Original Paper

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

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Key Words

Hydrogen sulfide • Diabetes-associated cognitive decline • IL-23/IL-17 axis • Mitochondria-mediated apoptosis

Abstract

Background: Diabetes-associated cognitive decline (DACD) is one of the complications of diabetes and leads to cognitive impairment and an increased risk of dementia. However, the exact mechanism of DACD has not been fully characterized, and a successful therapy for this issue has not been established. This study aimed to detect the anti-apoptotic and anti-inflammatory effects of hydrogen sulfide (H₂S) on DACD. **Methods:** We used a behavioural scoring method, Western blot, TUNEL staining and immunofluorescence staining to investigate the expression of the mitochondrial apoptotic pathway and the IL-23/IL-17 axis in db/db mice with or without sodium hydrosulfide (NaHS) administration. **Results:** NaHS administration mice exhibited reduced time to find the platform and a shorter swimming distance ($P < 0.05$), while the time spent in the target quadrant was increased compared to that of the db/db group ($P < 0.05$). Pro-apoptotic proteins, including cleaved Caspase-3, cleaved Caspase-9, Bax and cytochrome C, were elevated in the db/db group ($P < 0.01$) but were downregulated in the db/db+NaHS group ($P < 0.05$). Exogenous H₂S decreased the numbers of TUNEL-positive cells in the db/db mice ($P < 0.05$). The Western blot analysis showed that the expression levels of IL-23/IL-17 were lower in the NaHS administration group than in the db/db group ($P < 0.05$). **Conclusion:** We demonstrated that H₂S improved the spatial learning and memory abilities of the db/db mice by modulating the mitochondrial apoptotic pathway and the IL-23/IL-17 axis, which were found to be associated with DACD. H₂S treatment may help prevent the progression of apoptotic hippocampal neurons in db/db mice and inform the development of a new therapeutic target.

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Introduction

Because of the increase in the ageing population and rapid changes in life style, diabetes mellitus (DM), especially type 2 diabetes (T2DM), has become a common, clinical chronic metabolic disease. The prevalence of the disease has increased year by year and reached 9.7% among the population in China, which accounts for more than 6.4% of the worldwide average [1, 2]. Diabetes leads to microvascular and macrovascular complications, and diabetes-associated cognitive decline is one of the central nervous system complications, which induces advanced brain ageing, cognitive impairment and an increased risk of dementia [3, 4]. A meta-analysis showed that DM increased the relative risk of 1.56 (1.41-1.73) for Alzheimer's disease (AD) and 2.27 (1.94-2.66) for vascular dementia (VD) [5]. To investigate the association between DM and dementia, several mechanisms, including insulin resistance, insulin deficiency, inflammation, and cerebral vascular problems [6-8], have been proposed. In the clinic, the drugs currently available only slow the progression of symptoms in AD [9] and control the vascular risk factors for VD [10]. Thus, it is important to identify the exact underlying mechanism of DACD and effective therapeutic targets.

H₂S is now recognized as the third gaseous signalling molecule, along with nitric oxide and carbon monoxide [11-13]. Acting as a neuromodulator, H₂S exerts anti-inflammatory, anti-apoptotic and antioxidant effects on the central nervous system [14-16]. In the central nervous system, cystathionine β-synthase (CBS) is abundantly expressed, which initiates the trans-sulfuration pathway catalysing the amino acid cysteine (Cys) to H₂S [17]. It was reported that sodium hydrosulfide attenuated neuroinflammation in beta-amyloid (Aβ)-induced AD models by suppressing the expression of proinflammatory factors in the rat hippocampus [18]. In recent studies, H₂S improved spatial learning and memory abilities in VD, and the underlying mechanism identified in these studies is the Akt-GSK-3β signalling pathway [19]. Epidemiological studies have shown that the most common form of dementia is AD [20], and that the second most common form of dementia is VD [21]; however, the current animal models do not mimic the actual pathophysiological process of DACD. To determine the relationship between DM and dementia, in our experiments, we used db/db mice that exhibit hippocampal-dependent cognitive decline, as well as hyperglycaemia, hyperinsulinemia and insulin and leptin resistance [22-24].

Interleukin (IL)-17, the most effective cytokine of T helper 17 (Th17) cells, plays proinflammatory roles, especially in chronic inflammation in many inflammatory and autoimmune disorders [25, 26]. A previous study showed that IL-17 expression was elevated in the hippocampus, cerebrospinal fluid and serum of Aβ-injected rats, and indicated that IL-17 contributed to the neuroinflammatory response in AD [27]. IL-23, a heterodimeric cytokine, promotes the production of IL-17 and maintains the lineage [28, 29]. Our previous studies showed the IL-23/IL-17 axis played a role in the later phase of cerebral ischaemia [30, 31], which resulted in neuroinflammatory injuries in C57BL/6 mice. Hyperglycaemia leads to mitochondrial superoxide generation, which initiates endothelial dysfunction and oxidant production [32]. H₂S serves as an electron donor to the respiratory chain and plays a therapeutic role in DM and vascular diseases [33, 34]. A recent *in vitro* study also showed that H₂S inhibits Aβ-induced neuronal apoptosis by regulating mitochondrial function [35]. On these grounds, we proposed the hypothesis that H₂S could inhibit the expression of IL-23/IL-17 axis and mitochondrial apoptotic proteins to alleviate the cognitive decline caused by DM.

Materials and Methods

Animals

All 8-week-old male C57BLKs-J/db-db mice (n=24) and age-matched C57BLKs-J(WT) mice (n=12) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu Province, China). The animals were housed four per cage under specific

pathogen-free conditions and were free to eat and drink; the room temperature was 22°C with 50±5% humidity, and the room was on a 12-hour light/dark cycle. All animal protocols were approved by the Animal Research Ethics Committee at Harbin Medical University, and the study was conducted in accordance with the Animal Management Rules of the Ministry of Science and Technology of the People's Republic of China.

Preparation and administration of NaHS

The 10-week-old db/db mice were randomly divided into two groups: the db/db group (n=12) and NaHS administration group (db/db+H₂S group, n=12). According to body weight, NaHS solution (4.48 mg/kg), which served as an H₂S donor, was injected intraperitoneally once a day while the control group and db/db group received the same volume of sterile saline solution.

Random blood glucose and oral glucose tolerance test

We randomly measured the blood sugar levels of blood samples obtained from the tail every week to verify the development of DM, and we measured the body weight of all mice weekly. The mice were fasted for 12 hours and were administered a 50% glucose solution (2 g/kg) by gavage for the oral glucose tolerance test. Tail vein samples were collected at 0, 20, 40, 60, 80 and 120 minutes after administration. All blood samples were tested using glucose test strips.

Morris water maze test

To compare the spatial learning and memory abilities of mice, we conducted the Morris water maze (MWM) test [36, 37] seven weeks after the mice were assigned to their respective groups. The swimming pool (diameter: 120 cm; depth: 40 cm) was filled with water containing nontoxic milk powder (depth: 30 cm) and maintained at 25 ± 1°C. The experiment was conducted four times a day with 5-minute intervals for six consecutive days. On the first day, the mice were allowed to swim freely for 120 seconds without the platform (diameter 8 cm), which was set 1 cm below the surface of the water in the centre of the target quadrant. For the next four days, the platform was fixed, and the mice were allowed to find the platform from different starting points within the maximum observation time of 120 seconds. The time to find the platform (escape latency) and swimming distance required to find the platform were recorded by an automatic video recorder and an analysis system (EthoVision; Noldus Information Technology). On the last day, the mice were tested in a probe trial session without the platform and the time spent in the target quadrant was recorded.

Western blot

The 18-week-old mice of the control group (n=6), db/db group (n=6) and db/db+H₂S group (n=6) were deeply anesthetized and decapitated. Whole brain samples were collected, frozen in liquid nitrogen immediately and then stored at -80°C before protein extraction. We also isolated fresh hippocampal tissue from the CA1 region and used ice-cold RIPA buffer, which included components such as 1% protein inhibitor cocktail, 10 mg/ml phenylmethylsulfonyl fluoride and 100 mM sodium orthovanadate. The mixture was incubated on ice for 30 minutes and then centrifuged at 12,000×g at 4°C. The supernatant was collected and separated, the protein concentration was measured by the Lowry method, and the protein was then subjected to 12% SDS-PAGE. Afterwards, the protein was transferred to PVDF membranes (Millipore, Temecula, CA), which were blocked in 5% skim milk in TBS-T and incubated with antibodies directed against CBS (rabbit, 1:1000, Proteintech, China), Bax (rabbit, 1:1000, Proteintech, China), Bcl-2 (rabbit, 1:1000, Proteintech, China), cleaved Caspase-3 (rabbit, 1:500, Abcam, Cambridge, MA, USA), cleaved Caspase-9 (rabbit, 1:500, Abcam, Cambridge, MA, USA), cytochrome C (rabbit, 1:1000, Proteintech, China), IL-23 (rabbit, 1:500, Abcam, Cambridge, MA, USA), IL-17 (rabbit, 1:1000, Abcam, Cambridge, MA, USA) and GAPDH (rabbit, 1:1,000, Santa Cruz Biotechnology, Inc., USA). The membranes

were probed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., USA). ImageJ software (NIH, Bethesda, MD, USA) was used to analyse the bands, and the expression ratios were normalized to GAPDH.

Tissue preparation and H₂S probe incubation

The remaining six mice from the three groups were intraperitoneally anesthetized and perfused with cold sterile saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Half of the brain samples (n=3) were removed and were embedded in OCT compound (Sakura, Tokyo, Japan) at 4°C overnight and dehydrated in 10%, 20%, 30% sucrose solutions (one day for each concentration). The samples were then cryo-sectioned into 10-μm-thick serial coronal sections of the hippocampus with a freezing microtome (Leica CM1850) for immunofluorescence staining.

To detect H₂S expression, 7-azido-4-methylcoumarin (Sigma, Aldrich, Hong Kong, China) was used at a concentration of 0.3% in PBS. Frozen sections were fixed in cold acetone for 10 minutes and washed three times with PBS for 5 minutes. Afterwards, the sections were blocked in 5% bovine serum albumin (BSA) and washed with PBS followed by incubation with a H₂S probe. The fluorescence signals were detected by a Zeiss LSM 510 microscope.

HE staining and TUNEL staining

The other half of the brain samples (n=3) was immersed in 4% paraformaldehyde overnight and then embedded in paraffin. Coronal paraffin sections (2-4 μm) of the hippocampus of each mouse were stained with haematoxylin and eosin (HE). The TUNEL assay (In Situ Cell Death Detection kit, Roche Molecular Biochemicals) was carried out according to the manufacturer's instructions.

Immunofluorescence

The frozen sections were fixed in cold acetone, washed three times, and then permeabilized with 0.2 Triton X-100 at 4°C for 5 minutes. The slides were blocked with 5% BSA. The tissue sections were incubated with primary antibodies directed against IL-23 (rabbit, 1:100, Abcam, Cambridge, MA, USA) and IL-17 (rabbit, 1:100, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, the slides were washed four times and then incubated with the secondary antibody (FITC-conjugated anti-rabbit IgG, BA1105, Boster, Wuhan, China) at room temperature for 1 hour. After the tissue sections were washed with PBS, they were stained with DAPI. The control experiment consisted of staining brain sections but omitting the primary antibodies.

Statistical analysis

The data are expressed as the mean value ± standard deviation. The outcomes of MWM test were evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. One-way ANOVA followed by Bonferroni's post hoc multiple-comparison tests were performed to analyse differences among the three groups of mice. All analyses were performed using GraphPad Prism software (Version 5.00, CA, USA). P<0.05 was considered statistically significant.

Results

Biochemical variables

Random blood sugar levels, body weight, and oral glucose tolerance test results were recorded (Fig. 1) and were significantly increased in db/db mice (n=12) compared with the control group (n=12). However, there was no significant difference between the H₂S administration group (n=12) and the db/db group.

Fig. 1. Effect of H_2S on random blood sugar levels, body weight, and oral glucose tolerance test results. (A) Blood sugar levels were increased in db/db mice. (B) Body weight was elevated in db/db mice. (C) At the first time point for the glucose tolerance test, there was no significant difference between the db/db group and the control group. There was no significance between the H_2S administration group and db/db group (two-way ANOVA followed by Bonferroni's post hoc test). ### $P < 0.001$ compared between the db/db group ($n = 12$) and the control group ($n = 12$).

Fig. 2. Effects of exogenous H_2S on the MWM test. Spatial learning performance was analysed by escape latency (A) and swimming distance (B), and memory abilities were elucidated in the probe test (C). NaHS administration mice took less time to find the platform and exhibited a shorter swimming distance (two-way ANOVA followed by Bonferroni's post hoc test). The time spent in the target quadrant for the NaHS group was increased compared to that of the db/db group (one-way ANOVA followed by Bonferroni's post hoc test). # $P < 0.05$ and ### $P < 0.001$ compared between the db/db group ($n = 12$) and the control group ($n = 12$); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared between the NaHS administration group ($n = 12$) and the db/db group ($n = 12$).

Exogenous H_2S improves performance in the MWM

To observe the spatial learning and memory abilities of the db/db group and NaHS administration group, MWM tests were carried out. The data showed that the escape latency (Fig. 2, A) of the db/db group ($n = 12$) was significantly longer than that of the control group ($n = 12$) on the third, fourth and fifth days ($P < 0.001$). However, the NaHS administration group ($n = 12$) exhibited improved performance from the second day of training compared with the db/db group (2nd day, $P < 0.05$; 3rd day and 4th day, $P < 0.01$; 5th day $P < 0.001$). The swimming distance (Fig. 2, B) of the db/db mice was greater than that of the control mice ($n = 12$, $P < 0.001$), while NaHS treatment ($n = 12$) shortened the swimming distance compared with that of the db/db group ($n = 12$; 2nd day and 4th day, $P < 0.05$; 3rd day and 5th day, $P < 0.001$).

Fig. 3. H₂S levels and CBS expression levels among the different groups. (A) Exogenous supplementation of NaHS elevated the H₂S levels in the mouse brain (n=3, P<0.05), and endogenous H₂S was decreased in db/db mice compared with control mice (n=3, P<0.01). (B) Western blot analysis showed increased expression of CBS in the NaHS group (n=6, P<0.01) compared with the db/db group. The CBS expression levels of the db/db group were lower than that of control group (n=6, P<0.05) (one-way ANOVA followed by Bonferroni's post hoc test). ## P<0.01 compared between the db/db group and the control group; * P<0.05 compared between the NaHS administration group and the db/db group.

In the probe test (Fig. 2, C), the db/db (n=12) mice spent significantly less time in the target quadrant than the control mice (n=12, P<0.001). Exogenous H₂S improved the memory retrieval performance compared between the db/db+NaHS group (n=12) and the db/db group (P<0.01).

Measurement of H₂S levels

To test whether the animal models were established successfully and whether exogenous H₂S affected the intracranial generation of H₂S, we used an H₂S probe (Fig. 3, A) and Western blot analysis of CBS (Fig. 3, B). The relative fluorescence intensity of db/db mice (n=3) was decreased from that of control mice (n=3, P<0.01), indicating that expression of endogenous H₂S was lower in diabetic mice than in control mice, whereas, exogenous supplementation of NaHS elevated the levels of H₂S intracranially in the administration group (n=3) compared with the db/db group (P<0.05). Because CBS is key source of H₂S in the brain, the relative levels of CBS were calculated in the three groups (n=6). The CBS expression levels in the db/db mice were decreased compared with those in the control group (P<0.01), while the CBS protein levels were elevated in the NaHS administration group compared with those in the db/db group (P<0.05).

Exogenous H₂S reduces the apoptosis of neurons

To investigate whether exogenous NaHS could alleviate neuronal apoptosis, we also collected brain samples from the three groups and used Western blot to assess mitochondria-mediated apoptotic protein levels, which were normalized to GAPDH (Fig. 4). To better elucidate the underlying mechanism, we detected the expression levels of Bcl-2 and Bax in the hippocampal CA1 region (Fig. 5). The expression levels of the anti-apoptotic protein Bcl-2 were significantly reduced in the db/db mice (n=6, P<0.001); however, Bcl-2 expression levels were significantly elevated by NaHS administration (n=6, P<0.05). The expression levels of other apoptotic proteins, including cleaved Caspase-3, cleaved Caspase-9, Bax and cytochrome C, were also measured and found to be elevated in the db/db mice (n=6, ## P<0.01, ### P<0.001) and downregulated in the db/db+NaHS mice (n=6, P<0.05). The expression levels of Bcl-2 and Bax in the hippocampal CA1 region (n=6) showed trends

Fig. 4. Western blot analysis examining the levels of mitochondria-mediated apoptotic proteins. The expression levels of Bcl-2 were decreased in the db/db group (n=6, $P<0.001$) and elevated in the db/db+NaHS group (n=6, $P<0.05$). The protein expression levels of cleaved Caspase-3 (n=6, $P<0.001$), cleaved Caspase-9 (n=6, $P<0.01$), Bax (n=6, $P<0.001$) and cytochrome C (n=6, $P<0.01$) were increased in the db/db group. In addition, the expression levels of these proteins were decreased in the db/db+NaHS group (n=6, $P<0.05$) (one-way ANOVA followed by Bonferroni's post hoc test). ## $P<0.01$ and ### $P<0.001$ compared between the db/db group and the control group; * $P<0.05$ compared between the NaHS administration group and the db/db group.

Fig. 5. Western blot analysis of Bcl-2/Bax and IL-23/IL-17 in the hippocampal CA1 region. Cell lysates from the control group (n=6), db/db group (n=6) and db/db+NaHS group (n=6) were separated and transferred to PVDF membranes. Proteins were blotted with anti-Bcl-2, anti-Bax, anti-IL-23, anti-IL-17 and anti-GAPDH antibodies. The expression levels of Bcl-2 were lower in the hippocampus in the db/db group compared with the control group (n=6, $P<0.001$) and elevated in the db/db+NaHS group (n=6, $P<0.05$). The protein expression levels of Bax (n=6, $P<0.001$), IL-23 (n=6, $P<0.001$) and IL-17 (n=6, $P<0.01$) were increased in the db/db group. In addition, the expression levels of these proteins were decreased in the db/db+NaHS group (n=6, $P<0.05$) (one-way ANOVA followed by Bonferroni's post hoc test). ## $P<0.01$ and ### $P<0.001$ compared between the db/db group and the control group; * $P<0.05$ compared between the NaHS administration group and the db/db group.

similar to those in the whole brain ($P<0.001$ compared between the db/db group and the control group; $P<0.05$ compared between the NaHS administration group and the db/db

Fig. 6. Representative images of HE staining. (A) The image shows normal neurons in the control group (n=3). (B) Neuronal pyknosis can be seen in the db/db mice (n=3). (C) H_2S treatment reduced neuronal loss and pyknosis in the db/db mice (n=3).

Fig. 7. Percentage of TUNEL-positive cells.

(A) Low numbers of TUNEL-positive cells were present in the control group. (B) In the db/db group, TUNEL-positive cells were identified, and the percentage of positive cells was increased compared with the control group (n=6, $P<0.001$). (C) In the db/db+NaHS group, the number of TUNEL-positive cells was reduced (n=6, $P<0.05$), which suggested that exogenous H_2S ameliorates mitochondria-mediated apoptosis in the db/db mice. Arrows indicate the TUNEL-positive cells (one-way ANOVA followed by Bonferroni's post hoc test). ### $P<0.001$ compared between the db/db group and the control group; * $P<0.05$ compared between the NaHS administration group and the db/db group.

group). The data indicate that NaHS administration protected the neurons of the db/db mice from mitochondria-mediated apoptosis.

We also used HE staining (Fig. 6) and TUNEL staining (Fig. 7) to observe neuronal phenotypes and apoptosis. The morphology of neurons was normal in the control group (n=3); prominent nucleoli and clear nuclear membranes were observed. In the db/db group (n=3), there were a loss of neurons and pyknosis of the neurons in the hippocampal CA1 region. Neuronal pyknosis and cytoplasmic hyperchromasia were alleviated in the NaHS administration group (n=3).

The percentage of TUNEL-positive cells was significantly elevated in the db/db group ($P<0.001$, n=3) compared with the control group (n=3). Exogenous H_2S treatment decreased the numbers in the TUNEL-positive cells of db/db mice ($P<0.05$, n=3), indicating its anti-apoptotic effects.

Exogenous H_2S inhibits the expression of proinflammatory IL-23/IL-17

To test whether the IL-23/IL-17 axis played a proinflammatory role in pathophysiology of diabetes-associated cognitive decline, we used semi-quantitative Western blot analysis to measure the protein levels of the IL-23/IL-17 axis in both whole brain (Fig. 8, A) and the

Fig. 8. Western blot analysis and immunofluorescence of the IL-23/IL-17 axis. (A) The figure shows representative images of three independent experiments. The data indicate that the expression levels of IL-23/IL-17 increased in the db/db group (lane 2, $P < 0.001$ of IL-23, $P < 0.01$ of IL-17, $n = 6$) and that the expression levels of these proteins in the NaHS administration group were lowered than those of the db/db group (lane 3, $P < 0.05$, $n = 6$). (B) Localization of IL-23/IL-17-positive cells is shown for the three groups. Representative immunofluorescence images of IL-23 and IL-17 (green) and DAPI (blue) staining are displayed.

hippocampal CA1 region (Fig. 5). The expression levels of the IL-23/IL-17 axis were elevated in the whole brain and hippocampus of the db/db mice ($n = 6$, $##P < 0.01$, $###P < 0.001$) compared with the control group ($n = 6$). In the NaHS administration group ($n = 6$, $P < 0.05$), the expression levels of the axis were lower than those of the db/db group, suggesting that exogenous H_2S played anti-inflammatory roles by regulating IL-23/IL-17 expression levels.

The localization of IL-23-positive and IL-17-positive cells was observed by immunofluorescence staining. Representative images are displayed in Figure 8B. In the control group, IL-23/IL-17-positive cells were fewer in number than those in the db/db group, while NaHS treatment decreased the numbers of IL-23/IL-17-positive cells.

Discussion

This study verified that exogenous H_2S could supplement endogenous loss of H_2S and improve spatial learning and memory abilities in db/db mice. It was previously reported that diabetes patients had lower levels of H_2S in the plasma than normal volunteers, and a similar trend was also observed in rats with diabetes induced by streptozotocin [38]. The loss of H_2S , which acts as a physiological modulator, may contribute to the pathophysiological process of diabetes. The animal model of T2DM that we chose was the leptin-resistant db/db mouse, which also suffers from cognitive decline, as we corroborated in this study. Exogenous H_2S supplementation may provide an effective therapeutic strategy for diabetes-associated dementia. However, the underlying mechanism of this treatment is not fully understood. H_2S prevented neurodegeneration through inhibition of cleaving enzymes for amyloid

precursor protein (APP) [39], which downregulated A β generation both *in vivo* [40] and *in vitro*. H₂S was also shown to inhibit apoptosis in the hippocampus of a vascular dementia rat model that was induced by artery occlusion. Hence, H₂S exerts both anti-apoptotic and anti-inflammatory effects in multiple pathways, and we focused on mitochondrial apoptosis and the IL-23/IL-17 axis in this study.

When T2DM, hyperinsulinemia and insulin-resistance occur, chronic inflammation increases and affects disease development and related complications [41]. IL-17, a proinflammatory cytokine, plays a prominent role in neuroinflammation. Th17 cells were shown to infiltrate the rat brain through the blood-brain barrier, secrete proinflammatory IL-17 and IL-22, upregulate the expression of Fas and FasL and induce apoptosis in AD [27]. IL-23 produced by antigen-presenting cells promotes the differentiation of the lineage; thus, the IL-23/IL-17 axis gradually gained attention in the context of autoimmune-mediated tissue inflammation [42]. In this study, we detected upregulation of the IL-23/IL-17 axis in the db/db mouse brain and inferred its proinflammatory effects on diabetes-associated cognitive decline. H₂S treatment decreased the expression levels of the axis and prevented inflammatory injury. However, the origins of these cytokines and the specific pathways in which H₂S participates require further study. One of the limitations of this study is that specific pathways, such as MAPK/NF- κ B, p38/MAPK, and JNK, have not been investigated and still need to be explored.

The gasotransmitter H₂S can diffuse freely through the cell membrane, initiate multiple pathways at certain concentrations and serve as a hydrogen donor for mitochondrial respiration [43]. Maintaining endogenous H₂S at a nontoxic level could provide positive feedback for controlling the network. In our study, the expression levels of endogenous H₂S in the db/db mice decreased, the levels of pro-apoptotic proteins were elevated, and the number of TUNEL-positive cells increased, indicating the modulatory effect of H₂S on diabetes-associated dementia. The H₂S donors include sulfide salts such as sodium sulfide (Na₂S) and NaHS, garlic-derived compounds, and 1,2-dithiole-3-thiones [44]. Among these donors, NaHS has been widely used in neurodegenerative diseases such as Parkinson's disease (PD) and AD, and the concentration used in this study was selected according to previous results and other references [45, 46]. CBS mediates H₂S biosynthesis in the central nervous system of db/db mice, and there are several ways for CBS to generate H₂S [17]. It was reported that in the brains of rats with A β -induced Alzheimer's disease, the expression levels of CBS and H₂S were significantly decreased [18]. In diabetic patients, H₂S levels are significantly lower compared with healthy subjects [38]. Researchers in China determined that STZ-induced diabetic C57BL/6J mice showed lower levels of CBS activity and CBS expression than normal mice [47]. On these grounds, we propose the hypothesis that downregulation of endogenous H₂S may contribute to oxidative stress and neuroinflammation in the pathophysiological process of cognitive decline. Our results also demonstrated that endogenous H₂S levels in the brains of the db/db mice decreased, indicating a role for H₂S in diabetes-associated cognitive decline. H₂S could potentially prevent astrocyte cell death induced by oxidative stress and modify enzyme activity [48], and in the future, we may attempt to address how an exogenous H₂S donor affects the expression levels of CBS, which is mainly expressed in astrocytes [49, 50]. In the present experiment, CBS expression levels were increased in response to H₂S treatment and may increase H₂S levels in the brain to some extent. In future experiments, we may measure the levels of H₂S resulting from CBS synthesis or exogenous supplementation using a CBS activator or inhibitor. Furthermore, we did not confirm the source of CBS in this study, but we could use a CBS knockout mouse to answer this question in additional studies.

Conclusion

Altogether, the present study verified that H₂S improved the spatial learning and memory abilities of db/db mice by modulating the mitochondrial apoptotic pathway and the IL-23/IL-17 axis, which were related to diabetes-associated cognitive decline. This experiment

showed that H₂S treatment may help prevent the progression of apoptotic hippocampal neurons in db/db mice and may inform the development of a new therapeutic target.

Abbreviations

DM (diabetes mellitus); T2DM (type 2 diabetes); DACD (diabetes-associated cognitive decline); AD (Alzheimer's disease); VD (vascular dementia); H₂S (hydrogen sulfide); CBS (cystathionine β-synthase); Cys (amino acid cysteine); NaHS (sodium hydrosulfide); Aβ (beta-amyloid); IL (interleukin); Th17 (T helper 17); MWM (Morris water maze); PBS (phosphate-buffered saline); BSA (bovine serum albumin); HE (haematoxylin and eosin); ANOVA (analysis of variance); Na₂S (sodium sulfide); PD (Parkinson's disease).

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Disclosure Statement

There is no known conflict of interest associated with this paper.

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