для запобігання вікового неспецифічного запалення, мати захисний ефект при гіперглікемії.

Ключові слова: Газотрансмітери, сірководень, цистатіонін- β -синтази, цистатіонін- γ -ліаза, сульфат оксидаза, брижа, жирова тканина, клітинна цитопротекція, запалення, гомеостаз глюкози, вікові зміни

The prevalence of obesity and its comorbidities worldwide, including Ukraine, is increasing especially among adults in the last two decades (Fig.1). Recently this pathology was considered as a major medical problem which is attributed to the influence of the modern Western diet, rich in high caloric high sugar diet (HSD) [18].

Ectopic accumulation of visceral fat mass related to the HSD induces low grade inflammation [5] which has harmful effects on cellular survive, as well as inducing cell injury/death, changes in the microbiome and cancer development [1, 4, 12, 18].

The mesentery is one of recently described separate organs which functions and its ability to protect injury are still unclear [8]. Adipocytes are a part of mesentery, however, it is a little known about their age-related changes, reactive response during damage induced by hyperglycemia, stress, and their interaction, as well as association with low grade inflammation [15, 28].

Among several recently described key peripheral factors of cytoprotection is Hydrogen Sulfide (H_2S) system that endogenously generates gas by the pyridoxal 5'-phosphate (B_6)-dependent enzyme catalytic activities

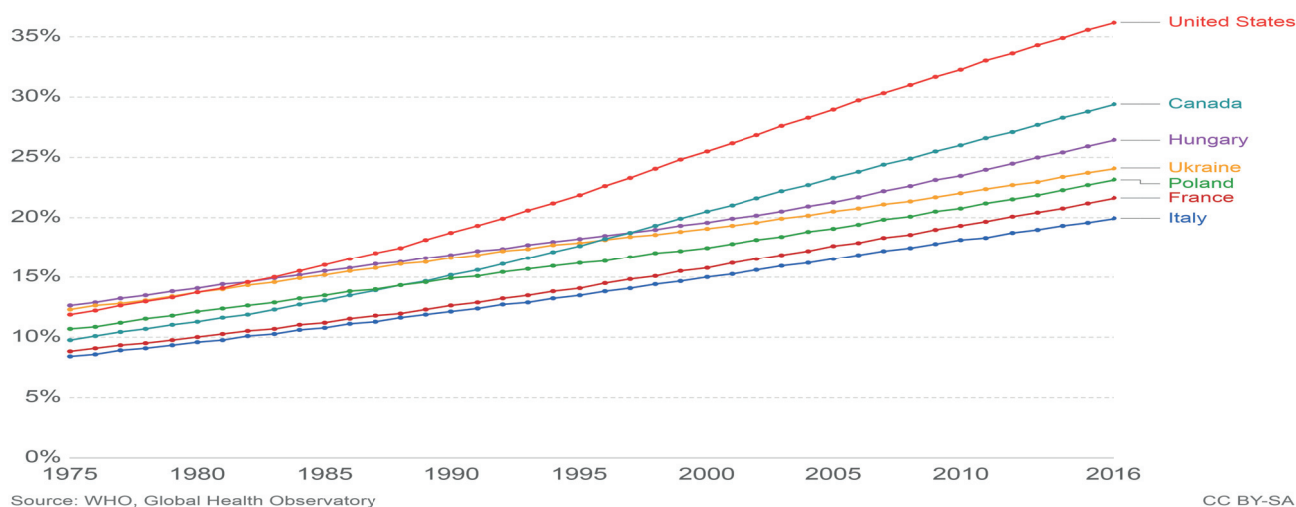


Fig. 1. The prevalence of obese adults aged 18+ in Ukraine, Hungary, Poland, Italy, France, Canada, and United States of America based on their body mass index which is greater or equal to 30 in WHO report (2017)

of Cystathionine- γ -lyase (CSE, EC 4.4.1.1), Cystathionine- β -synthase (CBS, EC 4.2.1.22), and 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) by Cysteine aminotransferase (CAT), which use L-cysteine as a substrate, and non-enzymatic production of H_2S by glucose [23], glutathione [19] which are essential for anti-inflammatory, anti-radical scavenged effects (e.g. by methemoglobin, metallo- or disulfide-containing molecules, and oxidized glutathione), vasodilatation, cytosolic methylation (*via* S-methyltransferase), mitochondrial oxidation (e.g. oxidation to thiosulfate, which is eventually converted to sulfite and sulfate by Sulfite Oxidase (EC 1.8.3.1, SOX) [2, 12, 20, 24], as well as from inorganic and organic polysulfides and elemental sulfur [13, 14] and microbiome [4, 16, 26]. The previous data of cytoprotective and anti-inflammatory effects of H_2S -containing compounds of non-steroidal anti-inflammatory drugs (NSAID) naproxen (ATB 346) on vascular tone, including vascular ageing [24, 27, 30], is very promising for future fat tissue investigation, which is an active source of endocrine factors according to the novel data [7]. Recently, it was demonstrated that relationship between H_2S pathway, adipogenesis, inflammation, and fat mass accumulation is controversial, as both stimulation and inhibition have been reported [28]. These observations, together with the known opposite effects of H_2S on glucose homeostasis (Tab.1) in the regulation of insulin secretion and insulin sensitivity, promoted us to further investigate the effect of H_2S by activities of its enzymes CBS, CSE, SUOX in the mesenteric adipocytes via determination their changes during induced injury in animal model of the pre-metabolic syndrome by hyperglycemia (HSD), induction of acute stress and modulation of cytoprotection by eicosanoid biosynthesis via NSAID and exogenous modification of H_2S pathway activities by H_2S -NSAID [17, 21].

Aim. To evaluate the CBS, CSE, SUOX activities in the mesenterium and ultrastructural changes of mesenteric adipocytes (MAC) in aged rats fed with HSD, at stress induction, and modification of H_2S synthesis.

Material and Methods. All experiments were carried out on the aged rats (N=48, body weight 350 ± 40 g; n=5-7) in accordance with the norms of the European Convention for the Protection

of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986) and in accordance with the Committee on Bioethics of Danylo Halytsky Lviv National Medical University agreement 4 from 23.04.2018. Animals were maintained under a constant 12 h light and dark cycle and an ambient temperature of C with $50 \pm 10\%$ relative humidity. All animals were kept in raised mesh-bottom cages to prevent coprophagy. Animal from the control group were allowed free access to tap water and kept on standard diet, other rats experimental groups on 28-days hypercaloric high-carbohydrate diet, as HSD by V. Kozar, 2008, with unrestricted access to 40% solution of fructose *ad libitum* within drinking-water [5,15]. The rats consequently underwent euthanasia under deep by ketamine anaesthesia (60 mkg/kg^{-1}) via intramuscular administration and sample of paired samples of MAC were obtained from the area contiguous with the ileum. Subcellular changes if MAC by morphological techniques via transmission electron microscopy (TEM) were used to detect the characteristics of the rat MAC in normal condition and during injury, and compare them with the characteristics of the control tissues [8]. Fixation of the material for electron microscopy was carried out with a 2% solution of osmium oxide (OsO_4) eV 0.1 mol/L phosphate buffer. Then the processing of the material was conducted according to generally accepted methods. Ultrathin cuts (30-60 nm) were produced on ultratome «UMTP-3M» and after coloring with Reynolds, they were photographed and studied with an electron microscope «UEMV-100K» (Ukraine) on an increase in 2,500, 3,000, 5,000, 6,000, 8,000, 10,000 and 15,000 times.

Table 1

Effects of H_2S on glucose homeostasis	
Hypoglycemia ($< 70 \text{ mg/dl}$)	Hyperglycemia ($> 200 \text{ mg/dl}$)
↓ Lipolys in adipocytes	↓ Insulin secretion from β -cells
↑ Glucose uptake into skeletal muscle	↑ Hepatic glucose production
↑ Glucose uptake into fat	↓ Glucose uptake into hepatocytes

For the objectives of the study and determination the role of H_2S pathway in the mechanisms of mesenteric adipocyte cytoprotection in old rats by activities of CSE, CBS and SUOX that

catalyzes the physiologically vital oxidation of sulfite to sulfate, the terminal reaction in the oxidative degradation of the sulfur-containing amino acids cysteine and methionine, were induced acute stress by water-immobilization stress model by Takagi, 1964, 1968 [21, 22], and animals were treated by hybrid H₂S-associated NSAIDs compounds with comparison to classical NSAIDs analogues [10, 11]. The design of study is represented on Fig.2.

The animals were subdivided into groups and pretreated *per os* by: 1) control group with standard diet and by vehicle (1,0 ml of saline), rats from 2-8 groups had HSD and for 2) 1,0 ml of saline; 3) NaHS in a dose of 100 mkmol/kg, 9 days 10 mg/kg/day; 4) 1,0 ml of saline and induction of stress; 5) NaHS at a dose of 100 mkmol/kg and induction of stress; 6) ASA at a dose of 10 mg/kg/day for 9 days, *per os*, NaHS at a dose of 100 mkmol/kg, *per os*, and induction of stress; 7) ASA, manufactured by Darnitsa, Ukraine, 10 mg/kg/day, *per os* for 9 days; 8) H₂S-aspirin (H₂S-ASA, ATB-340), manufactured by Antibes, Canada, 17,5 mg/kg/day, *per os* for 9 days). The administration of NaHS and ATB-340 was performed in doses tested by J.L. Wallace, 2012-2017 [24-26].

Evaluation of rat survival rate, basal conditions, expression of CSE, CBS, SUOX activities in MAC were done by standard biochemical meth-

ods [28]. The required statistical calculations and diagramming were prepared using IBM PC Pentium computer with STATISTICA for Windows 5.0 software and Microsoft Excel spreadsheets. The distribution of parametric indices in the samples was normal (Gaussian) – Shapiro-Wilk's W-test. Due to the presence of more than two study groups, parametric characteristics were compared using a post-test analysis (ANOVA unit – analysis of variance), where mean values were compared in pairs using the Newman-Keuls criterion. Data were considered valid when * – $p < 0.05$, # – $p < 0.01$.

Results and Discussion. Mass-metric studies in animals of experimental groups did not reveal significant differences (up to 10-12%) from the control group data. The rat glycemia data did not show any changes other than the control group. According to the results of investigation ultrastructural changes of MAC by electron microscopy (Fig. 3), it was determined that under normal conditions the small intestine mesentery is represented by adipose tissue of the regular histological structure with regional lymph nodes and well-preserved endothelial cells and the basal membrane of the habitual structure (Fig. 3,a).

In rats fed with HSD, the mesentery contained plethoric vessels, occasionally there was fluttering basal membrane of hemocapillaries. The

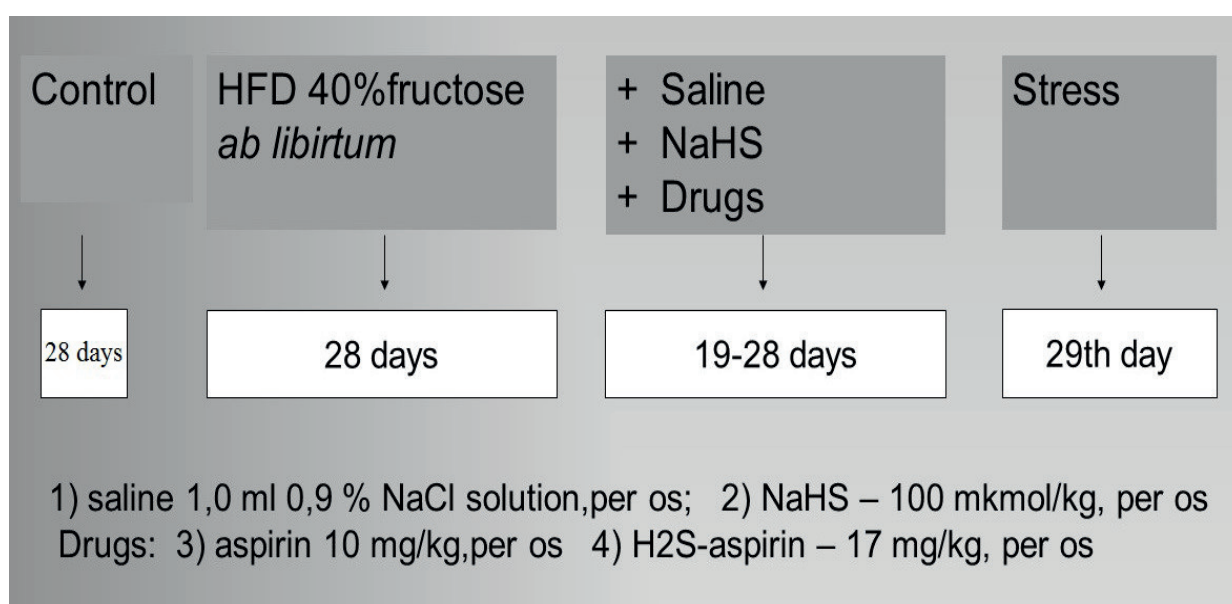


Fig.2. The scheme of study design; SD – standard diet; HSD – high sugar diet

white fat adipose tissue adipocytes have been unevenly enlarged. There was also observed a large number of heterogeneous mitochondria with an enlightened matrix, loosening of the basement membrane reflecting the adaptive-compensating adaptive reactions for hyperglycemia.

In animals with HSD and pretreatment of NaHS, non-organic compound that raises amount of H_2S in tissues, such signs were absent. In the nucleus of endothelial cells it was observed an uneven condensation of chromatin, while signs of endothelium and sub-endothelial structures violations were absent (Fig. 3,b).

Vacuating of fat inclusions in the MAC under conditions of ASA injection along with the vac-

uolated fragments of fat inclusions is observed (Fig. 3,c). There is also observed an accumulation of a large number of small electron dense mitochondria. This is an indirect sign that indicates the activation of mitochondrial breathing processes. In animals receiving aspirin, there were signs of swelling of the blood vessel walls, mesenteric adipocytes of considerable size and filled with lipid drops of average electron density. The use of a hydrogen sulfide natural synthesis donor in a hybrid H_2S -aspirin compound according to the electronic microscopy showed changes characterized by the preservation of intercellular contacts that could be interpreted as a protective action and reducing the size of adipocytes «slimming» (Fig 3,d). Similar results were obtained during other studies [1, 21].

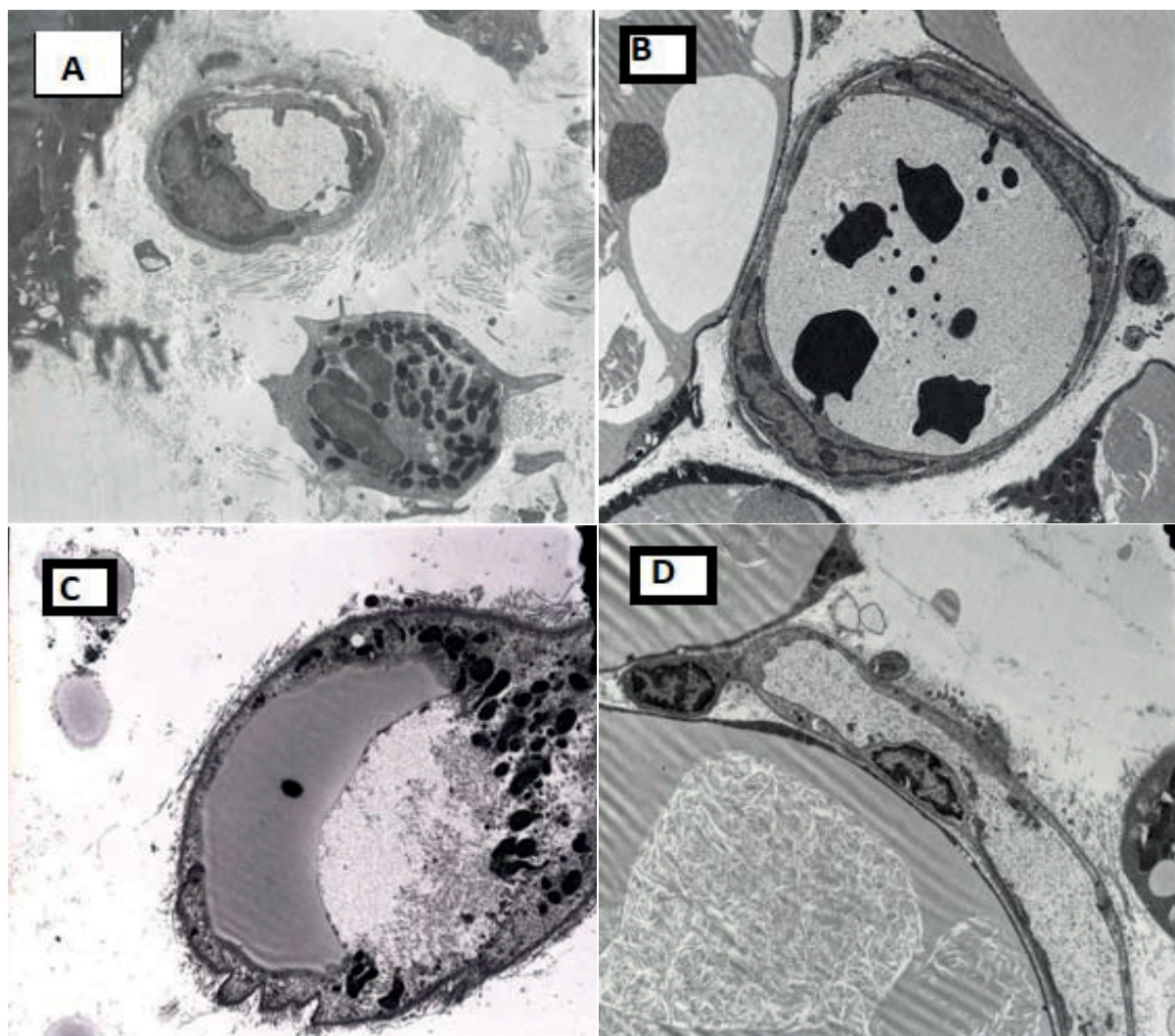


Fig. 3. Results of electron microscopy of rat mesenteric adipocytes with standard diet and pretreated by saline (a); NaHS (b); aspirin (c), H_2S -aspirin (d) during 28 days high fructose diet (b-d); Bar: in A, 1.6 μ m; and in B, 0.2 μ m

It is known that the characteristic feature of the H_2S effect is the modulation of protective qualities in the body that can be evaluated by the activity of CSE and CBS [2, 3, 19]. We examined how the regulatory influence of the hydrogen sulfide on MAC by the activities of CSE and CBS/ as well as SUOX under the modification of the hydrogen sulfide formation by pretreatment of the H_2S , hybrid H_2S -aspirin application versus the effect of classical aspirin (ASA) in rats with HSD and induction stress injury (Fig.4, 5].

The average values of CSE and CBS activities in the MAC of rat control group were 1.01 nmol H_2S /min•mg protein and 0.75 nmol H_2S /min•mg protein, respectively. In rats of the «relative control» group with 28-days hyperglycemia and saline administration the CSE activity was 1.21 nmol H_2S /min•mg protein for, and for CBS □ 0.89 nmol H_2S /min•mg protein ($p<0.05$). The pretreatment of NaHS caused an increase in CSE activity on 37% compared with the control group ($p<0.05$), while under stress it decreased to 27% ($p<0.05$). The activity of CBS did not undergo significant changes in the application of NaHS, the value remained at the level of benchmarks ($p<0.05$).

Under the influence of stress the CSE activity in the group that received saline decreased to 0.69 nmol H_2S /min•mg protein or to 44% compared to the «relative control» group of HSD ($p<0.05$) and for 32% to the control group ($p<0.05$) on SD. Under the influence of stress, the value of CBS activity in the group of animal that received saline was the opposite, it increased on 42% relatively to the HSD group and on 70% relatively to the control group ($p<0.05$).

The effect of aspirin on MAC under the influence of stress caused various effects on the activities of CSE and CBS. There was the increased of CSE on 33% compared to the saline group, while the activity of CBS decreased on 19% in comparison CSE activity on to animal with stress-induced damage and saline pretreatment. Obtained results could be explained by induction low-grade inflammation, regarding to the previous our data of pro- and anti-inflammatory cytokines expressions [24, 25, 29, 30].

The use of the hybrid aspirin led to even more significant increase activity of CSE on 81% and

a slight decrease on 16% for CBS in comparison to animal with stress-induced damage and saline pretreatment. The use of the aspirin and NaHS showed an increase in 2.7 times of CSE activity and the absence of changes in CBS activity compared to the data of the control group ($p<0.01$).

The result of the general activity of CSE and CBS in MAC showed the average data in the control group was 2 nmol H_2S /min•mg protein, in rats of the «relative control» group with HSD it was 2.11 nmol H_2S /min•mg protein. The use of NaHS caused an increase in general activity of H_2S /CSE and H_2S /CBS on 14% compared to the control group ($p<0.05$).

Under the influence of stress, the general activity of H_2S /CSE and H_2S /CBS did not undergo significant changes and remained on the value of the control group ($p<0.05$).

The effect of aspirin under the influence of stress at the general calculation did not reveal any changes in general activity of H_2S /CSE and H_2S /CBS vs saline pretreatment ($p<0.01$). The use of hybrid aspirin showed an increase on 15% of the general activity of H_2S /CSE and H_2S /CBS in composition to rats with ASA pretreatment ($p<0.05$). The use of aspirin and NaHS has shown an increase on 67% of the general activity of H_2S /CSE and H_2S /CBS compared to the values of the saline group.

The average value of Sulfite oxidase (SUOX) in the control serum of blood was 5.27 nmol/min•mg protein, in rats of the «relative» control group with 28-day hyperglycemia was 4.05 nmol/min•mg protein (Fig. 5). The use of NaHS under HSD and stress conditions did not cause SUOX changes comparable to the control. These results could be explained by metabolic changes in energy induced by acute stress [12, 18].

Under the influence of stress, SUOX in the saline group decreased on 26% compared with the control group.

The effect of aspirin under the influence of stress has led to a 35% reduction in SUOX compared to the control group. The use of the hybrid aspirin has led to an increase in SUOX values on

18% relatively to the control, while compared to the saline group to 60% growth. The use of aspirin and NaHS together showed a similar result.

Conclusions. This study suggests an important role for CSE/H₂S pathway in the mesenteric adipocyte cellular survival and control cytoprotection.

Novel findings relating to high expression activities in CSE/H₂S and SUOX pathways in mesenteric adipocytes compromised by age, hyperglycemia and stress might be associated with the development of low-grade in-

flammation and may be a potential therapeutic target for obesity treatment. H₂S aspirin could be an effective tool for prevention of age-related inflammation and exert defensive effect against hyperglycemia.

Disclosures. No conflicts of interest, financial or otherwise, are declared by the authors.

Author contribution. Oleh Revenko drafted manuscript; Natalia Zaichko edited and revised manuscript; John Wallace, Oksana Zayachkivska approved final version of the manuscript.

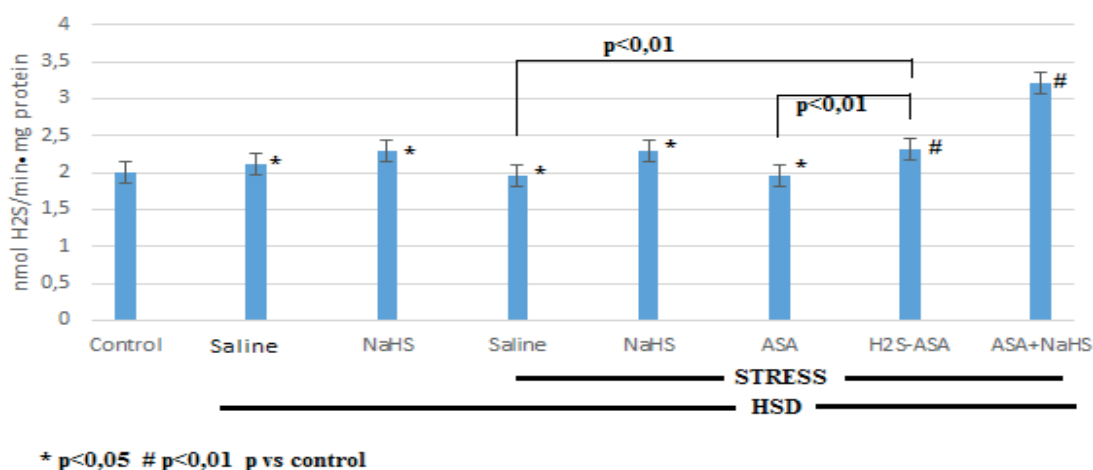


Fig. 4. Changes in activities of CSE and CBS in mesenterium adipocytes of control and experimental groups of rats

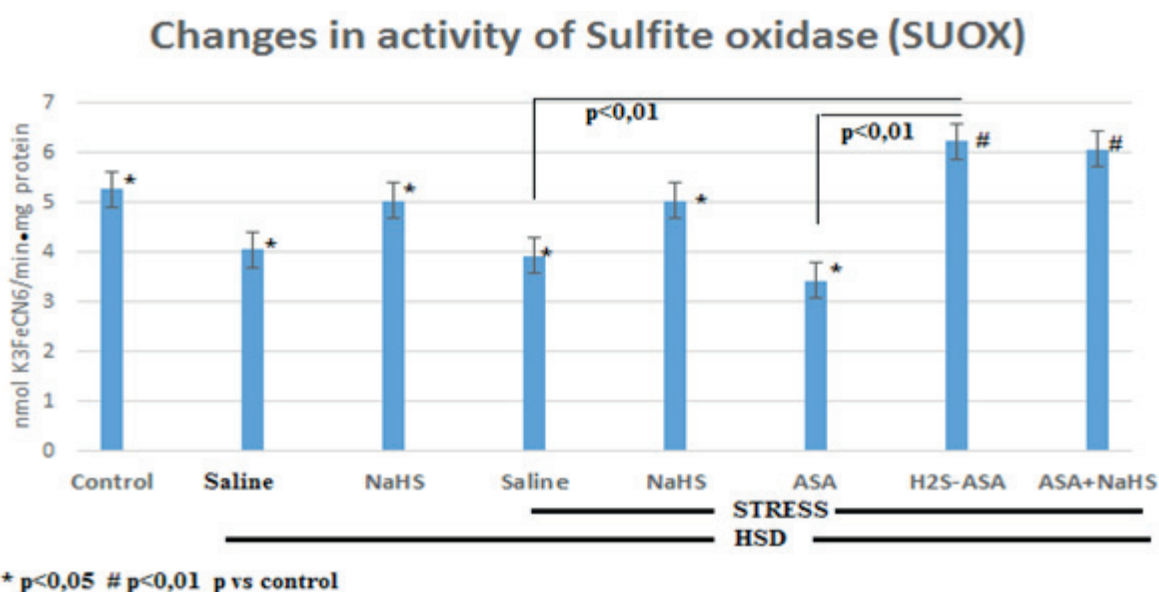


Fig. 5. Changes in activities of Sulfide oxidase in mesenterium adipocytes in control and experimental groups of rats

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