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Hydrogen sulfide potentiates the favorable metabolic effects of inorganic nitrite in type 2 diabetic rats

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ABSTRACT

Objective: Decreased nitric oxide (NO) bioavailability and hydrogen sulfide (H_2S) deficiency have been linked with the pathophysiology of type 2 diabetes (T2D). Restoration of NO levels by nitrite have been associated with favorable metabolic effects in T2D. Moreover, H_2S can potentiate the effects of NO in the cardiovascular system. The aim of this study was to determine the effects of long-term co-administration of sodium nitrite and sodium hydrosulfide (NaSH) on carbohydrate metabolism in type 2 diabetic rats.

Methods: T2D was induced using chronic high fat diet (HFD) feeding combined with low dose streptozotocin (STZ) regimen. Rats were divided into 5 groups (N = 10/group): Control, T2D, T2D + nitrite, T2D + NaSH, and T2D + nitrite + NaSH. Nitrite (50 mg/L in drinking water) and NaSH (0.28 mg/kg, daily i. p. injection) were administered for 9 weeks. Fasting serum glucose, insulin, lipid profile, liver function tests, and oxidative stress indices were measured. Intraperitoneal glucose tolerance test (GTT) was performed at the end of the eighth week, and three days later, intraperitoneal pyruvate tolerance test (PTT) was done. Protein levels and mRNA expression of glucose transporter type 4 (GLUT4) in soleus muscle and epididymal adipose tissue as well as mRNA expression of H_2S-producing enzymes in the liver, soleus muscle, and epididymal adipose tissue were measured at the end of the study.

Results: Compared to the controls, HFD and STZ treated rats developed metabolic dysfunction. Nitrite treatment improved carbohydrate metabolism, liver function, and oxidative stress indices whereas NaSH treatment *per se* had no significant effects. However, co-administration of NaSH and nitrite resulted in further improvement in serum insulin level, GTT, PTT, liver function, oxidative stress, protein level and mRNA expression of GLUT4, as well as mRNA expression of H_2S-producing enzymes in diabetic rats.

Conclusion: Low dose of NaSH *per se* had no effect on carbohydrate metabolism while it potentiated the favorable metabolic effects of inorganic nitrite in type 2 diabetic rats. These favorable effects were associated with decreased oxidative stress and increased GLUT4 expression in insulin-sensitive tissues as well as improvement of liver function.

1. Introduction

Decreased nitric oxide (NO) bioavailability [1] and deficiency of hydrogen sulfide (H_2S) [2,3] are considered to be involved in pathophysiology of type 2 diabetes (T2D). Supplementation with inorganic nitrate or nitrite, in order to restore NO levels, has been associated with many favorable metabolic effects in T2D [1,4–6]. The role of H_2S in pathophysiology of T2D is however controversial, as both inhibition and stimulation of the H_2S system [7,8] have been suggested to be potential therapeutic approaches.

There appear to be important interactions between NO and H_2S at the levels of biosynthesis and biological responses [5,9,10], yet clear an

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insight into this relation is lacking. The effectiveness of endothelial NO synthase (eNOS)/NO/PKG pathway is lower in H_2S-producing enzyme (cystathionine-gamma-lyase (CSE)) knockout mice and it has been shown that this pathway relies on endogenous H_2S production [11]. H_2S stimulates activity and synthesis of eNOS [12] as well as NO release from its storage pools [13]. Furthermore, H_2S at a concentration that does not produce vascular relaxation, it potentiates vasorelaxant effects of NO [14]. Likewise, NO increases the activity and expression of H_2S-producing enzymes, thus enhancing H_2S production [15,16]. Inhibition of NO production by L-NAME [17] and eNOS deficiency in mice [18] eliminates/attenuates the cardioprotective effects of H_2S.

With respect to these interactions, given the partial or absolute requirement of NO under some conditions like T2D, supplementation of NO alone would not be sufficient to overcome such deficiencies and any potential therapeutic effects of H_2S would be considered. In this situation, simultaneous stimulation of both H_2S and NO production may be more effective than the effect of NO alone. Thus, the aim of the present study is to determine the effects of long-term co-administration of sodium nitrite and sodium hydrosulfide (NaSH) on carbohydrate metabolism in a rat model of T2D.

2. Materials and methods

2.1. Animals and induction of diabetes

A total of 50 male Wistar rats (190–210 g) were housed under standard conditions (i.e. 23 ± 2 °C, relative humidity of 50 ± 6%, 12/12-h light-dark cycle). The protocols for animal care and use were approved by the ethics committee of the National Institute for Medical Research Development (IR.NIMAD.REC.1397.121), Tehran, Iran. Rats had free access to water and a normal rat pellet diet (total caloric value of ~3,100 kcal/kg) or a high fat diet (HFD) (total caloric value of ~4,900 kcal/kg), as previously reported [19].

T2D was induced using the HFD feeding for two weeks followed by a single intraperitoneal (i.p.) injection of low dose of streptozotocin (STZ, 30 mg/kg dissolved in 0.1 mM citrate buffer, pH 4.5; Sigma Aldrich, Hamburg, Germany). One week after STZ injection, serum glucose levels from the tail vein were measured and rats with a fasting glucose levels ≥150 mg/dL were considered to be diabetic [4]. The rats continued on their respective diets until the end of the study.

2.2. Experimental design

Rats were randomly divided into 5 experimental groups (n = 10 *per* group): Control group received regular chow and tap water with daily i. p. injection of normal saline; diabetic group was fed with HFD and tap water with daily i. p. injection of normal saline; diabetic + nitrite group was fed with the HFD and tap water supplemented with 50 mg/L sodium nitrite; diabetic + NaSH group was fed with HFD, tap water and treated daily with i. p. injection of NaSH (0.28 mg/kg dissolved in normal saline); diabetic + nitrite + NaSH group was fed with the HFD and tap water supplemented with 50 mg/L sodium nitrite as well as treated daily i. p. injection of NaSH (0.28 mg/kg) for 9 weeks. To minimize oxidation of hydrosulfide anion, the solution was prepared and injected within 10 min. The doses of nitrite and NaSH were selected based on our previous studies; nitrite at 50 mg/L had protective effects [4] and NaSH at higher doses (1.6–5.6 mg/kg) aggravated carbohydrate metabolism while at low doses (0.28 and 0.56 mg/kg) it had no effect [20]. To be able to attribute the metabolic effects of sodium nitrite to nitrite not sodium, sodium chloride at an equimolar concentration to sodium nitrite was added to drinking water and no metabolic effects were observed (unpublished data).

Body weight (Tefal Scale; sensitivity 1 g), food intake (g/day), calorie intake (kcal/day), and water consumption (mL/day) were recorded every week. Fasting serum glucose concentrations were measured every month. Fasting serum levels of insulin, nitrite + nitrate (NO_x), total cholesterol (TC), triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and total sulfide pool were measured before and after the treatments. Intraperitoneal glucose tolerance test (GTT) was done and serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), uric acid, total antioxidant capacity (TAC), total oxidant status (TOS), glutathione (GSH), oxidized glutathione (GSSG), thiobarbituric reactive substances (TBARS), and activities of superoxide dismutase (SOD) and catalase (CAT) were measured at the end of the eighth week of the treatment. Three days past the eighth week of the study, intraperitoneal pyruvate tolerance test (PTT) was performed and at the end of the ninth week the animals were anesthetized by sodium pentobarbital (60 mg/kg; Sigma Aldrich, Hamburg, Germany) and tissue levels of TAC, TBARS, GSH, GSSG, and CAT activity as well as mRNA expression of H_2S-producing enzymes (CSE, cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST)) were measured in the liver, soleus muscle, and epididymal adipose tissue. Protein levels and mRNA expression of glucose transporter type 4 (GLUT4) were measured in soleus muscle and epididymal adipose tissue. A schematic illustration of the timeline and the experimental procedures is shown in Fig. 1.

2.3. Measurement of serum glucose, insulin, lipid profile, and liver function tests

After 12–14 h of fasting, blood was collected from the tail vein and centrifuged at 1677 × *g* for 10 min (4 °C). Serum levels of glucose, TC, TG, LDL-C, HDL-C, ALT, AST, and ALP were measured using commercial kits (Pars Azmoon, Tehran, Iran) according to the manufacturer's instructions. Intra-assay coefficient of variations (CVs) for glucose, TC,

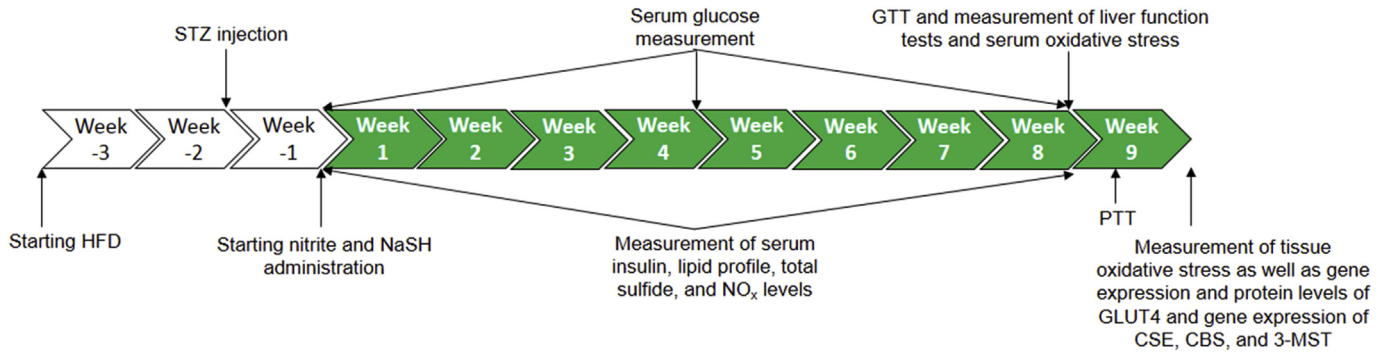

Fig. 1. Experimental timeline and procedures on five experimental groups (control, T2D, T2D + nitrite, T2D + NaSH, and T2D + nitrite + NaSH) during the study. The green chevrons indicate weeks of nitrite and NaSH administration. CBS, cystathionine-β-synthase; CSE, cystathionine gamma-lyase; GLUT4, glucose transporter 4; GTT, glucose tolerance test; HFD, high-fat diet; 3-MST, 3-mercaptopyruvate sulfurtransferase; NaSH, sodium hydrosulfide; NO_x, nitrite + nitrate; PTT, pyruvate tolerance test; STZ, streptozotocin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TG, LDL-C, HDL-C, ALT, AST, and ALP were 2.7%, 4.1%, 2.3%, 2.5%, 3.2%, 1.0%, 2.0%, and 1.0%, respectively and inter-assay CVs for glucose, TC, TG, LDL-C, and HDL-C were 3.9%, 6.4%, 3.7%, 5.1%, 4.9%, respectively. Serum insulin levels were measured using a rat ELISA kit with an assay sensitivity of 1 μU/mL (6 pmol/L) (Mercodia, Uppsala, Sweden); intra- and inter-assay CVs were 3.7% and 6.5%, respectively.

2.4. Measurement of serum total NO_x

Serum NO_x levels were measured using the Griess method [21] with slight modification. Briefly, serum was deproteinized with zinc sulfate (15 mg/mL) and NaOH (3.72 M), and then centrifuged at 6708 × *g* for 10 min (4 °C) after which the supernatants (100 μL each) were dispensed into microplate wells for measurement of NO_x levels. To reduce nitrate to nitrite, 100 μL vanadium trichloride (8 mg/mL prepared in 1 M HCl) was added to each well; then sulfanilamide (50 μL, 2%, dissolved in 5% HCl) and N-(1-naphthyl) ethylenediamine (50 μL, 0.1%, dissolved in ddH_2O) were added and incubated for 30 min at 37 °C; optical density was read at 540 nm by a microplate reader (BioTek, MQX2000R2, USA). NO_x concentrations were measured in the samples against a standard calibration curve (0–100 μM of sodium nitrate). Intra-assay CV for NO_x was 2.1% and inter-assay CV was 5.6%.

2.5. Measurement of serum total sulfide

The methylene blue method [22] was used for measuring total sulfide levels in the serum. Briefly, serum (100 μL) was added to a test tube containing zinc acetate (1% w/v, 200 μL), N, N-dimethyl-p-phenylenediamine sulfate (20 mM, 100 μL) in 7.2 M HCl, and $FeCl_3$ (30 mM, 133 μL) in 1.2 M HCl. The tubes were incubated at 37 °C for 30 min and centrifuged at 1677 × *g* for 10 min. After collecting the supernatants, total sulfide levels in the samples were determined using a standard calibration curve (0–200 μM of NaSH at a wavelength of 670 nm) using a microplate reader (BioTek, MQX2000R2, USA).

2.6. Measurement of serum oxidative stress indices

Serum TAC, TOS, SOD, GSH, and GSSG were measured using commercial kits (ZellBio GmbH, Germany); intra-assay CVs were 2.9%, 3.3%, 4.2%, 3.6%, and 2.9%, respectively and the sensitivity of the assays were 0.1 mM, 0.5 μM, 1.0 U/mL, 0.01 mM, and 0.01 mM, respectively. Serum levels of uric acid were measured using a commercial kit (Pars Azmoon, Tehran, Iran) and intra-assay CV was 1.0%.

The method described by Satoh [23] with slight modifications was used to measure serum TBARS as a product of lipid peroxidation. In brief, 100 μL of the serum was added to the test tubes containing trichloroacetic acid 20% (v/v) and the thiobarbituric acid reagent 0.67% (w/v) and incubated in a boiling water bath for 30 min. After cooling to room temperature, 800 μL of n-butanol was dispensed into each tube and following centrifugation, the absorbance of the upper layer was read at 530 nm. Serum TBARS levels were determined using a standard calibration curve (0–20 μM of 1, 1, 2, 3-tetraethoxypropane as malondialdehyde (MDA) precursor); intra-assay CV was 3.2%.

Serum CAT activity was measured by the method of Hadwan [24], in which the hydrogen peroxide decomposition is estimated by spectrophotometric assay using a complex reaction with dichromate/acetic acid reagent with an optimized serum CAT determination. Briefly, serum (20 μL) was added to a test tube containing hydrogen peroxide (200 μL) with the control tube containing distilled water (200 μL); the standard tube consisted of distilled water (20 μL) and hydrogen peroxide (200 μL). After incubation at 37 °C for 3 min, dichromate acetic acid reagent (400 μL) was dispensed into all tubes and incubated in boiling water for 10 min. After cooling and centrifugation, the supernatant was read at a wavelength of 570 nm and CAT activity was calculated using the following formula: 2.303/t × [log absorbance of standard tube/absorbance of test tube – absorbance of control test] × total volume of reagents in test tube/volume of serum; where t, is time of incubation. Intra-assay CV was 2.9%.

2.7. Measurement of tissue oxidative stress indices

Tissue samples from the liver, soleus muscle, and adipose tissues were homogenized in phosphate-buffered saline (100 mM, pH 7.4, 1:10 for liver and soleus muscle and 1:2 for adipose tissue, w/v) and then were centrifuged for 10 min at 10,000 × *g* at 4 °C; the supernatants were then used for determination of tissue oxidative stress.

The methods described by Satoh [23] and Hadwan [24] were used to measure tissue TBARS level and CAT activity, respectively (intra-assay CVs for TBARS in the soleus muscle, adipose tissue, and liver were 4.0%, 2.4%, and 4.1%, respectively and for CAT activity were 2.4%, 3.0%, and 2.6%, respectively). TAC were measured using the method of Ferric Reducing/Antioxidant Power (FRAP) assay [25]. Briefly, 50 μL of homogenate was added to 1.5 mL of freshly prepared and pre-warmed (37 °C) FRAP reagent in a test tube and incubated at 37 °C for 10 min. The FRAP reagent consists of 300 mM acetate buffer (pH = 3.6), 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$. The absorbance of the blue colored complex [Fe (II) (TPTZ) s]$^{2+}$ was read at 593 nm. Standard solutions of Fe^{2+} in the range of 0–2 mM were prepared from ferrous sulfate in distilled water. The data are presented as mM ferric ions reduced to ferrous form *per* liter (FRAP value). Intra-assay CVs for TAC in the soleus muscle, adipose tissue, and liver were 0.53%, 0.99%, and 1.1%, respectively.

GSH and total glutathione were measured by the Sedlak and Lindsay method [26]. In brief, to determine the GSH + GSSG content, 20 μL of homogenate and 20 μL distilled water were added to each well of a 96-well flat bottom plate. To this, equal volumes of freshly prepared DTNB (5, 5′-dithiobis-(2-nitrobenzoic acid)) and glutathione reductase solutions were mixed and added (120 μL) to each well. After 30 s (for conversion of GSSG to GSH), 60 μL of β-NADPH was added and the mixture was incubated at room temperature for 5 min. The absorbance was read at a wavelength of 412 nm. To determine the GSH content, 20 μL of the homogenate and 200 μL of DTNB were added to each well. After incubation at room temperature for 5 min, the absorbance was read at 412 nm. The GSSG content was calculated as the difference between the total glutathione and GSH contents. Intra-assay CVs for GSH in the soleus muscle, adipose tissue, and liver were 1.9%, 1.2%, and 2.7%, respectively and for total glutathione were 2.1%, 1.9%, and 1.3%, respectively.

2.8. Intraperitoneal glucose tolerance and pyruvate tolerance tests

After 12–14 h of fasting, the rats were anesthetized with an i. p. injection of sodium pentobarbital (60 mg/kg; Sigma Aldrich, Hamburg, Germany). Glucose (2 g/kg from 50% glucose solution) or pyruvate (2 g/kg) was injected intraperitoneally and blood samples for glucose measurements were obtained from the tail vein before and again at 10, 20, 30, 60, and 120 min after glucose or pyruvate injection [5].

2.9. Measurement of GLUT4 protein levels in soleus muscle and epididymal adipose tissue

GLUT4 protein levels in soleus muscle and epididymal adipose tissue were measured by a rat ELISA kit (ZellBio GmbH, Germany). Tissues were homogenized in ice-cold phosphate buffer (100 mM, pH 7.4, 1 mL/100 mg tissue) containing a protease inhibitor cocktail (Roche, Germany) and centrifuged at 1677 × *g* for 10 min at 4 °C. Collected supernatant was diluted according to the relevant manufacturer's instructions.

2.10. RNA extraction, cDNA synthesis and real-time PCR

Soleus muscle, epididymal adipose tissue, and liver were removed,

Table 1
Primers used for real-time PCR analysis.

Primer name	Gene bank Accession No.	Primer sequence (5′→3′)	Product length (bp)
CBS	NM_012522.2	Forward: TGGTGACTCTCGGGAACATG Reverse: AGGTGGATCGGCTTGAACTG	103
CSE	NM_017074.1	Forward: TTGTATACAGCCGCTCTGGA Reverse: CGAGCGAAGGTCAAACAGTG	94
3-MST	NM_138843.1	Forward: GGCATCGAACCTGGACACATC Reverse: ACTGGCGTTGGATCTCCTCTG	103
GLUT4	NM_012751.1	Forward: CGCACCACAGAAAGTGATTG Reverse: GGTAGTGAGTGTGCCTTGTG	100
β-actin	NM_031144.3	Forward: GCGTCCACCTGCTAGTACAAC Reverse: CGACGACTAGCTCAGCGATA	100

CBS, cystathionine-β-synthase; CSE, cystathionine gamma-lyase; GLUT4, glucose transporter 4; 3-MST, 3- mercaptopyruvate sulfurtransferase.

frozen in liquid nitrogen and then stored at −80 °C. Total RNA was extracted from the homogenized liver, soleus muscle, and epididymal adipose tissue using the RNX-Plus solution kit (Cinagen Co., Tehran, Iran) according to the manufacturer's instructions. Nanodrop spectrophotometer (NanoDrop-1000, Thermo Scientific, USA) was used to measure the quantity and purity of RNA samples. cDNA synthesis was performed using Thermo Scientific RevertAid Reverse Transcriptase in accordance with manufacturer instructions. In brief, after DNase treatment, 3 μg of total RNA was reversed to cDNA using M-MuLV RevertAid Reverse Transcriptase (1 μL of 200 U/μL), dNTPS (2 μL of 10 mM), random hexamer primers (1 μL of 100 μM), and RiboLock RNase-inhibitor (0.5 μL of 40 U/μL), incubated for 10 min at 25 °C, followed by 60 min at 42 °C in a total volume of 20 μL. The reaction was stopped by heating the reactions at 70 °C for 10 min.

Primers were designed using the primer 3 and GeneRunner programs; primer sequences are shown in Table 1. Amplifications were done in a Rotor-Gene 6000 Real time PCR machine (Corbett, Life Science, Sydney, Australia). Target genes were normalized with reference gene (β-actin) and the relative mRNA levels for GLUT4, CSE, CBS, and 3-MST were calculated by the $2^{-\delta\delta Ct}$ method where ΔC_t (cycle threshold) = $C_t$of target gene- C_t of reference gene and $\Delta\Delta C_t = \Delta C_t$ of the target gene in the experimental groups - ΔC_t of the target gene in the control group [27].

2.11. Statistical analysis

Statistical analysis was done using GraphPad Prism software (Version 6). All values are presented as mean ± SEM. For analyzing the data for water consumption, food intake, calorie intake, body weight, GTT, PTT, and serum glucose, two-way mixed (between-within) analysis of variance (ANOVA), followed by Bonferroni post-hoc test was used. One-way ANOVA was used for comparing the area under the curves (AUC) and GLUT4 protein levels as well as serum/tissue levels of insulin, lipid profile, SOD, CAT, MDA, TAC, TOS, GSH, GSSG, AST, ALT, ALP, uric acid, total sulfide, and NO_x as well as GSH/GSSG ratio. The Mann-Whitney *U* test was used for comparing fold changes in mRNA expression of GLUT4, CSE, CBS, and 3-MST between groups. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of nitrite and NaSH on serum sulfide and NO_x levels

Compared to the controls, before nitrite and NaSH treatment, diabetic rats had lower total serum sulfide levels ($p < 0.001$) (see Supplementary Fig. 1A). Compared to non-treated diabetic rats, chronic nitrite supplementation increased ($p = 0.098$) while NaSH administration alone had no significant effect on serum total sulfide levels. Compared to nitrite-treated diabetic rats, co-administration of nitrite and NaSH had no further effect on serum total sulfide levels (Fig. 2A).

Before nitrite and NaSH treatments, there was no significant difference in serum NO_x levels between the control and diabetic rats (see Supplementary Fig. 1B). Nitrite and NaSH administration alone increased serum NO_x levels in the diabetic rats ($p < 0.001$), co-administration however resulted in higher serum NO_x levels than nitrite administration alone ($p = 0.071$) (Fig. 2B).

3.2. Effect of nitrite and NaSH on mRNA expression of H_2S-producing enzymes

As shown in Fig. 3, compared to the controls, mRNA expression of the CSE and CBS in diabetic rats were significantly lower in the soleus muscle ($p < 0.001$), epididymal adipose tissue ($p < 0.001$ and $p < 0.05$, respectively), and liver ($p < 0.001$). mRNA expression of 3-MST in diabetic rats were higher in the soleus muscle and liver ($p < 0.001$ and $p < 0.01$, respectively) while lower in the adipose tissue ($p < 0.001$). Nitrite supplementation increased mRNA expression of CSE ($p < 0.001$) and CBS ($p < 0.01$) in the soleus muscle and CBS in the epididymal adipose tissue ($p < 0.001$) and liver ($p = 0.063$). Nitrite supplementation also decreased elevated mRNA expression of 3-MST in the soleus muscle ($p < 0.001$) but had no effect in the liver and adipose tissue. NaSH administration decreased elevated mRNA expression of 3-MST in the soleus muscle but had no effect on the mRNA expression of the other H_2S-producing enzymes. Compared to nitrite-treated diabetic rats, co-administration of nitrite and NaSH resulted in further increases in mRNA expression of CSE and CBS in the liver ($p < 0.05$ and $p < 0.01$, respectively).

3.3. Effect of nitrite and NaSH on body weight, water consumption, and food intake

Before starting the treatment with HFD, body weights were comparable amongst all groups. Two weeks of HFD consumption significantly ($p < 0.001$) increased body weight, and STZ injection was associated with transient reduction of body weight in all HFD treated groups. At the time of initiating treatments with NaSH and nitrite, the body weights of all groups were similar. Simultaneous treatment with nitrite but not NaSH attenuated body weight gain in diabetic rats ($p < 0.05$). Co-administration of nitrite and NaSH had no further effect on body weight (Fig. 4).

Compared to the controls, diabetic rats had higher water consumption ($p < 0.001$) and calorie intake ($p < 0.01$) while lower food intake ($p < 0.001$) (see Supplementary Fig. 2). NaSH and nitrite administration as well as co-administration had no effect on these parameters.

3.4. Effect of nitrite and NaSH on serum glucose and insulin concentration

As shown in Fig. 5A, before NaSH and nitrite treatment, diabetic rats had higher serum glucose levels than the controls ($p < 0.001$). Compared to the non-treated diabetic rats, nitrite supplementation decreased serum glucose level ($p = 0.06$); NaSH administration alone

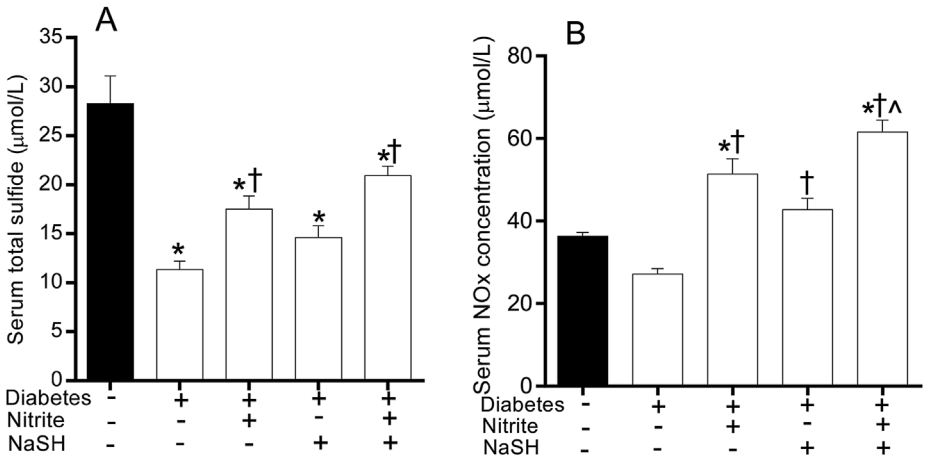

Fig. 2. Effect of 9 weeks nitrite and NaSH administration on serum total sulfide (A) and NO_x (B) levels in the diabetic rats. Results are mean ± SEM (n = 9/group). *Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ^Significant difference compared to nitrite-treated diabetic rats.

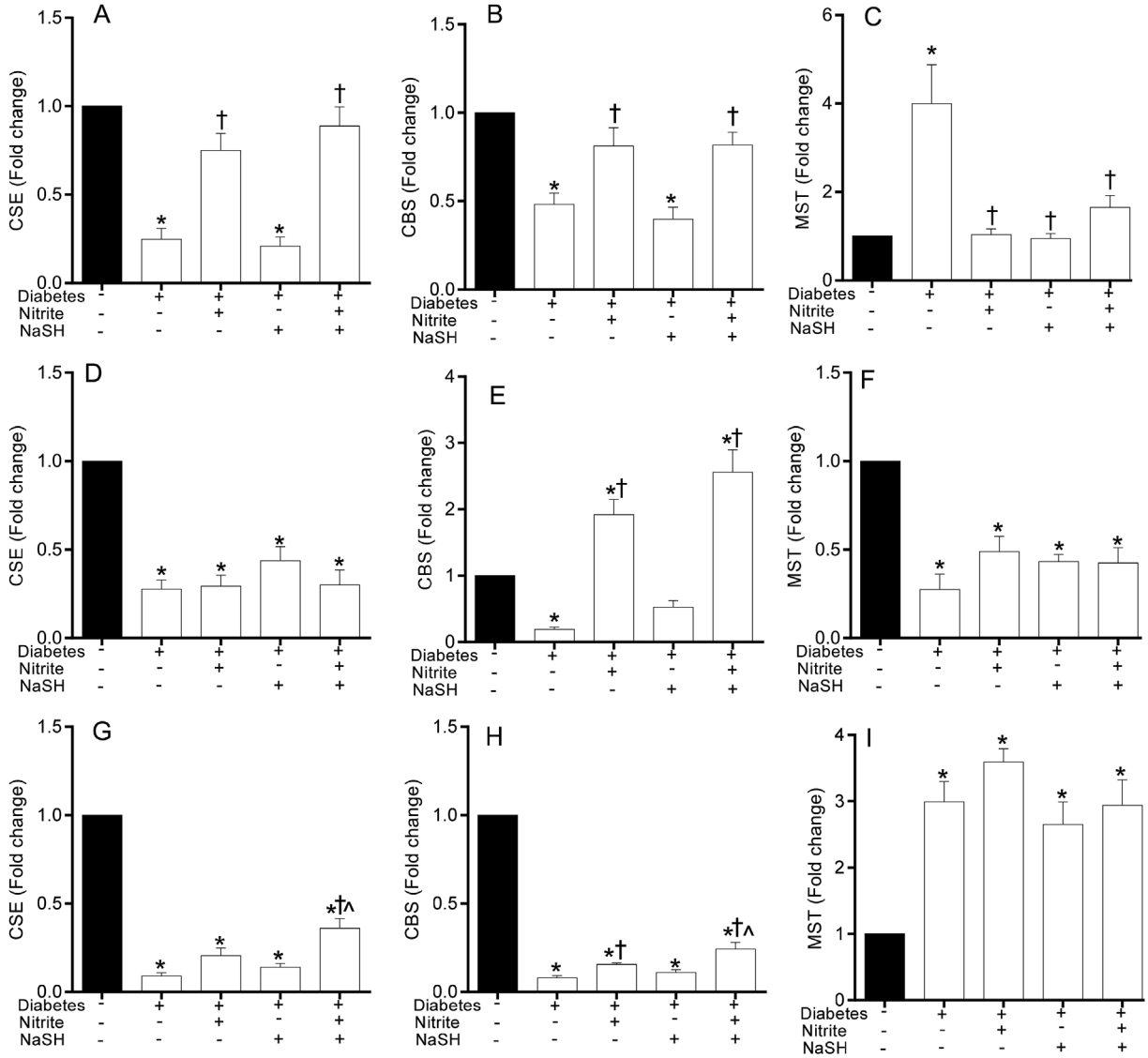

Fig. 3. Effect of 9 weeks nitrite and NaSH administration on mRNA expression of cystathionine gamma-lyase (CSE), cystathionine-β-synthase (CBS), and mercaptopyruvate sulfurtransferase (3-MST) in soleus muscle (A–C); adipose tissue (D–F), and liver (G–I). Results are mean ± SEM (n = 8/group). *Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ^Significant difference compared to nitrite-treated diabetic rats.

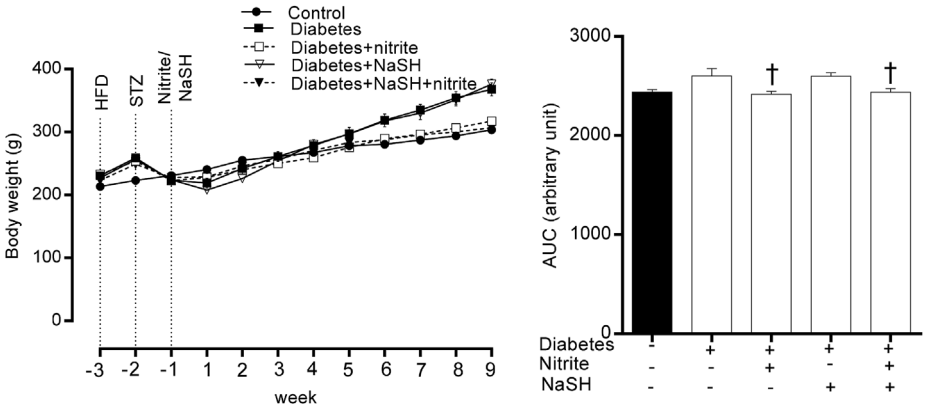

Fig. 4. Effect of 9 weeks nitrite and NaSH administration on body weight in the diabetic rats. Right column indicates the area under the curves (AUC) from week −1 to week 9. Results are mean ± SEM (n = 10/group). †Significant difference compared to non-treated diabetic rats.

had no effect on serum glucose levels and when administered in combination with nitrite, the effects were not different compared to that of nitrite alone (Fig. 5A and B).

Before nitrite and NaSH treatments, serum insulin levels were comparable between the control and diabetic rats (see Supplementary Fig. 3). Continuing the HFD resulted in higher serum insulin levels in the diabetic rats ($p < 0.001$), which indicates insulin resistance. Compared to the non-treated diabetic rats, nitrite supplementation decreased elevated serum insulin levels ($p < 0.001$). NaSH administration alone had no effect on serum insulin levels in diabetic rats but in co-administration with nitrite, it resulted in a further decrease in serum insulin levels ($p < 0.01$), suggesting improved insulin sensitivity (Fig. 5C).

3.5. Effect of nitrite and NaSH on glucose and pyruvate tolerance

As shown in Fig. 6A, diabetic rats had impaired glucose tolerance compared to the controls ($p < 0.001$). Chronic nitrite supplementation improved glucose clearance in the diabetic rats ($p < 0.001$) while NaSH administration alone had no effect. Compared to nitrite-treated diabetic rats, co-administration of nitrite and NaSH resulted in further improvement in glucose tolerance in the diabetic rats ($p < 0.05$).

To investigate potential effects of nitrite and NaSH in modulating gluconeogenesis, PTT was performed. Compared to the controls, pyruvate administration, as a gluconeogenic substrate precursor, resulted in higher glucose production in the diabetic rats ($p < 0.001$). Compared to the non-treated diabetic rats, chronic nitrite and NaSH administration alone had no effect on serum glucose concentration during PTT, whereas co-administration resulted in a significant decrease in serum glucose concentration ($p < 0.05$) (Fig. 6B), indicating reduced gluconeogenesis.

3.6. Effect of nitrite and NaSH on protein levels and mRNA expression of GLUT4 in insulin sensitive tissues

As shown in Fig. 7, compared to the control group, mRNA

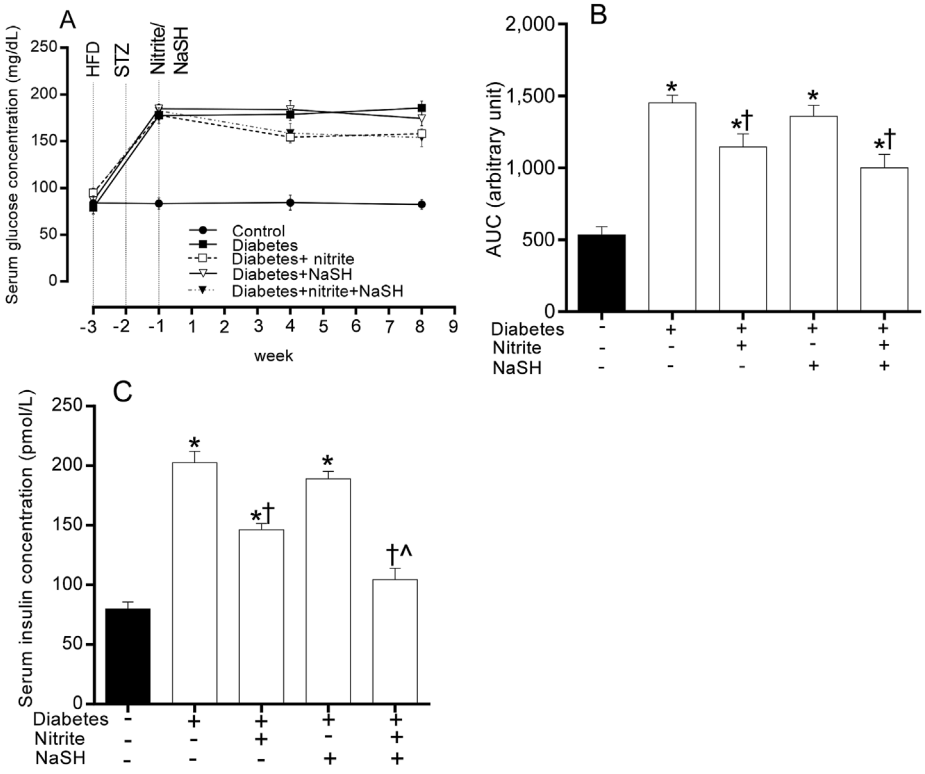

Fig. 5. Effect of nitrite and NaSH administration on fasting serum glucose (n = 10/group) (A), area under the curves (AUC) of serum glucose from week −1 to week 9 (n = 10/group) (B), and serum insulin levels (n = 8/group) (C) in the diabetic rats. Results are mean ± SEM. *Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ^Significant difference compared to nitrite-treated diabetic rats.

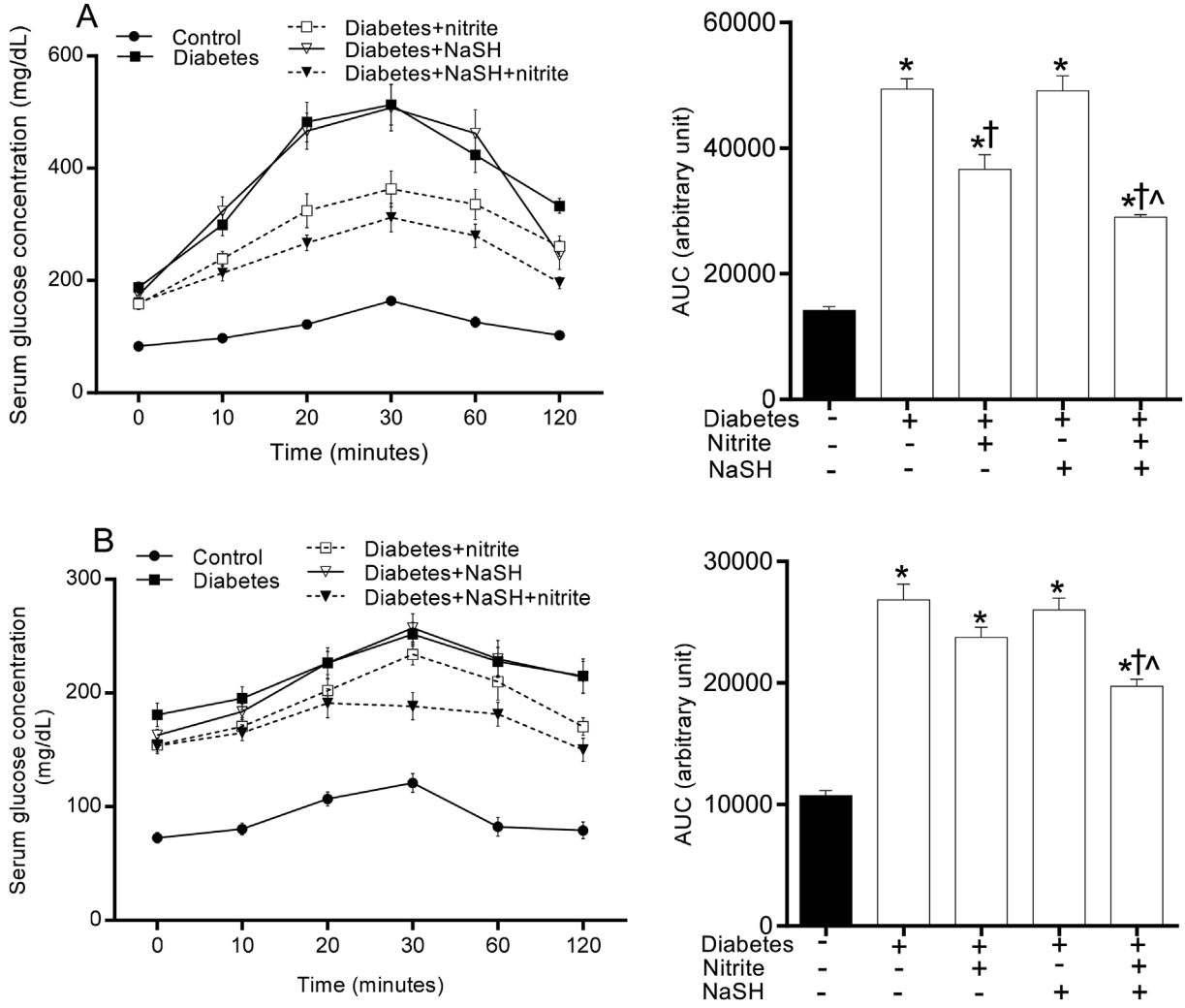

Fig. 6. Effect of nitrite and NaSH administration on glucose tolerance (A) and pyruvate tolerance (B). Area under the curves (AUC) are shown in the columns on the right. Results are mean ± SEM (n = 10/group). *Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ^Significant difference compared to nitrite-treated diabetic rats.

expression of GLUT4 in diabetic rats was significantly lower in both the soleus muscle (Fig. 7A and 44% of control values, $p < 0.01$) and epididymal adipose tissue (Fig. 7B and 28% of control values, $p < 0.001$). Nitrite supplementation alone significantly increased mRNA expression of GLUT4 in both the soleus muscle ($p < 0.05$) and epididymal adipose tissue ($p < 0.05$). NaSH administration alone had no effect on GLUT4 mRNA expression but in combination with nitrite, it resulted in a further increase in GLUT4 mRNA expression in both the soleus muscle ($p < 0.05$) and significantly more in epididymal adipose tissue ($p < 0.001$) compared to nitrite supplementation alone.

Compared to the controls, diabetic rats had lower GLUT4 protein levels in both the soleus muscle (Fig. 7C, $p < 0.001$) and epididymal adipose tissue (Fig. 7D, $p < 0.001$). Nitrite and NaSH administration alone had no effect on GLUT4 protein levels in the non-treated diabetic rats but in the combination treatment groups, GLUT4 protein levels were increased in both the soleus muscle ($p < 0.01$) and epididymal adipose tissue ($p < 0.01$).

3.7. Effect of nitrite and NaSH on serum lipid concentrations

Before nitrite and NaSH treatment, diabetic rats had higher serum concentrations of TC, TG, and LDL-C than the control rats ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively), whereas HDL-C was not changed (see Supplementary Table 1). Nitrite supplementation restored serum LDL-C levels to near normal value; NaSH administration neither alone nor in combination with nitrite had no effect on serum TC, TG, LDL-C, and HDL-C concentrations in diabetic rats (Table 2).

3.8. Effect of nitrite and NaSH on liver function

As shown in Fig. 8, compared to the control group, diabetic rats had higher serum levels of ALT, AST, and ALP (all $p < 0.001$), indicating liver dysfunction. Compared to the non-treated diabetic rats, nitrite supplementation significantly lowered the elevated serum levels of ALT, AST, and ALP (all $p < 0.01$). NaSH administration alone decreased serum ALP level ($P < 0.05$) but had no effect on AST and ALT levels. Compared to nitrite-treated diabetic rats, co-administration of NaSH and nitrite resulted in a further decrease in serum levels of AST ($p = 0.074$) and ALP ($p < 0.05$).

3.9. Effect of nitrite and NaSH on serum and tissue oxidative stress indices

Compared to the control group, diabetic rats showed a significant increase ($p < 0.001$) in serum TOS, MDA, GSSG, and uric acid while a significant decrease ($p < 0.001$) in serum TAC levels and GSH/GSSG ratio as well as serum SOD and CAT activities but there was no change in serum GSH level. Nitrite supplementation alone restored serum TAC level and SOD activity. NaSH administration *per se* had no effect on

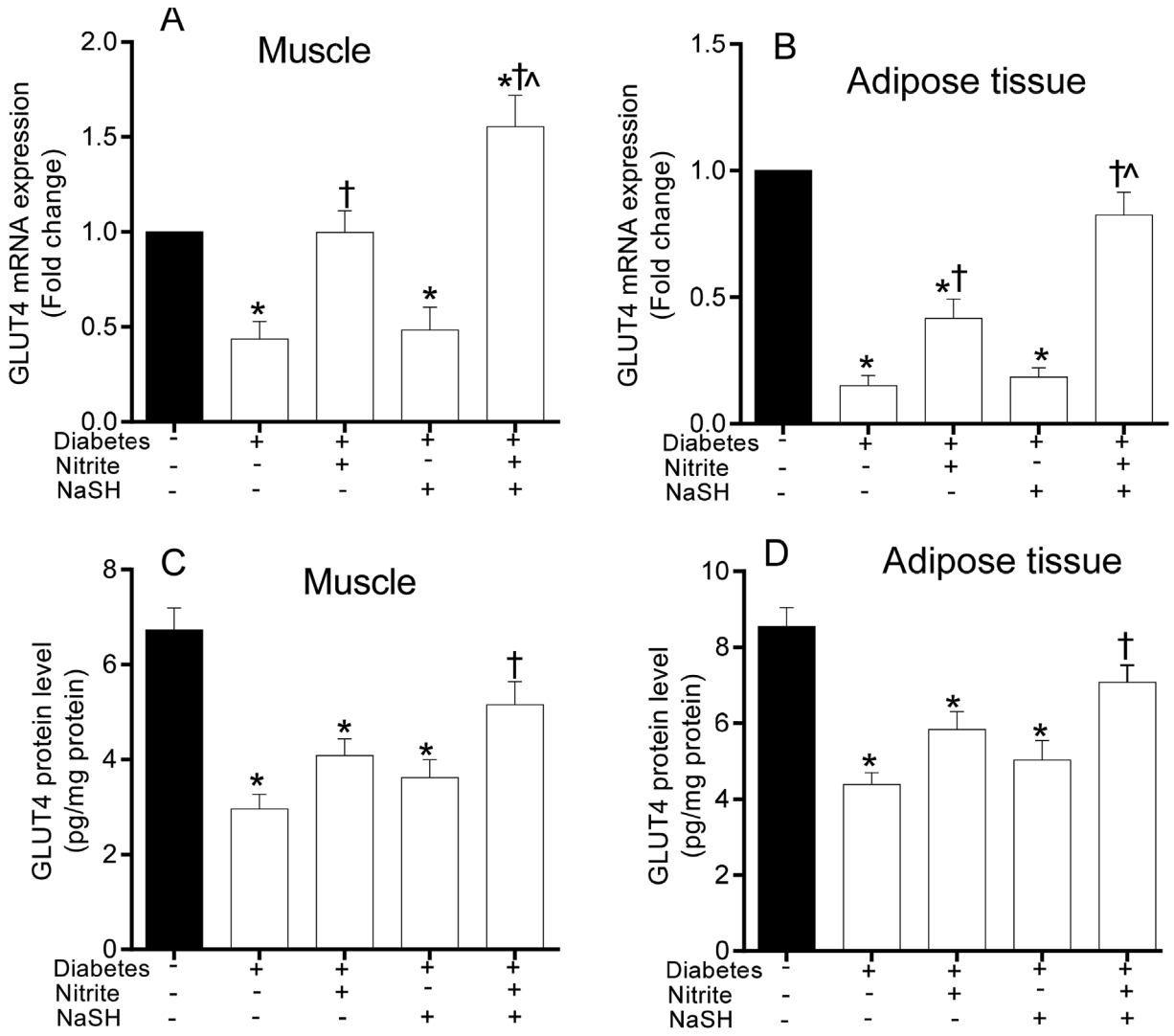

Fig. 7. Effect of nitrite and NaSH administration on the mRNA expression and protein levels of the glucose transporter 4 (GLUT4). Results are mean ± SEM (n = 9/group).
*Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ˆSignificant difference compared to nitrite-treated diabetic rats.

serum oxidants and antioxidants levels but co-administration resulted in a further improvement in the serum TAC levels ($p < 0.01$) as well as SOD ($p < 0.01$) and CAT ($p = 0.08$) activities but had no further effect on serum TOS, MDA, GSH, GSH/GSSG, and uric acid levels (Table 3).

Compared to the control group, diabetic rats showed a significant increase in levels of GSSG in the liver, soleus muscle, and adipose tissue ($p < 0.01$); increase in MDA levels in the liver and soleus muscle ($p < 0.001$ and $p < 0.05$, respectively) while a significant decrease in TAC levels ($p < 0.001$, $p < 0.01$, and $p < 0.01$, respectively) and GSH/GSSG ratio ($p < 0.01$) in the liver, soleus muscle, and adipose tissue as well as CAT activity in the liver and soleus muscle ($p < 0.001$ and $p < 0.01$, respectively) and GSH in the liver ($p < 0.01$). Nitrite supplementation in the liver increased levels of TAC and GSH as well as CAT activity and GSH/GSSG ratio ($p < 0.01$), while it decreased MDA level ($p < 0.01$). In the soleus muscle, the nitrite group had restored levels of TAC and GSH ($p < 0.05$) as well as CAT activity and

Table 2
Changes in serum lipid profile following 9 weeks of nitrite and NaSH administration in control and diabetic rats.

	Animal group				
	Control	Diabetes	Diabetes + nitrite	Diabetes + NaSH	Diabetes + nitrite + NaSH
TC (mg/dL)	64.7 ± 5.0	117.3 ± 6.4*	98.8 ± 4.9*	112.9 ± 8.6*	100.5 ± 6.7*
TG (mg/dL)	60.6 ± 4.6	112.1 ± 7.7*	92.5 ± 6.6*	108.1 ± 5.5*	93.2 ± 9.9*
LDL-C (mg/dL)	36.0 ± 3.0	64.3 ± 4.8*	52.3 ± 4.3	62.5 ± 5.2*	52.4 ± 6.9
HDL-C (mg/dL)	30.7 ± 1.0	32.3 ± 1.2	32.1 ± 1.4	31.6 ± 1.1	32.4 ± 1.2

Data are mean ± SEM.
TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
* Significant difference compared to the control group. n = 10.

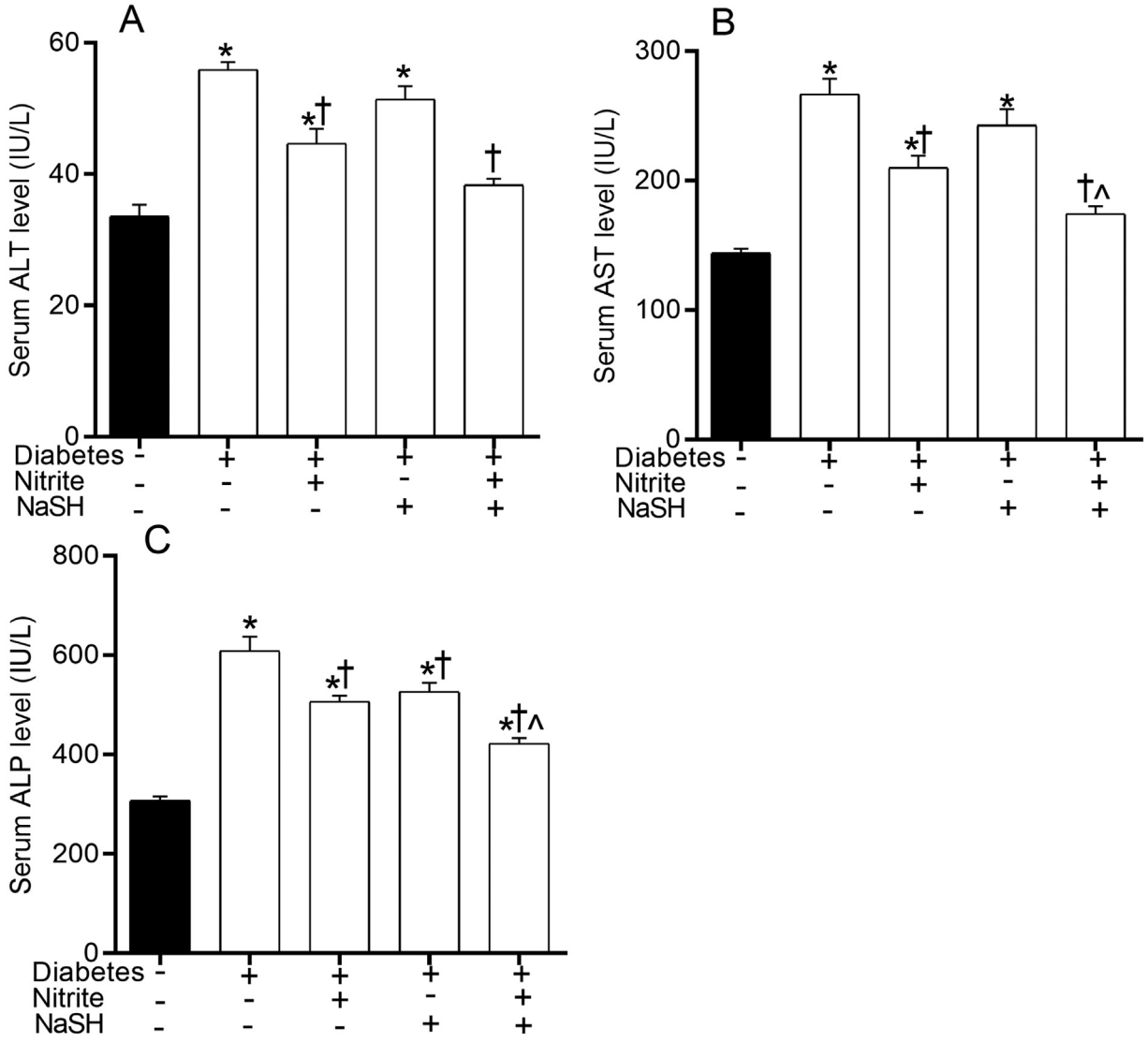

Fig. 8. Effect of nitrite and NaSH administration on serum levels of alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B), and alkaline phosphatase (ALP) (C). Results are mean ± SEM (n = 10/group). *Significant difference compared to the control group, †Significant difference compared to non-treated diabetic rats, ^Significant difference compared to nitrite-treated diabetic rats.

Table 3
Changes in serum oxidants and antioxidants levels following 9 weeks of nitrite and NaSH administration.

	Animal group				
	Control	Diabetes	Diabetes + nitrite	Diabetes + NaSH	Diabetes + nitrite + NaSH
TAC (μM)	163.7 ± 10.9	107.7 ± 3.7*	132.8 ± 8.1	120.5 ± 6.9*	155.9 ± 9.5[a]
SOD (U/mL)	48.79 ± 3.56	27.76 ± 2.90*	36.81 ± 4.50	31.05 ± 3.07*	45.09 ± 2.34[a]
CAT (U/L)	9.0 ± 0.8	3.5 ± 0.5*	5.2 ± 0.6*	3.9 ± 0.4*	7.1 ± 0.6[a, b]
Uric acid (mg/dL)	1.67 ± 0.08	2.17 ± 0.09*	2.02 ± 0.04*	2.07 ± 0.09*	1.91 ± 0.07
GSH (μM)	68.1 ± 7.6	85.0 ± 9.3	95.8 ± 9.7	91.5 ± 8.6	92.5 ± 6.0
GSSG (μM)	18.2 ± 1.0	52.8 ± 4.6*	61.0 ± 4.5*	54.1 ± 5.9*	55.9 ± 5.8*
GSH/GSSG	3.7 ± 0.4	1.6 ± 0.1*	1.5 ± 0.1*	1.8 ± 0.2*	1.7 ± 0.1*
TOS (μM)	35.8 ± 3.7	164.2 ± 13.8*	137.4 ± 14.9*	153.0 ± 12.7*	140.7 ± 13.5*
MDA (μM)	2.9 ± 0.21	7.09 ± 0.78*	5.24 ± 0.53	4.82.0 ± 0.55	5.28 ± 0.63

Data are mean ± SEM.
TAC, total antioxidant capacity; SOD, superoxide dismutases; CAT, catalase; GSH, glutathione; GSSG, oxidized glutathione; TOS, total oxidant status; MDA, malondialdehyde.
*Significant difference compared to the control group.
[a] Significant difference compared to non-treated diabetic rats.
[b] Significant difference compared to nitrite-treated diabetic rats. n = 10/group.

Table 4
Changes in tissue oxidants and antioxidants levels following 9 weeks of nitrite and NaSH administration.

	Animal group				
	Control	Diabetes	Diabetes + nitrite	Diabetes + NaSH	Diabetes + nitrite + NaSH
Liver					
TAC (mM)	1.10 ± 0.08	0.45 ± 0.04*	0.80 ± 0.05*,[a]	0.49 ± 0.05*	0.82 ± 0.05*,[a]
CAT (U/L)	20.40 ± 1.70	5.60 ± 0.46*	13.33 ± 1.07*,[a]	8.60 ± 0.94*	17.01 ± 1.57[a]
GSH (μM)	214.7 ± 12.2	121.4 ± 7.1*	204.8 ± 10.3[a]	176.7 ± 6.6	360.6 ± 13.4*,[a,b]
GSSG (μM)	142.4 ± 9.4	246.5 ± 11.3*	159.2 ± 8.9[a]	227.5 ± 12.7*	235.2 ± 10.8*,[b]
GSH/GSSG	1.5 ± 0.1	0.48 ± 0.04*	1.3 ± 0.2[a]	0.83 ± 0.1*	1.4 ± 0.1[a]
MDA (μM)	3.0 ± 0.4	8.6 ± 0.7*	5.12 ± 0.65[a]	7.4 ± 1*	3.0 ± 0.3[a]
Soleus muscle					
TAC (mM)	0.82 ± 0.08	0.44 ± 0.04*	0.63 ± 0.04	0.42 ± 0.04*	0.74 ± 0.05[a]
CAT (U/L)	15.38 ± 1.3	7.37 ± 0.67*	12.48 ± 1.30	6.83 ± 1.13*	20.89 ± 2.37[a, b]
GSH (μM)	199.1 ± 9.9	209.0 ± 12.4	309.9 ± 14.1*,[a]	237.2 ± 11.6	314.9 ± 14.2*,[a]
GSSG (μM)	113.8 ± 7.8	225.0 ± 9.9*	239.7 ± 12.0*	233.2 ± 12.7*	183.6 ± 10.6*,[b]
GSH/GSSG	1.8 ± 0.09	0.90 ± 0.09*	1.3 ± 0.15	1.0 ± 0.10*	1.8 ± 0.20[a,b]
MDA (μM)	5.92 ± 0.49	8.98 ± 0.76*	5.04 ± 0.64[a]	6.54 ± 0.66[a]	4.49 ± 0.50[a]
Adipose tissue					
TAC (mM)	0.18 ± 0.02	0.09 ± 0.01*	0.13 ± 0.04	0.10 ± 0.02*	0.12 ± 0.04
CAT (U/L)	6.96 ± 1.06	4.81 ± 0.56	5.94 ± 0.61	6.08 ± 0.69	10.77 ± 0.79*,[a, b]
GSH (μM)	163.3 ± 9.5	192.1 ± 10.3	191.2 ± 8.9	198.3 ± 11.7	319.1 ± 14.9*,[a,b]
GSSG (μM)	106.1 ± 5.94	235.2 ± 15.84*	191.5 ± 8.42*,[a]	176.5 ± 11.54*,[a]	156.3 ± 11.53*,[a]
GSH/GSSG	1.5 ± 0.12	0.79 ± 0.08*	0.88 ± 0.08*	0.76 ± 0.11*	1.5 ± 0.11[a,b]
MDA (μM)	4.72 ± 0.71	5.6 ± 0.5	4.3 ± 0.5	4.92 ± 0.36	4.2 ± 0.5

Data are mean ± SEM. n = 9/group.
TAC, total antioxidant capacity; CAT, catalase; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde.
*Significant difference compared to the control group.
[a] Significant difference compared to non-treated diabetic rats.
[b] Significant difference compared to nitrite-treated diabetic rats.

decreased MDA level ($p < 0.01$). In the adipose tissue, nitrite restored TAC level while decreased GSSG level ($p = 0.061$). NaSH administration *per se* decreased MDA level ($p = 0.081$) in the soleus muscle and GSSG level ($p < 0.01$) in the adipose tissue but had no effect on other tissue oxidants and antioxidants levels. Co-administration of NaSH and nitrite resulted in an improvement in TAC levels in the soleus muscle, GSH levels in the liver and adipose tissue ($p < 0.001$); CAT activity in the liver and adipose tissue ($p < 0.01$) and GSH/GSSG ratio in the soleus muscle and adipose tissue ($p = 0.064$ and $p < 0.01$, respectively) with a further decrease in liver GSSG levels ($p < 0.05$) (Table 4).

4. Discussion

This study is the first to show that long-term NaSH administration can potentiate the protective metabolic effects of nitrite in obese type 2 diabetic rats. Mechanistically, these favorable effects on carbohydrate metabolism, at least in part, are associated with increased expression of GLUT4 in insulin sensitive tissues as well as improved antioxidant status and liver function.

In this study, nitrite but not NaSH decreased weight gain in diabetic rats. Decrease in body weight gain has been reported following nitrate/nitrite administration in diabetic mice [28] and rats [4,5], eNOS-deficient mice [1], and white rabbits [29]. NO derived from nitrite stimulates mitochondria biogenesis and browning of white adipose tissues [30,31], both of which contribute to lowering of body weight. The inability of NaSH to decrease weight gain may be due to the dose used, as well as species and sex differences as reported by Drorman et al. [32].

In this study, diabetic rats had lower serum total sulfide and unchanged serum NO_x levels. Despite decreased eNOS-derived NO in diabetes [28,33], literature reports regarding the changes in serum NO_x concentrations are controversial as increased [34,35], no change [36,37] or even decreased [38] levels have been reported. Using circulating NO_x levels as means to estimate NO synthesis is disputable [21,39], as the true contribution of the NOS isoforms to circulating nitrite and nitrate concentration has not yet been clarified [39,40]. This controversy also applies to serum sulfide levels as, increased [41,42], decreased [3,43], as well as unchanged [44] blood levels of H_2S have been reported in diabetic patients [3,41,43], and animals [42,44,45].

In this study, mRNA expression of CSE and CBS decreased in the liver, skeletal muscle, and adipose tissue while mRNA expression of 3-MST increased in the liver and skeletal muscle. There are conflicting reports regarding mRNA expression and protein levels of H_2S-producing enzymes in diabetes as increased [42], decreased [46,47], and no change [48] in CSE [42,47] and CBS [42] expression have been reported in diabetic rats [42,47] and mice [46]. A possible explanation for the increased mRNA expression of 3-MST in diabetic rats may be due to compensation; which has previously been proposed [49]. Hyperglycemia and increased reactive oxygen species (ROS) decrease CSE expression [47] and increase H_2S consumption [50], leads to lower H_2S levels, as supported by our findings. In this study, nitrite increased serum NO_x and total sulfide while NaSH *per se* had no effect on serum total sulfide levels but increased serum NO_x. However, combined treatment further increased NO_x but not sulfide. Although controversial, it has been reported that H_2S increases eNOS activity [12] and NO release from nitrite [13]. Likewise, NO increases H_2S production by increasing the activity and expression of CSE [15,16] that may explain increased serum sulfide in diabetic rats following nitrite supplementation, observed in our study.

In this study, nitrite decreased elevated serum glucose and insulin, improved glucose tolerance, and increased GLUT4 mRNA expression, but NaSH *per se* had no effects on these parameters. In line with our results, decreased blood glucose and insulin [4,28], improved glucose tolerance [4,51], and increased GLUT4 expression [4,5] have been reported following nitrate/nitrite administration. The favorable effects of nitrite on carbohydrate metabolism is also attributed to increased islet blood flow [52] as well as *S*-nitrosylation of glucokinase and syntaxin 4, which increase insulin secretion [53].

Few studies have evaluated effects of H_2S on carbohydrate metabolism in T2D. These reports are essentially dichotomous in nature as

worsened [20,54], improved [54], or even no changes [20,55] in blood glucose levels or in glucose tolerance have been documented. These discrepancies may be due to the differences in the duration of treatment, species employed, the dose and type of H_2S donor used, and the animal models studied. We recently demonstrated that NaSH at high doses (1.6–5.6 mg/kg) aggravates carbohydrate metabolism while at low doses (0.28 and 0.56 mg/kg) it had no effect on these parameters using the same animal model of T2D [20]. In pancreatic β-cells, H_2S decreases insulin secretion by opening K_{ATP} channels [56], which is consistent with our recent report that H_2S decreases insulin secretion dose-dependently [20]. Based on our previous observations [20] and given that H_2S at low doses has no effect on vascular relaxation, but that it potentiates the vasorelaxant effects of NO [14], we selected the lowest dose of NaSH (0.28 mg/kg/day equals to ~0.75 μmol/rat/day) which had no effect on carbohydrate metabolism to evaluate its effects in combination with nitrite.

In our study, NaSH potentiated the favorable effects of nitrite on serum insulin, GTT, PTT, and GLUT4 expression. Although there is no study addressing the co-administration effects of NO and H_2S on carbohydrate metabolism, some studies have however shown interactions between these two gassotransmitter at the levels of both biosynthesis and biological responses in the cardiovascular system [11,57]. H_2S increases eNOS activity [12] and stimulates NO release from nitrite via increasing xanthine oxidoreductase activity [13]. In addition, H_2S maintains soluble guanylate cyclase (sGC) in an NO-activatable form [58] and decreases degradation of cyclic guanosine monophosphate (cGMP) via inhibition of phosphodiesterase [10,11], and therefore increases cGMP, which increases insulin secretion. Hence, it can be suggested that H_2S at low concentrations stimulates NO production and potentiates the favorable effects of nitrite on carbohydrate metabolism.

In the present study, nitrite but not NaSH *per se* improved liver function; however, NaSH potentiated the favorable effects of nitrite. In line with our results, decreased serum AST [59–61] and ALT [59,61] have been reported following nitrite administration in a murine model of hepatic ischemia reperfusion injury. In addition, increased plasma AST and ALT in cirrhotic rats is associated with decreased plasma H_2S levels [62], which could be improved by NaSH treatment [63]. By contrast, NaSH aggravates LPS-induced liver damage and increases serum AST and ALT levels [64]. The protective effects of NO and H_2S on liver function is attributed to their anti-oxidative and anti-inflammatory actions [6,63,65]. Increased oxidative stress, as well as aberrant inflammatory responses are crucial factors in liver damage [63]. The vasodilatory activities of NO [5] and H_2S [15] may also contribute to this protective property, as NO/H_2S-induced vasorelaxation can improve microcirculation within the liver, which will help the liver to remove excessive lipid peroxides. In addition, since H_2S potentiates the vasorelaxetory effects of NO [14], further improvement in liver function by co-administration of nitrite and NaSH can be expected. Regarding association between liver function and carbohydrate metabolism, improved liver function with co-administration of nitrite and NaSH may contribute to improved carbohydrate metabolism in our study.

Nitrite supplementation in our study partially restored elevated serum levels of LDL-C in the diabetic rats, which is in agreement with previous studies [1,38]. This may be due to increased fat metabolism or energy utilization and inhibition of acetyl CoA carboxylase by NO [66]. H_2S deficiency is also involved in pathogenesis of lipid disorders as CSE-knockout mice have elevated plasma levels of TC and LDL-C [67].

Oxidative stress has been crucially linked to the development of T2D and associated complications. In the present study, NaSH potentiated the anti-oxidative effects of nitrite in the diabetic rats, whereas NaSH *per se* had no effect. These favorable effects on redox balance following nitrate/nitrite supplementation are in agreement with previous reports in obese T2D models [5,6,68]. Unlike our study, improvements in the anti-oxidant status have been reported following treatments using H_2S-donors in both lean (Goto-Kakizaki rat) and obese (db/db mouse) models of T2D models [45,54]. H_2S increases NO release from nitrite [13] and also increases sulfinyl nitrite (an NO donor) production [69], suggesting that nitrite in the presence of H_2S becomes more biologically active.

Regarding strengths of this study, as rodents do not as efficiently concentrate circulating nitrate in saliva as in humans [70], a low dose of sodium nitrite supplementation instead of sodium nitrate was used. In addition, the HFD-STZ model of T2D, which exhibits the metabolic characteristics of human T2D was used; although no animal model is perfect, HFD causes insulin resistance and the low-dose of STZ causes partial β-cells dysfunction [4]. This study has however some limitations: First, we did not measure eNOS expression/activity, cGMP levels, GLUT4 translocation, or inflammatory cytokines, all of which can be affected by nitrite and NaSH. Second, the methylene blue method, which is commonly used for determination of H_2S levels in biological systems [71], was used. This method measures all sulfur compounds and does not specifically quantify H_2S levels, which may have impacted on the interpretation of the data. In fact, there is accumulating evidence indicating that the physiological levels of H_2S are actually in the submicromolar range or even lower [72]. Finally, some favorable effects of co-administrations of nitrite and NaSH may be due to the producing some intermediate reactants [73–75], which we did not measure in the current study.

In conclusion, long-term administration of low dose NaSH, potentiated the beneficial effects of nitrite on metabolic functions and associated complications in rats with T2D. Although further mechanistic studies are warranted, these favorable effects were associated with dampening of oxidative stress and increased GLUT4 expression in insulin-sensitive tissues as well as improvement of liver function.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.08.006.

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