

Article

# Effects of Hydrogen Sulfide on Carbohydrate Metabolism in Obese Type 2 Diabetic Rats

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**Abstract:** Hydrogen sulfide (H<sub>2</sub>S) is involved in the pathophysiology of type 2 diabetes. Inhibition and stimulation of H<sub>2</sub>S synthesis has been suggested to be a potential therapeutic approach for type 2 diabetes. The aim of this study was therefore to determine the effects of long-term sodium hydrosulfide (NaSH) administration as a H<sub>2</sub>S releasing agent on carbohydrate metabolism in type 2 diabetic rats. Type 2 diabetes was established using high fat-low dose streptozotocin. Rats were treated for 9 weeks with intraperitoneal injections of NaSH (0.28, 0.56, 1.6, 2.8, and 5.6 mg/kg). Serum glucose was measured weekly for one month and then at the end of the study. Serum insulin was measured before and after the treatment. At the end of the study, glucose tolerance, pyruvate tolerance and insulin secretion were determined and blood pressure was measured. In diabetic rats NaSH at 1.6–5.6 mg/kg increased serum glucose (11%, 28%, and 51%, respectively) and decreased serum insulin, glucose tolerance, pyruvate tolerance and in vivo insulin secretion. In controls, NaSH only at 5.6 mg/kg increased serum glucose and decreased glucose tolerance, pyruvate tolerance and insulin secretion. Chronic administration of NaSH in particular at high doses impaired carbohydrate metabolism in type 2 diabetic rats.

**Keywords:** diabetes; H<sub>2</sub>S; glucose tolerance; blood pressure

## 1. Introduction

Hydrogen sulfide (H<sub>2</sub>S), a novel gasotransmitter, is involved in many (patho)physiological processes [1]. H<sub>2</sub>S exerts its well-known effect, i.e., decreasing blood pressure, mostly by opening ATP-sensitive potassium channels (K<sub>ATP</sub>) in vascular smooth muscle cells [2]. K<sub>ATP</sub> channels also play a key role in insulin secretion in pancreatic  $\beta$ -cells [3], where opening of the channels by H<sub>2</sub>S decreases insulin secretion [4]. Both endogenous and exogenous H<sub>2</sub>S inhibit insulin secretion from  $\beta$ -cells by activation of K<sub>ATP</sub> channels and inhibition of L-type voltage-dependent calcium channels [5]. In addition, through inhibition of glucose transporter-4 (GLUT-4), H<sub>2</sub>S inhibits insulin-stimulated glucose uptake in white adipocytes in rat, indicating that H<sub>2</sub>S decreases insulin sensitivity of adipocytes [6]. In Zucker diabetic fatty rats, the rate of endogenous H<sub>2</sub>S production is higher than non-diabetic rats and inhibition of H<sub>2</sub>S production restores hyperglycemia to near normal levels [7]. Inhibition of H<sub>2</sub>S synthesis has been therefore suggested to be a potential therapeutic approach for insulin resistance states and also for protecting  $\beta$ -cells during the induction phase of diabetes [8].

On the other hand, type 2 diabetes is a state of H<sub>2</sub>S deficiency [9–12] and H<sub>2</sub>S protects  $\beta$ -cells against glucotoxicity-induced apoptosis [13]. Chondroitin sulfate prebiotic diet, which increases H<sub>2</sub>S levels in colon, stimulates glucagon like peptide-1 (GLP-1) secretion, which has anti-diabetic

actions [14]. H<sub>2</sub>S up-regulates insulin signaling pathways essential for glucose utilization [15] and mediates the effects of vitamin D on translocation of GLUT-4 in adipocytes [16]. These data are in favor of boosting H<sub>2</sub>S as a treatment for type 2 diabetes.

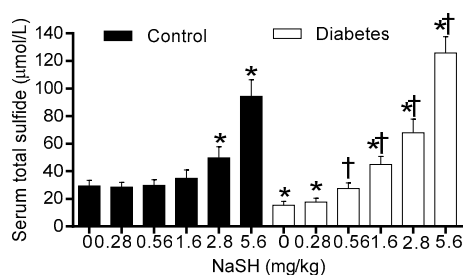
To our knowledge, there is only one in vivo study with the aim of assessing H<sub>2</sub>S effects on carbohydrate metabolism in type 2 diabetic rats [17]; results indicate decreased plasma glucose at a low dose of sodium hydrosulfide (NaSH) and increased plasma glucose at a high dose. Other studies, all involving db/db mice and assessing the effects of H<sub>2</sub>S administration on cardiac function [18–20] or learning and memory [21], have reported beneficial effects on glucose metabolism [20], no effect on blood glucose levels [18,19,21], or glucose tolerance [19,21].

Whether H<sub>2</sub>S has beneficial or deleterious effects on carbohydrate metabolism in type 2 diabetes has not been answered yet and before suggesting strategies to augment/inhibit H<sub>2</sub>S levels in clinic, further in vivo animal studies are needed considering long-term and dose-dependent effects of exogenous H<sub>2</sub>S. The aim of this study was therefore to determine effects of long-term administration of different doses of NaSH, a H<sub>2</sub>S donor, on carbohydrate metabolism in type 2 diabetic rats.

## 2. Results

### 2.1. Effect of NaSH on Serum Total Sulfide Levels

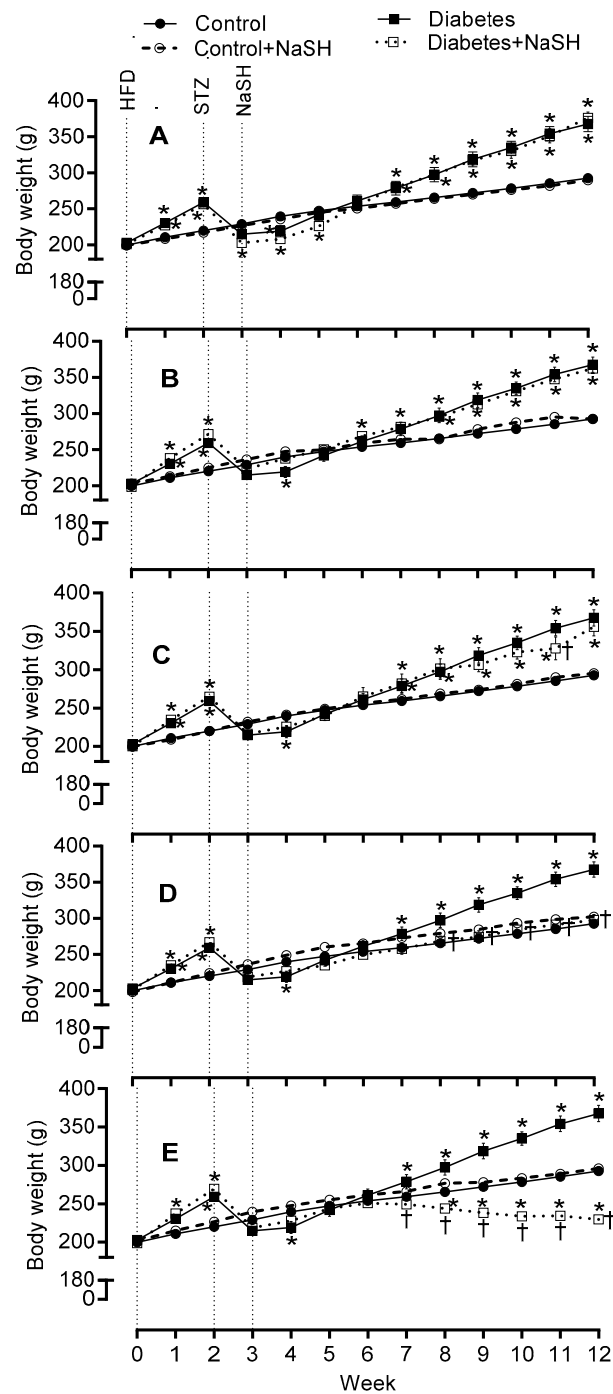
Diabetic rats had lower total serum sulfide levels than controls ( $16.5 \pm 2.8$  vs.  $28.3 \pm 2.4$   $\mu\text{mol/L}$ ,  $p = 0.001$ ). NaSH administration increased total serum sulfide levels in control rats at doses 2.8 and 5.6 mg/kg and in diabetic rats at doses 0.56 mg/kg and higher (Figures 1 and S1).



**Figure 1.** Effect of NaSH on serum total sulfide levels in control and diabetic rats. Rats were treated daily with different concentrations of NaSH for 9 weeks. Results are mean  $\pm$  SEM ( $n = 10$ /group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

### 2.2. Effect of NaSH on Body Weight, Water Consumption, and Food Intake

Body weights were comparable in all groups before starting the high fat diet (HFD). HFD consumption for 2 weeks caused a significant ( $p < 0.001$ ) increase in body weights, but streptozotocin (STZ) injection to the HFD-fed rats resulted in a significant ( $p < 0.001$ ) reduction in the body weights. Before starting the NaSH administration, the body weights were similar between all groups. During 9 weeks of NaSH administration, the body weights decreased gradually in the NaSH-treated diabetic rats and these decreases were significant at the doses of 2.8 and 5.6 mg/kg. The differences in body weight gain were significant from weeks 7 and 8 in NaSH-treated diabetic rats at 5.6 and 2.8 mg/kg, respectively, and were observed throughout the remainder of the study (Figures 2 and S2).

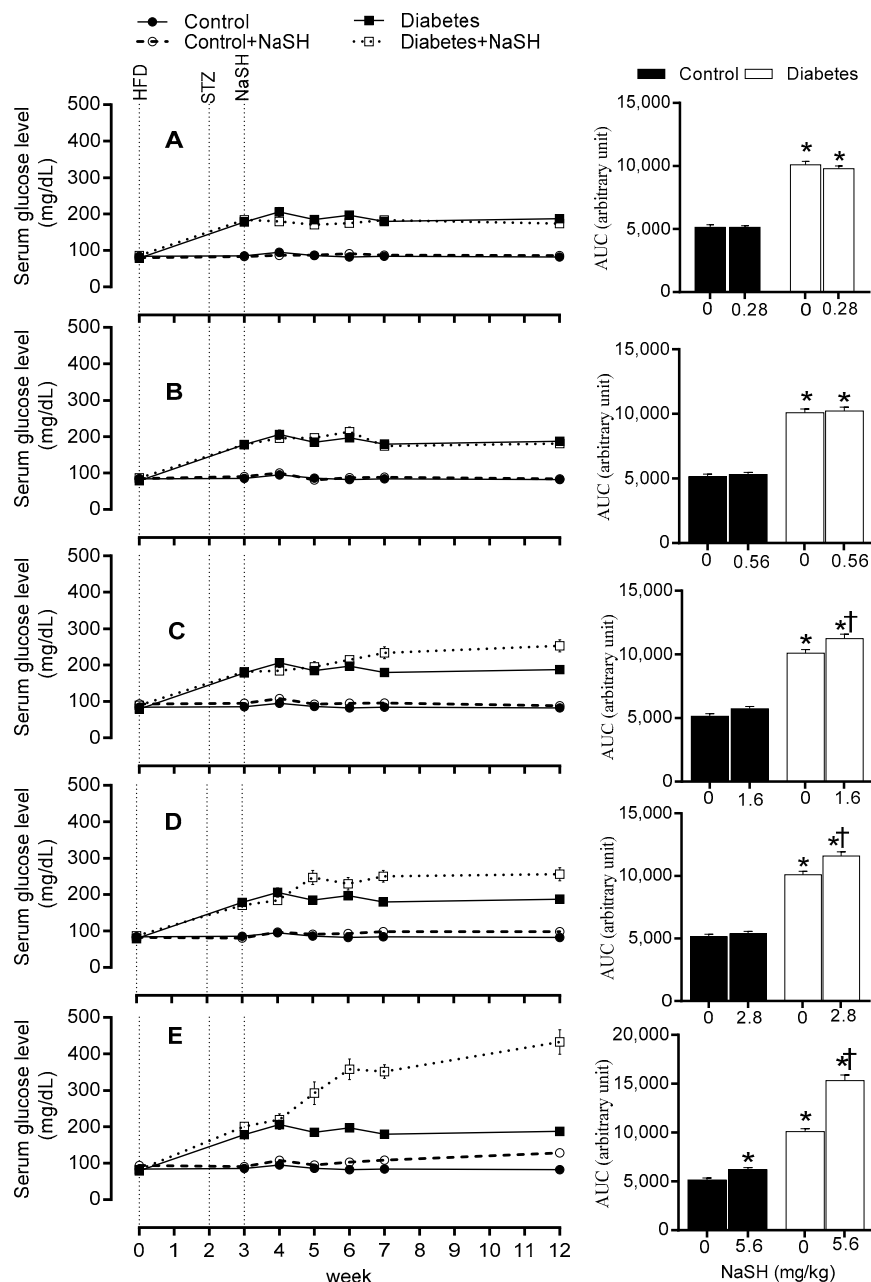


**Figure 2.** Effects of NaSH on body weight at 0.28 (A), 0.56 (B), 1.6 (C), 2.8 (D), and 5.6 (E) mg/kg. Results are mean  $\pm$  SEM ( $n = 10$ /group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

As expected, in the diabetic rats, water consumption (Figures S3 and S4) and calorie intake (Figures S5 and S6) were significantly higher, while food intake (Figures S7 and S8) was significantly lower than the control group. NaSH-treated diabetic rats at 2.8 and 5.6 mg/kg displayed higher water consumption and lower food and calorie intake. NaSH administration in control rats had no effects on body weight, food and calorie intakes, or water consumption.

### 2.3. Effect of NaSH on Serum Glucose Concentration

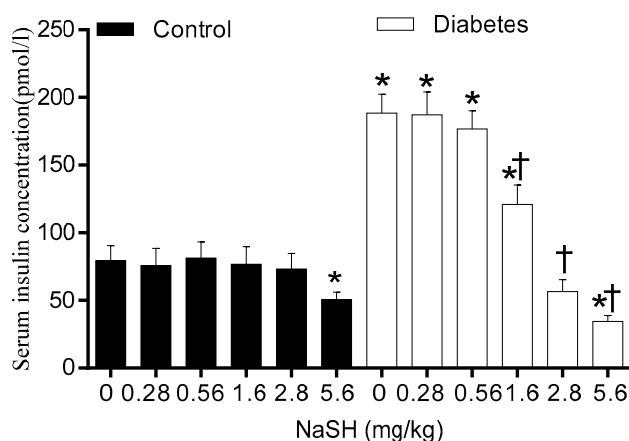
Compared to the control group, diabetic rats had higher serum glucose levels before NaSH treatment (178.6 vs. 85.5 mg/dL,  $p < 0.001$ ). After 9 weeks of NaSH administration at 1.6, 2.8, and 5.6 mg/kg, serum glucose concentrations increased significantly (253.0, 255.9, and 432.8 vs. 187.6 mg/dL, respectively,  $p < 0.001$ ) and these became evident at weeks 4 (233.9 vs. 180.0 mg/dL,  $p = 0.001$ ), 2 (247.3 vs. 184.9 mg/dL,  $p < 0.001$ ), and 2 (292.4 vs. 184.9 mg/dL,  $p < 0.001$ ), respectively. NaSH at 0.28 and 0.56 mg/kg, had no effect on serum glucose levels in diabetic rats. In the control rats, only the highest dose of NaSH (5.6 mg/kg) (128.3 vs. 82.5 mg/dL,  $p = 0.001$ ) increased serum glucose concentrations at week 9 (Figures 3 and S9).



**Figure 3.** Effects of NaSH at 0.28 (A), 0.56 (B), 1.6 (C), 2.8 (D), and 5.6 (E) mg/kg on serum glucose concentrations. Area under the curves (from week 3 to week 12) are shown in columns on the right. Results are mean  $\pm$  SEM (n = 10/group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

#### 2.4. Effect of NaSH on Serum Insulin Concentrations

Before starting NaSH treatment, serum insulin concentrations were comparable between the control and the diabetic rats (Figure S10). Continuing HFD increased serum insulin concentrations in non-treated diabetic rats ( $188.40 \pm 14.09$  vs.  $79.31 \pm 11.31$  pmol/L,  $p < 0.001$ ). In the diabetic rats, NaSH administration at 1.6, 2.8, and 5.6 mg/kg significantly decreased serum insulin levels ( $120.9 \pm 14.3$ ,  $56.6 \pm 8.7$ , and  $34.5 \pm 4.3$  vs.  $188.4 \pm 14.1$ , pmol/L  $p < 0.001$ , respectively), whereas this was not changed at 0.28 and 0.56 mg/kg. In the control rats, only the highest dose of NaSH (5.6 mg/kg) significantly decreased serum insulin levels ( $50.4 \pm 5.8$  vs.  $79.3 \pm 11.3$  pmol/L,  $p < 0.001$ ) (Figure 4).



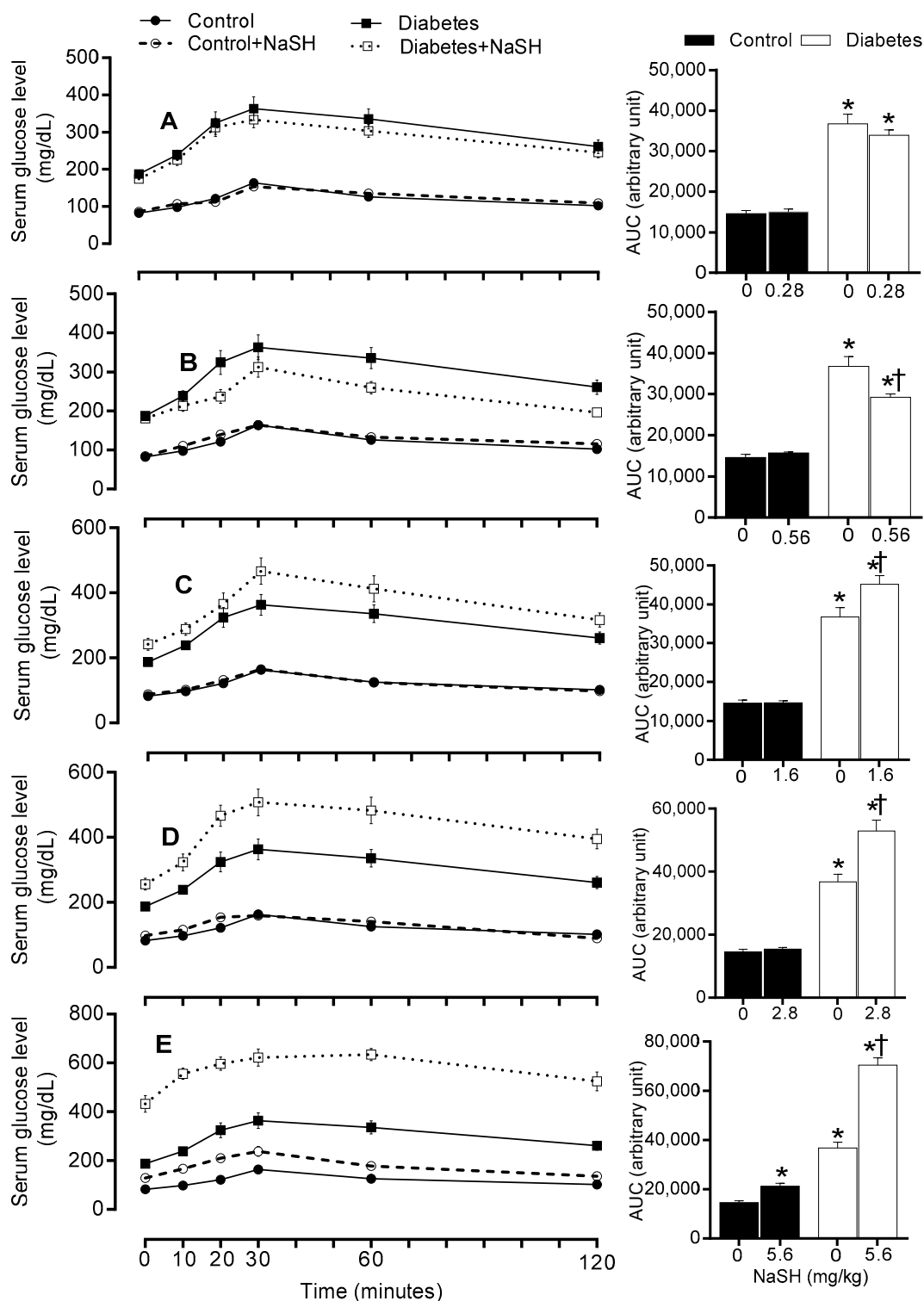
**Figure 4.** Effect of NaSH administration on fasting serum insulin levels at the end of the study. Results are mean  $\pm$  SEM ( $n = 8$ /group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

#### 2.5. Effect of NaSH on Glucose Tolerance

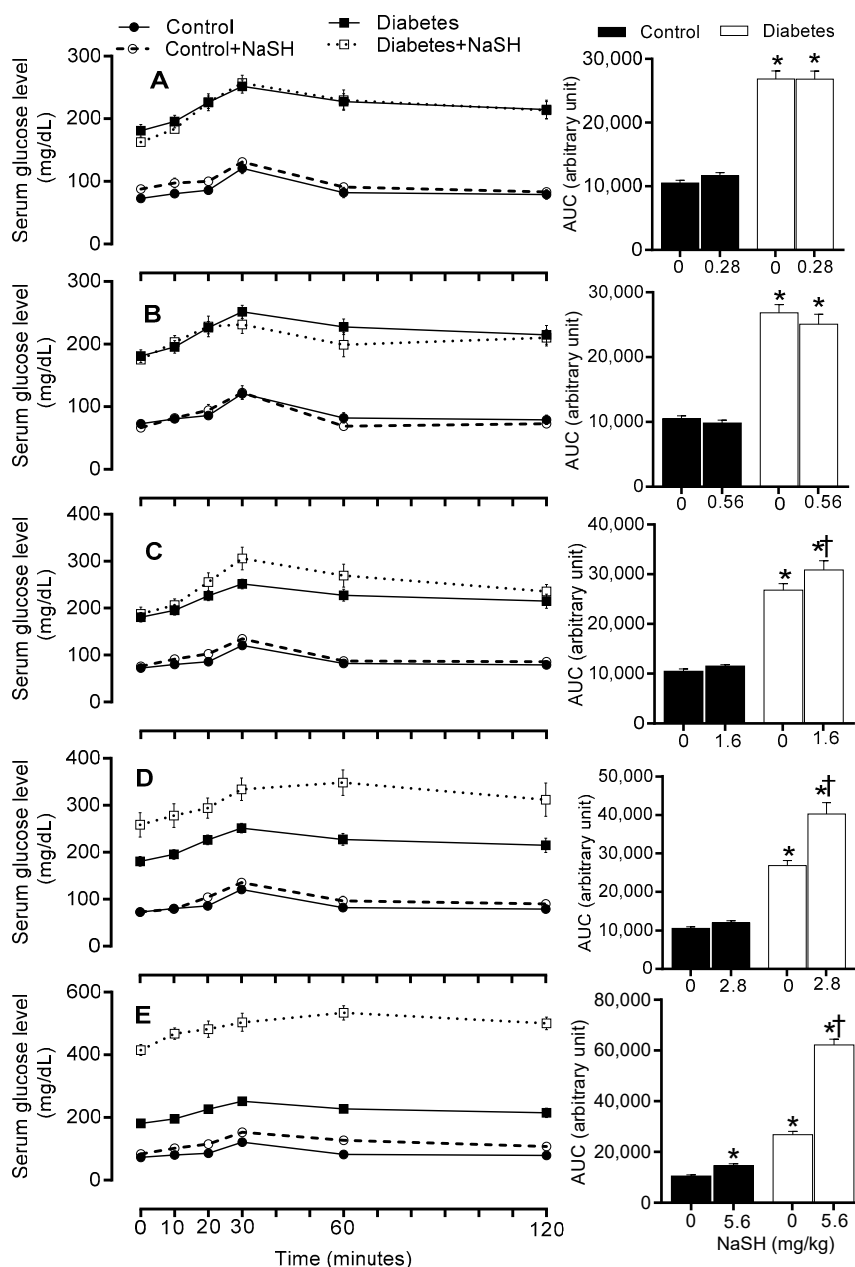
As shown in Figures 5 and S11, compared to the control rats, non-treated diabetic rats had impaired glucose tolerance ( $p < 0.001$ ). Chronic NaSH administration at 1.6, 2.8, and 5.6 mg/kg, impaired glucose tolerance in diabetic rats ( $p < 0.001$ ), while improved it at 0.56 mg/kg ( $p = 0.009$ ) and had no effect at 0.28 mg/kg. In the control rats, only the highest dose of NaSH impaired glucose tolerance ( $p < 0.001$ ).

#### 2.6. Effect of NaSH on Gluconeogenesis

To investigate the ability of NaSH treatment to modulate gluconeogenesis, pyruvate tolerance test (PTT) was performed. In non-treated diabetic rats, pyruvate administration, a gluconeogenic substrate precursor, caused higher glucose production compared to the control rats (AUC:  $26844 \pm 1286.0$  vs.  $10523 \pm 435.1$ ,  $p < 0.001$ ). Chronic NaSH administration at 1.6 (AUC:  $30876 \pm 1848$  vs.  $26844 \pm 1286$ ,  $p = 0.048$ ), 2.8 (AUC:  $40241 \pm 2955$  vs.  $26844 \pm 1286$ ,  $p < 0.001$ ), and 5.6 (AUC:  $62190 \pm 2303$  vs.  $26844 \pm 1286$ ,  $p < 0.001$ ) mg/kg, increased serum glucose concentration during PTT in diabetic rats, while it had no effect at 0.28 and 0.56 mg/kg. In controls, NaSH only at 5.6 mg/kg significantly (AUC:  $14598 \pm 730.2$  vs.  $10523 \pm 435.1$ ,  $p = 0.046$ ) impaired pyruvate tolerance (Figures 6 and S12).



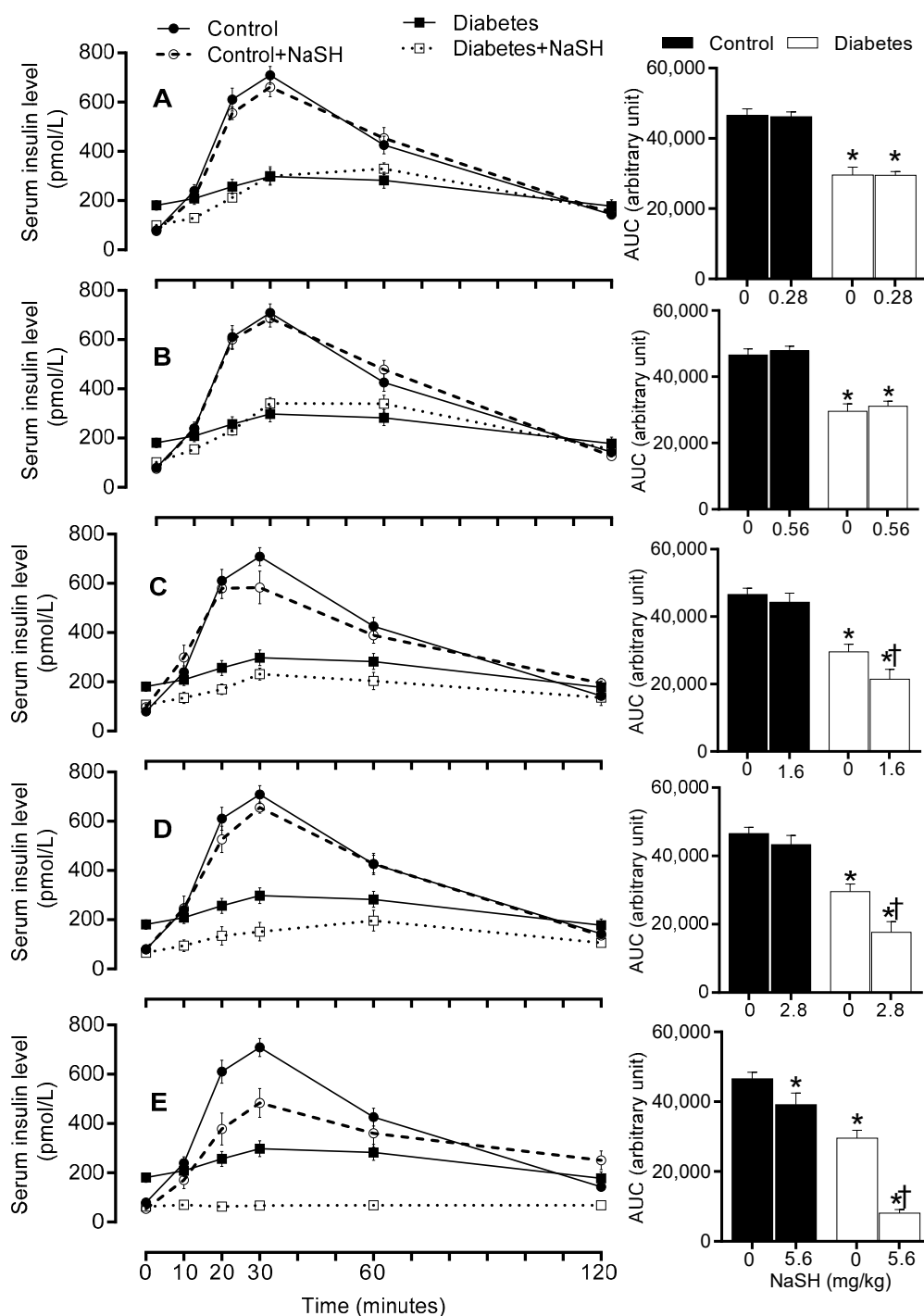
**Figure 5.** Effects of NaSH on glucose tolerance at 0.28 (A), 0.56 (B), 1.6 (C), 2.8 (D), and 5.6 (E) mg/kg. Area under the curves are shown in columns on the right. Results are mean  $\pm$  SEM (n = 10/group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.



**Figure 6.** Effects of NaSH on pyruvate tolerance at 0.28 (A), 0.56 (B), 1.6 (C), 2.8 (D), and 5.6 (E) mg/kg. Area under the curves are shown in columns on the right. Results are mean  $\pm$  SEM (n = 10/group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

### 2.7. Effect of NaSH on In Vivo Insulin Secretion

We next measured serum insulin levels during glucose tolerance test (GTT) to determine the effects of NaSH on in vivo insulin secretion. In non-treated diabetic rats, increases in insulin secretion during GTT, were lower than the control rats (AUC:  $29582 \pm 2235$  vs.  $46553 \pm 1857$ ,  $p < 0.001$ ); NaSH administration for 9 weeks at 1.6 (AUC:  $21459 \pm 2938$  vs.  $29582 \pm 2235$ ,  $p = 0.026$ ), 2.8 (AUC:  $17668 \pm 3085$  vs.  $29582 \pm 2235$ ,  $p = 0.002$ ), and 5.6 (AUC:  $8112 \pm 1000$  vs.  $29582 \pm 2235$ ,  $p < 0.001$ ) mg/kg, decreased insulin response to glucose injection in the diabetic rats. In controls, NaSH only at 5.6 mg/kg significantly (AUC:  $39150 \pm 3323$  vs.  $46553 \pm 1857$ ,  $p = 0.028$ ) decreased insulin response to glucose injection (Figures 7 and S13).

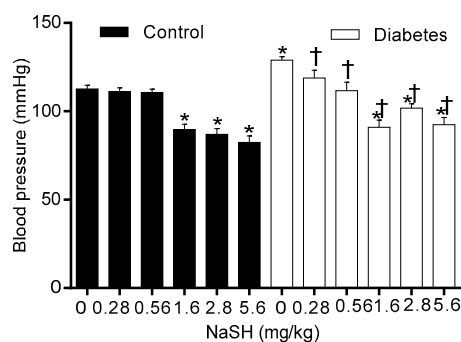


**Figure 7.** Effects of NaSH on in vivo insulin secretion at 0.28 (A), 0.56 (B), 1.6 (C), 2.8 (D), and 5.6 (E) mg/kg. Areas under the curves are shown in columns on the right. Results are mean  $\pm$  SEM ( $n = 8$ /group). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

### 2.8. Effect of NaSH on Blood Pressure

As shown in Figure 8, compared to the controls, non-treated diabetic rats had higher systolic blood pressure (SBP) ( $129.0 \pm 1.9$  vs.  $112.6 \pm 2.1$ ,  $p = 0.007$ ); NaSH administration for 9 weeks at all doses decreased SBP in diabetic rats. In controls, NaSH at 1.6, 2.8, and 5.6 mg/kg significantly decreased SBP.





**Figure 8.** Effects of NaSH on blood pressure. Results are mean  $\pm$  SEM ( $n = 9/\text{group}$ ). \*  $p < 0.05$  compared to non-treated control rats, †  $p < 0.05$  compared to non-treated diabetic rats.

### 3. Discussion

This study showed that chronic administration of high doses of NaSH aggravated carbohydrate metabolism in obese type 2 diabetic rats. NaSH administration at high doses increased serum glucose and impaired glucose tolerance and pyruvate tolerance, as well as decreased insulin secretion and serum insulin levels.

In this study, NaSH administration increased total sulfide levels in the serum of both control and diabetic rats, however, this increase was about 40% lower in the diabetic rats as compared to the corresponding controls. In this regard, literature reports are quite controversial as increased [22,23], decreased [10,18,24], and even no change [25] in blood  $\text{H}_2\text{S}$  levels have been reported in diabetic patients [10,22,24] and animals [18,23,25]. Our results may be explained in light of what has been reported in the literature where it has been shown that hyperglycemia results in an increase in  $\text{H}_2\text{S}$  consumption due to increases in reactive oxygen species [26]; moreover, the activities of  $\text{H}_2\text{S}$ -generating enzymes have been reported to be lower in diabetes [18] which results in lower  $\text{H}_2\text{S}$  levels which is in line with our observations reported here.

In this study, chronic NaSH administration in diabetic rats only at higher doses (2.8 and 5.6 mg/kg) inhibited weight gain, decreased food intake, and increased water consumption; NaSH administration, had however no effect on these parameters in the control rats. Few studies have reported effects of  $\text{H}_2\text{S}$  on body weight gain in type 2 diabetes; in line with our results in control rats and low dose of NaSH in diabetic rats, no change in body weight have been reported following  $\text{H}_2\text{S}$  donors administration in type 2 diabetic animals [18,20,21]. In addition, there was no significant differences in food intake in cystathionine  $\gamma$ -lyase-knockout (CSE-KO) mice [27] as well as in HFD-induced insulin resistance mice treated with the slow  $\text{H}_2\text{S}$ -donating compound, GYY4137 [28].

In the present study, higher doses (1.6, 2.8, and 5.6 mg/kg) of NaSH increased fasting serum glucose and decreased serum insulin levels in the diabetic rats. Few studies have assessed the effects of  $\text{H}_2\text{S}$  on glucose metabolism in type 2 diabetes; in line with our study, administration of high dose of NaSH (6.72 mg/kg for 6 weeks) to Goto-Kakizaki (GK) rats increased plasma glucose levels by 86.5% [17] (cf. with 115% increase in our study following 9 weeks of NaSH at 5.6 mg/kg).

In our study, diabetic rats exhibited insulin resistance as confirmed by 137% increase of fasting serum insulin in the presence of moderate stable hyperglycemia. Administration of NaSH dose-dependently decreased serum insulin levels in the presence of aggravated hyperglycemia, indicating decreased insulin secretion by pancreatic  $\beta$ -cells as previously reported in *in vitro* studies [4,29]. This result is also in agreement with decreased *in vivo* insulin secretion during GTT observed in our study. In our study, the highest dose of NaSH increased serum glucose and decreased serum insulin levels and *in vivo* insulin secretion in control rats. This is consistent with pathophysiology of type 2 diabetes with partial dysfunction of  $\beta$ -cells.  $\text{H}_2\text{S}$ -induced hyperglycemia is associated with decreased glucose-induced  $\text{Ca}^{2+}$  influx [29], activating pancreatic  $\beta$ -cell  $\text{K}_{\text{ATP}}$  channels [4], and decreased ATP production in the pancreatic  $\beta$ -cells [29]. Other explanations for

H<sub>2</sub>S-induced hyperglycemia include a decrease in peripheral glucose uptake and an increase in glycogenolysis [30].

In contrast to our results, Rong et al. reported a decrease in serum glucose concentrations following administration of NaSH at 1.68 mg/kg in GK type 2 diabetic rats [17]. Our model of obese type 2 diabetes (HFD-STZ) is characterized with a stable hyperglycemia (~15% fasting serum glucose fluctuation during 8 weeks) along with insulin resistance. However, non-obese GK rats in the study of Rong et al. did not show stable hyperglycemia and their fasting plasma glucose levels fluctuated by as much as 75% during the 10 weeks of their study. In addition, in the Rong et al. study, fasting plasma insulin at the end of their study was approximately one-third of the corresponding control rats, indicating the absence of insulin resistance as the main characteristic of type 2 diabetes. Indeed, it has been reported that  $\beta$ -cell defect is the primary defect in the GK rats [31]. These observations strongly suggest that the blood-glucose lowering effects of a low dose of H<sub>2</sub>S as has been reported by Rong et al. needs further verification in other models of type 2 diabetes.

Other studies that assessed the effects of H<sub>2</sub>S donors on blood glucose levels in type 2 diabetic animals are all in male db/db mice and have reported no effect on blood glucose concentrations using NaSH (4.5 mg/kg for 8 weeks) [21], Na<sub>2</sub>S (0.1 mg/kg for one week) [18], and NaSH (5.6 mg/kg for 12 weeks) [19]. These data showing that high doses of NaSH have no effect on blood glucose levels is in contrast to our results reported here. One potential explanation for this apparent discrepancy is the use of different species. In our study we used rats whereas others used mice. The lethal dose (LD<sub>50</sub>) of NaSH following intraperitoneal (IP) injection has been reported to be lower in male rats (i.e., 15 mg/kg) [32] compared to male mice (i.e., 25.2 mg/kg) [33]. In addition, mouse models may not be suitable for studying H<sub>2</sub>S effects, as has been reported in assessing neurological sequel of H<sub>2</sub>S [34]; this however remains to be determined as it pertains to the metabolic effects of H<sub>2</sub>S.

In our study, NaSH at high doses impaired glucose tolerance and increased gluconeogenesis (as measured by PTT) in diabetic rats. Unlike our results, no change [21] or even an increase [17,20] in glucose tolerance have been reported in db/db mice [20,21] and GK rats [17] following NaSH administration. In our study, decreased insulin secretion in response to exogenous glucose resulted in poor glucose clearance during GTT. Our study is the first to show increased gluconeogenesis in type 2 diabetic rats; this finding is in line with both in vitro and in vivo studies in non-diabetic conditions. In CSE-KO mice, glucose production in hepatocytes is lower [30] and NaSH administration (2.18 and 3.53 mg/kg) increases gluconeogenesis [35]. H<sub>2</sub>S donors increased gluconeogenesis in vitro by increasing activities of gluconeogenic enzymes [30,35].

In the present study, all doses of NaSH (0.28–5.6 mg/kg) decreased SBP in diabetic rats. In line with our results, antihypertensive properties of NaSH at 0.56–5.6 mg/kg have been reported in animal models of hypertension [36–40] and in one study in type 2 diabetic rats [17]. H<sub>2</sub>S decreases blood pressure mainly by the opening of vascular K<sub>ATP</sub> channels [41]; opening of these channels in pancreatic  $\beta$ -cells also decreases insulin secretion [4]. Our data indicate that effects of H<sub>2</sub>S on carbohydrate metabolism should be taken into account when favorable effects including its antihypertensive effect are intended.

Regarding strengths of this study, different doses of NaSH were administered for a long time. In addition, an animal model of diabetes (HFD-STZ) which closely reflects characteristics of human type 2 diabetes was used; in this model, feeding a HFD induces insulin resistance and a low dose of STZ partially destroys  $\beta$  cells [42]. This study however has some limitations; serum total sulfide level was measured by the methylene blue method which measures all sulfur compounds rather than only free H<sub>2</sub>S; this method is however one of the most commonly used methods for measuring H<sub>2</sub>S in biological systems [43]. In addition, we did not measure oxidative stress and GLUT-4 protein levels in insulin sensitive tissues; these factors affect glucose metabolism in type 2 diabetes.

In conclusion, the unfavorable effects of chronic administration of NaSH in particular at high doses on carbohydrate metabolism in type 2 diabetic rats are reported. Further studies are indeed

needed to clear the *in vivo* effects of different doses of H<sub>2</sub>S on carbohydrate metabolism in type 2 diabetes.

## 4. Materials and Methods

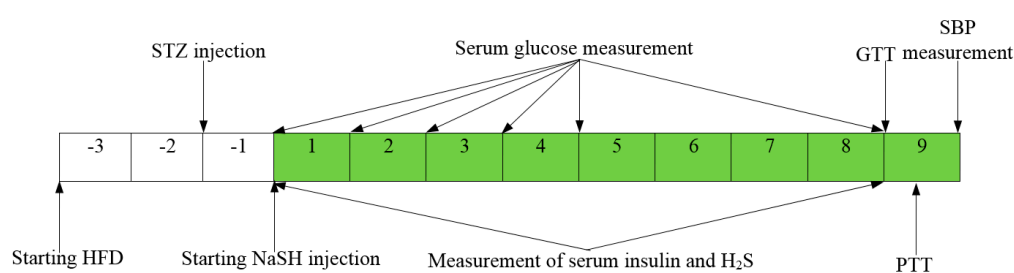
### 4.1. Animals and Induction of Diabetes

The care and use of laboratory animals was approved by the ethics committee of the National Institute for Medical Research Development (IR.NIMAD.REC.1396.307), Tehran, Iran. All animal treatments were carried out according to internationally approved guidelines for the use and care of laboratory animals. A total of 120 male Wistar rats (190–210 g) were housed under controlled conditions (23 ± 2 °C, relative humidity of 50 ± 6%, 12/12-h light-dark cycle). Rats had free access to water and a normal rat pellet diet with a total caloric value of ~3,100 kcal/kg, or a HFD with a total caloric value of ~4,900 kcal/kg.

Diabetes was induced using HFD-STZ as described previously [42]. After feeding the HFD for two weeks, a single IP injection of low dose of STZ (30 mg/kg dissolved in 0.1 mM citrate buffer, pH = 4.5; Sigma Aldrich, Hamburg, Germany) was done. One week after STZ injection, serum glucose levels were measured and rats with a fasting glucose levels ≥150 mg/dL were considered to be diabetic. The rats were allowed to continue on their respective diets for 9 weeks, when the study ended.

### 4.2. Experimental Design

Rats were randomly divided into 2 groups of 60 each: a control group and a diabetic group. Each group was sub-divided into 6 groups (n = 10) and treated daily with IP injection of NaSH (0.28, 0.56, 1.6, 2.8, and 5.6 mg/kg) or vehicle (normal saline) for 9 weeks. For minimizing oxidation of SH<sup>-</sup> (hydrosulfide anion) the solutions were prepared and injected immediately (within 15 min). Body weight (using Tefal Scale; sensitivity 1 g), food intake (g/day), and water consumption (mL/day) were recorded every week. Fasting serum glucose concentrations were measured weekly for one month and then at the end of the study. Fasting serum levels of insulin and total sulfide levels were measured before and after the treatment. Intraperitoneal GTT was performed at the end of the eighth week of treatment and *in vivo* insulin secretion was assessed during GTT. PTT was done 3 days after GTT and blood pressure was measured at the end of the study. The experimental design is shown in Figure 9.



**Figure 9.** Experimental design; the dark squares indicate weeks of NaSH injection. HFD, high-fat diet; STZ, streptozotocin; GTT, glucose tolerance test; PTT, pyruvate tolerance test; NaSH, sodium hydrosulfide; SBP, systolic blood pressure.

### 4.3. Measurement of Serum Glucose and Insulin Levels

After 12–14 h fasting, blood samples were collected from tip of the tail and centrifuged at 5000 g for 10 min. Serum levels of glucose were determined using commercially available kit (Pars Azmoon, Tehran, Iran). Intra- and inter-assay coefficient of variations (CVs) for glucose were 2.1 and 3.4%, respectively. A rat ELISA kit (Rat insulin ELISA; Mercodia, Uppsala, Sweden) was used for measuring serum insulin levels; Intra- and inter-assay CVs were 3.3% and 7.6%, respectively. The assay sensitivity was 1 µU/mL (6 pmol/L).

#### 4.4. Measurement of Serum Total Sulfide

Total sulfide levels in serum were measured using the methylene blue method [44]. Briefly, 100  $\mu$ L serum was added into a test tube containing zinc acetate (1% *w/v*, 200  $\mu$ L), *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl (20 mM, 100  $\mu$ L), and FeCl<sub>3</sub> in 1.2 M HCl (30 mM, 133  $\mu$ L). After incubation at 37 °C for 30 min, the tubes were centrifuged at 5000 g for 10 min. Supernatants were then collected for measurement of total sulfide. Total sulfide concentration was determined in the samples using a standard calibration curve established by 0–200  $\mu$ M of NaSH at a wavelength of 670 nm using a microplate reader (BioTek, MQX2000R2, Winooski, VT, USA).

#### 4.5. Intraperitoneal Glucose Tolerance Test, Pyruvate Tolerance Test, and In Vivo Insulin Secretion

For GTT and PTT, after 12–14 h of fasting, animals were anesthetized with an IP injection of sodium pentobarbital (60 mg/kg; Sigma Aldrich, Hamburg, Germany) and glucose (50% solution, 2 g/kg) or pyruvate (2 g/kg) was injected intraperitoneally. Blood samples for glucose measurements were collected from the tail vein immediately before, and at 10, 20, 30, 60, and 120 min after glucose or pyruvate administration [45]. Blood samples during GTT were used for assessment of in vivo insulin secretion.

#### 4.6. Measurement of Systolic Blood Pressure

Systolic blood pressure was measured in anesthetized rats using a noninvasive tail-cuff method (AD Instruments, MLT125R, New South Wales, Australia) at room temperature as previously reported [46]. SBP values were averaged from three consecutive recordings obtained from each rat.

#### 4.7. Statistical Analysis

GraphPad Prism software (Version 6, La Jolla, San Diego CA, USA) was used for data analysis. All values are presented as mean  $\pm$  SEM. Two-way mixed (between-within) analysis of variance (ANOVA), followed by Fisher post-hoc test was used for analyzing data of water consumption, food intake, calorie intake, body weight, GTT, PTT, in vivo insulin secretion, and serum glucose. One-way ANOVA was used for comparing the area under the curves (AUC), SBP, and serum levels of insulin and H<sub>2</sub>S. Two-sided *p* values < 0.05 were considered to be statistically significant.

**Supplementary Materials:** The following are available online: Figure S1. Serum total sulfide levels before NaSH administration; Figure S2. Effects of NaSH on body weight; Figures S3 and S4. Effects of NaSH on water consumption; Figures S5 and S6. Effects of NaSH on calorie intake; Figures S7 and S8. Effects of NaSH on food intake; Figure S9. Effects of NaSH on serum glucose; Figure S10. Serum insulin levels before NaSH administration; Figure S11. Effects of NaSH on glucose tolerance; Figure S12. Effects of NaSH on pyruvate tolerance; Figure S13. Effects of NaSH on in vivo insulin secretion.

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## References

1. Zhang, L.; Wang, Y.; Li, Y.; Li, L.; Xu, S.; Feng, X.; Liu, S. Hydrogen sulfide (H<sub>2</sub>S)-releasing compounds: Therapeutic potential in cardiovascular diseases. *Front. Pharmacol.* **2018**, *9*, 1066. [[CrossRef](#)] [[PubMed](#)]
2. Hosoki, R.; Matsuki, N.; Kimura, H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 527–531. [[CrossRef](#)] [[PubMed](#)]

3. Bonfanti, D.H.; Alcazar, L.P.; Arakaki, P.A.; Martins, L.T.; Agustini, B.C.; de Moraes Rego, F.G.; Frigeri, H.R. ATP-dependent potassium channels and type 2 diabetes mellitus. *Clin. Biochem.* **2015**, *48*, 476–482. [[CrossRef](#)] [[PubMed](#)]
4. Yang, W.; Yang, G.; Jia, X.; Wu, L.; Wang, R. Activation of KATP channels by H<sub>2</sub>S in rat insulin-secreting cells and the underlying mechanisms. *J. Physiol.* **2005**, *569*, 519–531. [[CrossRef](#)] [[PubMed](#)]
5. Tang, G.; Zhang, L.; Yang, G.; Wu, L.; Wang, R. Hydrogen sulfide-induced inhibition of L-type Ca<sup>2+</sup> channels and insulin secretion in mouse pancreatic beta cells. *Diabetologia* **2013**, *56*, 533–541. [[CrossRef](#)] [[PubMed](#)]
6. Feng, X.; Chen, Y.; Zhao, J.; Tang, C.; Jiang, Z.; Geng, B. Hydrogen sulfide from adipose tissue is a novel insulin resistance regulator. *Biochem. Biophys. Res. Commun.* **2009**, *380*, 153–159. [[CrossRef](#)] [[PubMed](#)]
7. Wu, L.; Yang, W.; Jia, X.; Yang, G.; Duridanova, D.; Cao, K.; Wang, R. Pancreatic islet overproduction of H<sub>2</sub>S and suppressed insulin release in Zucker diabetic rats. *Lab. Invest.* **2009**, *89*, 59–67. [[CrossRef](#)]
8. Szabo, C. Roles of hydrogen sulfide in the pathogenesis of diabetes mellitus and its complications. *Antioxid. Redox. Signal.* **2012**, *17*, 68–80. [[CrossRef](#)] [[PubMed](#)]
9. Suzuki, K.; Sagara, M.; Aoki, C.; Tanaka, S.; Aso, Y. Clinical implication of plasma hydrogen sulfide levels in Japanese patients with type 2 diabetes. *Intern. Med.* **2017**, *56*, 17–21. [[CrossRef](#)] [[PubMed](#)]
10. Jain, S.K.; Bull, R.; Rains, J.L.; Bass, P.F.; Levine, S.N.; Reddy, S.; McVie, R.; Bocchini, J.A. Low levels of hydrogen sulfide in the blood of diabetes patients and streptozotocin-treated rats causes vascular inflammation? *Antioxid. Redox. Signal.* **2010**, *12*, 1333–1337. [[CrossRef](#)]
11. Whiteman, M.; Gooding, K.M.; Whatmore, J.L.; Ball, C.I.; Mawson, D.; Skinner, K.; Tooke, J.E.; Shore, A.C. Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulphide. *Diabetologia* **2010**, *53*, 1722–1726. [[CrossRef](#)] [[PubMed](#)]
12. Durante, W. Hydrogen sulfide therapy in diabetes-accelerated atherosclerosis: A whiff of success. *Diabetes* **2016**, *65*, 2832–2834. [[CrossRef](#)] [[PubMed](#)]
13. Okamoto, M.; Yamaoka, M.; Takei, M.; Ando, T.; Taniguchi, S.; Ishii, I.; Tohya, K.; Ishizaki, T.; Niki, I.; Kimura, T. Endogenous hydrogen sulfide protects pancreatic beta-cells from a high-fat diet-induced glucotoxicity and prevents the development of type 2 diabetes. *Biochem. Biophys. Res. Commun.* **2013**, *442*, 227–233. [[CrossRef](#)]
14. Pichette, J.; Fynn-Sackey, N.; Gagnon, J. Hydrogen sulfide and sulfate prebiotic stimulates the secretion of GLP-1 and improves glycemia in male mice. *Endocrinology* **2017**, *158*, 3416–3425. [[CrossRef](#)] [[PubMed](#)]
15. Manna, P.; Jain, S.K. Hydrogen sulfide and L-cysteine increase phosphatidylinositol 3,4,5-trisphosphate (PIP3) and glucose utilization by inhibiting phosphatase and tensin homolog (PTEN) protein and activating phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase (AKT)/protein kinase C $\zeta$ /lambda (PKC $\zeta$ /lambda) in 3T3L1 adipocytes. *J. Biol. Chem.* **2011**, *286*, 39848–39859. [[CrossRef](#)] [[PubMed](#)]
16. Manna, P.; Jain, S.K. Vitamin D up-regulates glucose transporter 4 (GLUT4) translocation and glucose utilization mediated by cystathionine-gamma-lyase (CSE) activation and H<sub>2</sub>S formation in 3T3L1 adipocytes. *J. Biol. Chem.* **2012**, *287*, 42324–42332. [[CrossRef](#)]
17. Xue, R.; Hao, D.-D.; Sun, J.-P.; Li, W.-W.; Zhao, M.-M.; Li, X.-H.; Chen, Y.; Zhu, J.-H.; Ding, Y.-J.; Liu, J.; et al. Hydrogen sulfide treatment promotes glucose uptake by increasing insulin receptor sensitivity and ameliorates kidney lesions in type 2 diabetes. *Antioxid. Redox. Signal.* **2013**, *19*, 5–23. [[CrossRef](#)] [[PubMed](#)]
18. Peake, B.F.; Nicholson, C.K.; Lambert, J.P.; Hood, R.L.; Amin, H.; Amin, S.; Calvert, J.W. Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating Nrf2 signaling in an Erk-dependent manner. *Am. J. Physiol. Heart Circ. Physiol.* **2013**, *304*, 1215–1224. [[CrossRef](#)] [[PubMed](#)]
19. Wu, J.; Tian, Z.; Sun, Y.; Lu, C.; Liu, N.; Gao, Z.; Zhang, L.; Dong, S.; Yang, F.; Zhong, X.; et al. Exogenous H<sub>2</sub>S facilitating ubiquitin aggregates clearance via autophagy attenuates type 2 diabetes-induced cardiomyopathy. *Cell. Death Dis.* **2017**, *8*, 2992. [[CrossRef](#)]
20. Sun, Y.; Tian, Z.; Liu, N.; Zhang, L.; Gao, Z.; Sun, X.; Yu, M.; Wu, J.; Yang, F.; Zhao, Y.; et al. Exogenous H<sub>2</sub>S switches cardiac energy substrate metabolism by regulating SIRT3 expression in db/db mice. *J. Mol. Med.* **2018**, *96*, 281–299. [[CrossRef](#)]
21. Ma, S.; Zhong, D.; Ma, P.; Li, G.; Hua, W.; Sun, Y.; Liu, N.; Zhang, L.; Zhang, W. Exogenous hydrogen sulfide ameliorates diabetes-associated cognitive decline by regulating the mitochondria-mediated apoptotic pathway and IL-23/IL-17 expression in db/db mice. *Cell. Physiol. Biochem.* **2017**, *41*, 1838–1850. [[CrossRef](#)] [[PubMed](#)]

22. Saha, P.; Banerjee, P.; Pal, P.; Auddya, L.; Sen, S.; Sau, T.J.; Kumar, A.; Biswas, U.K. Enhanced plasma H<sub>2</sub>S levels associated with fasting blood glucose in type-2 diabetes mellitus. *Asian J. Med. Sci.* **2015**, *6*, 5. [[CrossRef](#)]
23. Yusuf, M.; Kwong Huat, B.T.; Hsu, A.; Whiteman, M.; Bhatia, M.; Moore, P.K. Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 1146–1152. [[CrossRef](#)] [[PubMed](#)]
24. Li, H.; Feng, S.J.; Zhang, G.Z.; Wang, S.X. Correlation of lower concentrations of hydrogen sulfide with atherosclerosis in chronic hemodialysis patients with diabetic nephropathy. *Blood. Purif.* **2014**, *38*, 188–194. [[CrossRef](#)] [[PubMed](#)]
25. Ng, H.H.; Yildiz, G.S.; Ku, J.M.; Miller, A.A.; Woodman, O.L.; Hart, J.L. Chronic NaHS treatment decreases oxidative stress and improves endothelial function in diabetic mice. *Diab. Vasc. Dis. Res.* **2017**, *14*, 246–253. [[CrossRef](#)]
26. Suzuki, K.; Olah, G.; Modis, K.; Coletta, C.; Kulp, G.; Gero, D.; Szoleczky, P.; Chang, T.; Zhou, Z.; Wu, L.; et al. Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13829–13834. [[CrossRef](#)]
27. Mani, S.; Yang, G.; Wang, R. A critical life-supporting role for cystathionine gamma-lyase in the absence of dietary cysteine supply. *Free Radic. Biol. Med.* **2011**, *50*, 1280–1287. [[CrossRef](#)]
28. Geng, B.; Cai, B.; Liao, F.; Zheng, Y.; Zeng, Q.; Fan, X.; Gong, Y.; Yang, J.; Cui, Q.H.; Tang, C.; et al. Increase or decrease hydrogen sulfide exert opposite lipolysis, but reduce global insulin resistance in high fatty diet induced obese mice. *PLoS ONE* **2013**, *8*, 73892. [[CrossRef](#)]
29. Kaneko, Y.; Kimura, Y.; Kimura, H.; Niki, I. L-cysteine inhibits insulin release from the pancreatic beta-cell: Possible involvement of metabolic production of hydrogen sulfide, a novel gasotransmitter. *Diabetes* **2006**, *55*, 1391–1397. [[CrossRef](#)]
30. Zhang, L.; Yang, G.; Untereiner, A.; Ju, Y.; Wu, L.; Wang, R. Hydrogen sulfide impairs glucose utilization and increases gluconeogenesis in hepatocytes. *Endocrinology* **2013**, *154*, 114–126. [[CrossRef](#)]
31. Picarel-Blanchot, F.; Berthelie, C.; Bailbe, D.; Portha, B. Impaired insulin secretion and excessive hepatic glucose production are both early events in the diabetic GK rat. *Am. J. Physiol.* **1996**, *271*, 755–762. [[CrossRef](#)] [[PubMed](#)]
32. Warena, M.W.; Goodwin, L.R.; Benishin, C.G.; Reiffenstein, R.J.; Francom, D.M.; Taylor, J.D.; Dieken, F.P. Acute hydrogen sulfide poisoning. Demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. *Biochem. Pharmacol.* **1989**, *38*, 973–981. [[CrossRef](#)]
33. Truong, D.H.; Mihajlovic, A.; Gunness, P.; Hindmarsh, W.; O'Brien, P.J. Prevention of hydrogen sulfide (H<sub>2</sub>S)-induced mouse lethality and cytotoxicity by hydroxocobalamin (vitamin B(12a)). *Toxicology* **2007**, *242*, 16–22. [[CrossRef](#)] [[PubMed](#)]
34. Cronican, A.A.; Frawley, K.L.; Ahmed, H.; Pearce, L.L.; Peterson, J. Antagonism of acute sulfide poisoning in mice by nitrite anion without methemoglobinemia. *Chemical Res. Toxicol.* **2015**, *28*, 1398–1408. [[CrossRef](#)] [[PubMed](#)]
35. Untereiner, A.A.; Wang, R.; Ju, Y.; Wu, L. Decreased gluconeogenesis in the absence of cystathionine gamma-lyase and the underlying mechanisms. *Antioxid. Redox. Signal.* **2016**, *24*, 129–140. [[CrossRef](#)]
36. Sun, L.; Jin, H.; Chen, S.; Huang, Y.; Liu, J.; Li, Z.; Zhao, M.; Sun, Y.; Tang, C.; Zhao, B.; et al. Hydrogen sulfide alleviates myocardial collagen remodeling in association with inhibition of TGF-beta/Smad signaling pathway in spontaneously hypertensive rats. *Mol. Med.* **2015**, *20*, 503–515. [[CrossRef](#)] [[PubMed](#)]
37. Huang, P.; Chen, S.; Wang, Y.; Liu, J.; Yao, Q.; Huang, Y.; Li, H.; Zhu, M.; Wang, S.; Li, L.; et al. Down-regulated CBS/H<sub>2</sub>S pathway is involved in high-salt-induced hypertension in Dahl rats. *Nitric Oxide* **2015**, *46*, 192–203. [[CrossRef](#)] [[PubMed](#)]
38. Ahmad, F.U.; Sattar, M.A.; Rathore, H.A.; Tan, Y.C.; Akhtar, S.; Jin, O.H.; Pei, Y.P.; Abdullah, N.A.; Johns, E.J. Hydrogen sulphide and tempol treatments improve the blood pressure and renal excretory responses in spontaneously hypertensive rats. *Ren. Fail.* **2014**, *36*, 598–605. [[CrossRef](#)]
39. Lu, M.; Liu, Y.H.; Goh, H.S.; Wang, J.J.; Yong, Q.C.; Wang, R.; Bian, J.S. Hydrogen sulfide inhibits plasma renin activity. *J. Am. Soc. Nephrol.* **2010**, *21*, 993–1002. [[CrossRef](#)]

40. Shi, Y.X.; Chen, Y.; Zhu, Y.Z.; Huang, G.Y.; Moore, P.K.; Huang, S.H.; Yao, T.; Zhu, Y.C. Chronic sodium hydrosulfide treatment decreases medial thickening of intramyocardial coronary arterioles, interstitial fibrosis, and ROS production in spontaneously hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *293*, 2093–2100. [[CrossRef](#)]
41. Zhao, W.; Zhang, J.; Lu, Y.; Wang, R. The vasorelaxant effect of H<sub>2</sub>S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J.* **2001**, *20*, 6008–6016. [[CrossRef](#)] [[PubMed](#)]
42. Gheibi, S.; Kashfi, K.; Ghasemi, A. A practical guide for induction of type-2 diabetes in rat: Incorporating a high-fat diet and streptozotocin. *Biomed. Pharmacother.* **2017**, *95*, 605–613. [[CrossRef](#)] [[PubMed](#)]
43. Chattopadhyay, M.; Kodela, R.; Duvalsaint, P.L.; Kashfi, K. Gastrointestinal safety, chemotherapeutic potential, and classic pharmacological profile of NOSH-naproxen (AVT-219) a dual NO<sup>-</sup> and H<sub>2</sub>S-releasing hybrid. *Pharmacol. Res. Perspect.* **2016**, *4*. [[CrossRef](#)] [[PubMed](#)]
44. Shen, X.; Pattillo, C.B.; Pardue, S.; Bir, S.C.; Wang, R.; Kevil, C.G. Measurement of plasma hydrogen sulfide in vivo and in vitro. *Free Radic. Biol. Med.* **2011**, *50*, 1021–1031. [[CrossRef](#)] [[PubMed](#)]
45. Gheibi, S.; Jeddi, S.; Carlström, M.; Gholami, H.; Ghasemi, A. Effects of long-term nitrate supplementation on carbohydrate metabolism, lipid profiles, oxidative stress, and inflammation in male obese type 2 diabetic rats. *Nitric Oxide* **2018**, *75*, 27–41. [[CrossRef](#)] [[PubMed](#)]
46. Ghanbari, M.; Jeddi, S.; Bagheripour, F.; Ghasemi, A. The effect of maternal hypothyroidism on cardiac function and tolerance to ischemia-reperfusion injury in offspring male and female rats. *J. Endocrinol. Invest.* **2015**, *38*, 915–922. [[CrossRef](#)] [[PubMed](#)]

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