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ORIGINAL ARTICLE

# Subcutaneous injection of hydrogen gas is a novel effective treatment for type 2 diabetes

Xiaolong Zhang[1], Jiaming Liu[2], Keke Jin[3], Haifeng Xu[3], Chuang Wang[4], Zhuang Zhang[3], Mimi Kong[3], Zhengzheng Zhang[3], Qingyi Wang[5], Fangyan Wang[3]*

[1]Department of Anesthesia and Critical Care, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, [2]Department of Preventive Medicine, School of Environmental Science and Management, Wenzhou Medical University, [3]Department of Pathophysiology, School of Basic Medicine Science, Wenzhou Medical University, Wenzhou, [4]Ningbo Key Laboratory of Behavioral Neuroscience, Ningbo University School of Medicine, Ningbo, Zhejiang Province, China, and [5]Center for Computational and Integrative Biology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

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***Correspondence**
Fangyan Wang
Tel.: +86-577-8668-9817
Fax: +86-577-8668-9817
E-mail address:
wzyxywfy@126.com

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## ABSTRACT

**Aims/Introduction:** In previous studies, hydrogen gas ($H_2$) administration has clearly shown effectiveness in inhibiting diabetes. Here, we evaluated whether subcutaneous injection of $H_2$ shows enhanced efficacy against type 2 diabetes mellitus induced in mice by a high-fat diet and low-dose streptozotocin treatment.

**Material and Methods:** $H_2$ was injected subcutaneously at a dose of 1 mL/mouse/week for 4 weeks. Type 2 diabetes mellitus-associated parameters were then evaluated to determine the effectiveness of subcutaneous $H_2$ administration.

**Results:** The bodyweight of $H_2$-treated mice did not change over the course of the experiment. Compared with the untreated control animals, glucose, insulin, low-density lipoprotein and triglyceride levels in the serum were significantly lower in treated mice, whereas high-density lipoprotein cholesterol in the serum was significantly higher. Glucose tolerance and insulin sensitivity were both improved in $H_2$-treated mice. Diabetic nephropathy analysis showed significant reductions in urine volume, urinary total protein and β2-microglobulin, kidney/bodyweight ratio, and kidney fibrosis associated with subcutaneous injection of $H_2$.

**Conclusions:** Subcutaneous injection of $H_2$ significantly improves type 2 diabetes mellitus and diabetic nephropathy-related outcomes in a mouse model, supporting further consideration of subcutaneous injection as a novel and effective route of clinical $H_2$ administration.

## INTRODUCTION

Type 2 diabetes mellitus, the predominant form of diabetes, is characterized by high levels of blood sugar and insulin resistance. Approximately 170 million people in the world suffer from diabetes, a number expected to double by 2030[1]. Although effective drugs are available for clinical use, including insulin, metformin and glucagon-like peptide-1, the World Health Organization reported 1.5 million deaths from diabetes in 2012, making it the eighth most prevalent cause of death[2]. Most diabetes deaths occur in developing countries[3], and were associated with an estimated cost of $612 billion in 2014[4].

The anti-oxidant properties of hydrogen gas ($H_2$) have been recognized in recent years[5,6], and it has been used extensively to treat various conditions including ischemia/reperfusion[7], sepsis[8] and acute lung injury[9]. $H_2$ has also shown to inhibit diabetes[10,11] and related diseases[12]. The low solubility of $H_2$ renders therapeutic administration, and various routes have been used for specific treatments including high-content (saturated) $H_2$ water[10,12], inhalation[7,13,14], electrically reduced water[11] and $H_2$-producing intestinal bacteria[15].

In the present study, we used a classical mouse model of type 2 diabetes mellitus to investigate the therapeutic effects of subcutaneously injected $H_2$ by examining blood glucose, insulin and lipid levels, oxidative stress, and kidney function.

## MATERIALS AND METHODS

### Drugs and chemicals

Streptozotocin (STZ) was purchased from Sigma (St. Louis, Missouri, USA). The 40% high-fat diet was obtained from Slac Laboratory Animal Ltd. (Shanghai, China). The radioimmunoassay kits of β2-microglobulin and insulin were purchased

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from Beijing North Institute of Biological Technology (Beijing, China). Urine total protein assay kit was purchased from Baoding Great Wall Clinical Reagents Co., Ltd. (Baoding, China). Detection kits for total superoxide dismutase (T-SOD), catalase (CAT) and malondialdehyde (MDA) were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Masson staining kit was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

### Animals

The 4-week-old male C57/BL6J mice were obtained from Slac Laboratory Animal Ltd. The animals were bred under standard conditions (12-h light–dark cycle, 24°C), with free access to water and standard laboratory chow. All mice were carefully fed according to the standards of the Guide for the Care and Use of Laboratory Animals.

### Diabetic induction and animal grouping

Diabetes induction was carried out according to a previously reported protocol[16]. Briefly, mice were fed a high-fat diet for 4 weeks and then intraperitoneally injected with 100 mg/kg STZ (Sigma) or an equal volume of vehicle as the control ($n = 8$). After 2 weeks of high-fat diet feeding, glucose levels in the plasma were determined by a blood glucose meter (Andon Health Co., Ltd., Tianjin, China). Mice with glucose levels ≥10 mmol/L were considered diabetic, and were used for experiments if they continuously maintained hyperglycemia (≥10 mmol/L) over 10 days.

Mice were divided into three groups: mice without diabetes induction (normal control [NC], $n = 8$); diabetic mice receiving subcutaneous injection of air (DM; $n = 15$); diabetic mice receiving subcutaneous injections of $H_2$ (SAH; $n = 15$). The SAH group was injected subcutaneously with $H_2$ at a dose of 1 mL/mouse/week for 4 weeks. The same dose of air was given to the DM group by subcutaneous injection. In each group, the bodyweight was monitored daily, and blood glucose level was checked weekly.

### Insulin tolerance test and glucose tolerance test

The insulin tolerance test (ITT) and glucose tolerance test (GTT) were carried out at the end of the experiment. For ITT, the mice were intraperitoneally injected with insulin at a dose of 0.5 U/kg bodyweight (Wanbang Biopharmaceuticals, Jiangsu, China) after a 15-h fast. Blood samples (10 μL) were collected at 0, 30, 60 and 120 min after insulin administration. For GTT, the mice received oral glucose at 2 g/kg bodyweight of glucose after a 12-h fast. Blood samples (10 μL) were taken at 0, 30, 60 and 120 min after glucose treatment. The ITT and GTT were carried out on mice without anesthetization. Glucose levels in the plasma were determined as described above.

### Measurements of biochemical parameters

All measurements were carried out after 6 h of fasting. Blood samples from the common carotid artery were collected under anesthesia into chilled tubes treated with ethylenediamine tetraacetic acid disodium salt, immediately centrifuged and supernatants stored at −80. Plasma low-density lipoprotein (LDL), triglyceride (TG), total cholesterol and high-density lipoprotein levels were measured with the automatic biochemistry analyzer. Plasma insulin level was detected according to the instructions of the kit.

### 24-h urine volume collection, and measurement of urinary total protein and β2-microglobulin

Mice were fasted and supplied with water *ad libitum* for 24-h urine collection. Urinary total protein and β2-microglobulin were determined following the instructions supplied with the commercial kits.

### Calculation of kidney weight/bodyweight ratio and examination of renal fibrosis

Under anesthesia, the kidneys were isolated and weighed to calculate the kidney weight/bodyweight ratio (Kw/Bw [mg/g]). The right kidney was fixed in 4% paraformaldehyde for paraffin embedding. Then, 4-μm sections were stained with Masson and observed by microscopy.

### Detection of oxidative stress indicator

The MDA content, T-SOD, and CAT activities in the plasma and kidney tissue were determined by the thiobarbituric acid method[17], xanthine oxidase[18] and the ammonium molybdate colorimetric method[19], respectively. The assays were carried out according to the instructions supplied with the commercial kits. The bicinchoninic acid assay was used to normalize the levels in kidney tissue.

### Statistical analysis

All data are expressed as mean ± SD. Significant differences were determined by the Bonferroni test. A $P$-value <0.05 was considered statistically significant.

## RESULTS

### Subcutaneous administration of $H_2$ improved hyperglycemia in diabetic mice induced by high-fat diet and STZ

To initially investigate the effects of $H_2$ on diabetic mice, bodyweight was tracked throughout the experimental period. Weight gains in all groups were similar without statistical significance ($P > 0.05$), suggesting that $H_2$ administration does not affect bodyweight (Figure 1a).

We carried out biochemical analysis on blood samples to further investigate the antidiabetic effects of $H_2$. Glucose plasma levels were significantly reduced by $H_2$ injection ($P < 0.01$ or $P < 0.05$; Figure 1b). ITT and GTT were carried out at day 28. As shown in Figure 1c,d, blood glucose levels in the diabetic group reached the maximum at 30 min, and then gradually decreased. In contrast, blood glucose in the $H_2$-treated mice declined faster. Serum glucose levels in the diabetic mice were significantly increased compared with the control animals, and

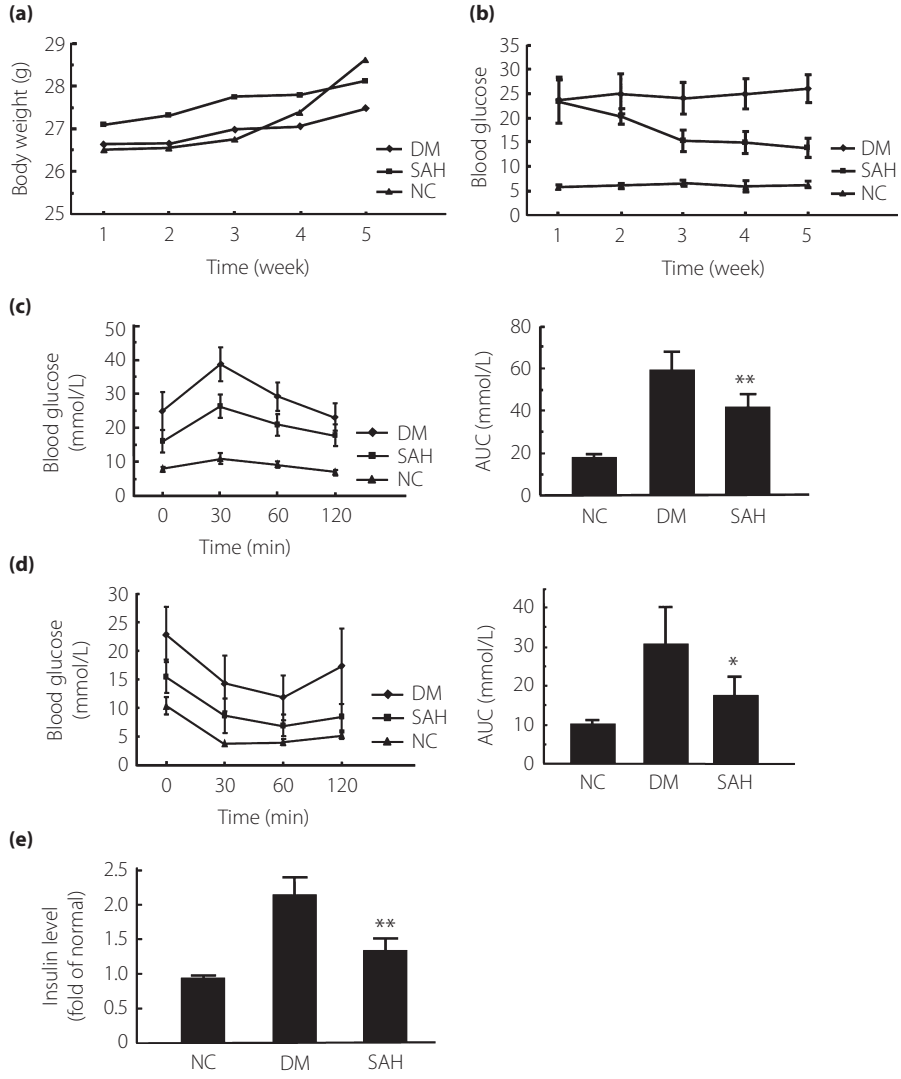


**Figure 1** | Subcutaneous administration of hydrogen gas ($H_2$) suppressed hyperglycemia in mice with diabetes mellitus (DM) induced by a high-fat diet and a low dose of streptozotocin. Diabetic mice were subcutaneously injected $H_2$ at 1 mL/mouse/week for 4 weeks. The same dose of air was given as a control. Biochemical analysis was carried out to obtain plasma parameters. (a) Bodyweight after $H_2$ treatment for each group was recorded weekly. There was no significant difference in bodyweight between the SAH group and DM group. (b) The level of blood glucose after $H_2$ treatment for each group was recorded weekly. At the 4-week time-point, the level of blood glucose was significantly reduced in the subcutaneous administration of $H_2$ group (SAH) compared with the DM group. (c,d) At day 28, the glucose tolerance test and insulin tolerance test were carried out to check the levels of blood glucose for each group at the time-points of 0, 30, 60 and 120 min. The area under the curve (AUC) for each group is also shown. (e) The level of plasma insulin was measured after 4-week $H_2$ treatment. The data are expressed as mean ± SD ($n$ = 8–16). *$P$ < 0.05, **$P$ < 0.01. NC, normal control group.

this was clearly inhibited by subcutaneous administration of $H_2$ (Figure 1d). Thus, glucose tolerance and insulin sensitivity in diabetic mice were significantly improved by subcutaneous $H_2$ treatment. Additionally, the area under the corresponding curve of GTT and ITT in the $H_2$ group was significantly decreased compared with the DM group ($P < 0.01$ or $P < 0.05$; Figure 1c,d). Plasma insulin levels also showed a dramatic reduction in the $H_2$-treated group compared with the DM group (Figure 1e). These data suggest that subcutaneous injection of $H_2$ improves hyperglycemia in diabetic mice.

### Subcutaneous administration of $H_2$ improved hyperlipemia in diabetic mice induced by a high-fat diet and a low dose of STZ

Hyperlipemia is an important feature of type 2 diabetes mellitus. We examined plasma lipids in diabetic mice after subcutaneous $H_2$ administration. Levels of LDL and TG, but not total cholesterol, were significantly attenuated, whereas the level of high-density lipoprotein was increased after $H_2$ treatment in the SAH group (**$P < 0.01$; Figure 2). These observations show significant improvement of hyperlipemia by subcutaneous

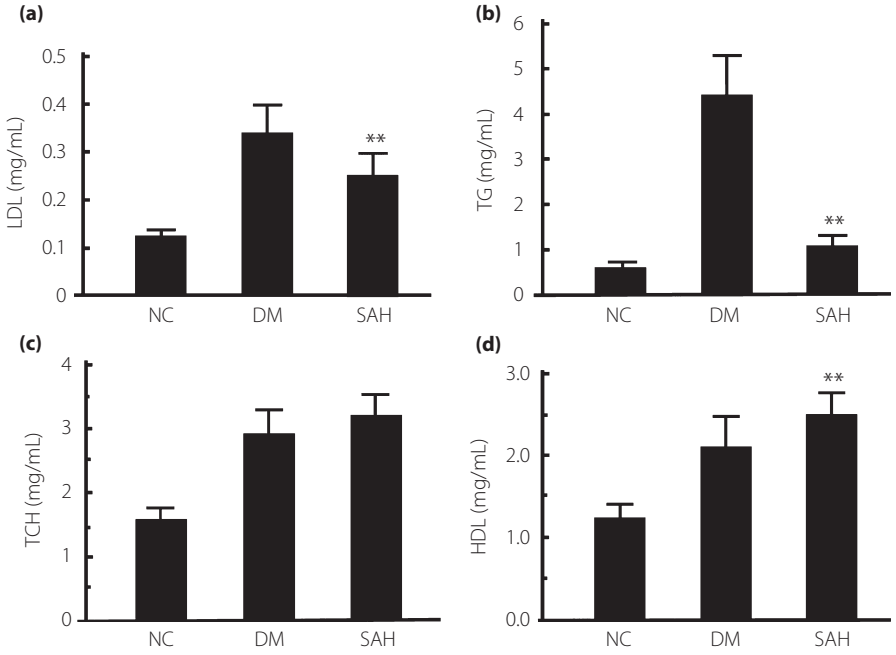


**Figure 2** | Subcutaneous administration of hydrogen gas ($H_2$) suppressed hyperlipemia in the mice with diabetes mellitus (DM) induced by a high-fat diet and a low dose of streptozotocin. After 4 weeks of $H_2$ treatment, plasma samples were collected from each group to measure the levels of plasma lipids including (a) low-density lipoprotein (LDL), (b) triglyceride (TG), (c) total cholesterol (TCH) and (d) high-density lipoprotein (HDL). The levels of plasma LDL and TG in the subcutaneous administration of $H_2$ group (SAH) group were significantly lower than those in the DM group. The data are expressed as mean ± SD ($n$ = 8–16). **$P < 0.01$. NC, normal control group.

$H_2$ administration in diabetic mice induced by a high-fat diet and a low dose of STZ.

### Subcutaneous administration of $H_2$ reduced the oxidative stress of diabetic mice induced by a high-fat diet and a low dose of STZ

The effect of $H_2$ on oxidative stress was examined by measuring the levels of T-SOD, CAT and MDA in plasma (Figure 3a–c). The MDA level in plasma was significantly reduced in the $H_2$-administered mice ($P < 0.01$). The activity of T-SOD and CAT in plasma was prominently reduced in the diabetic mice compared with the NC group, whereas these changes were reversed by subcutaneous administration of $H_2$ ($P < 0.01$ or $P < 0.05$). Thus, $H_2$ suppressed oxidative stress in the diabetic mice. We also measured the levels of T-SOD, CAT and MDA to evaluate the oxidative stress in the kidney after $H_2$ administration (Figure 3d–f). H2 administration significantly reduced MDA compared with the DM group. The activity of T-SOD and CAT in the kidney tissue of diabetic mice was prominently attenuated, which was enhanced by subcutaneous administration of $H_2$. These data show that $H_2$ inhibited renal oxidative stress in the diabetic mice.

### Subcutaneous administration of $H_2$ reduced diabetic renal injury

Because the kidney was sensitive to injury induced by diabetes, we evaluated 24-h urine volume, total urine protein and β2-microglobulin, Kw/Bw, and renal fibrosis in the experimental and control groups. As shown in Figure 4a, the 24-h urine volume of mice in the DM group was significantly increased, and this was ameliorated by subcutaneous injection of $H_2$ ($P < 0.01$). The levels of total urinary protein and β2-microglobulin, and the ratio of Kw/Bw were significantly higher in the DM vs NC mice, and subcutaneous administration of $H_2$ attenuated all DM-associated parameters ($P < 0.01$; Figure 4b–d). The fibrosis observed in the kidneys of diabetic mice (blue) was alleviated by $H_2$ treatment (Figure 4e). These data strongly show that subcutaneous administration of $H_2$ is able to reduce renal injury in diet- and STZ-induced diabetic mice.

## DISCUSSION

Previous studies showed that $H_2$-water is able to attenuate oxidative stress in patients with type 2 diabetes mellitus and metabolic syndrome[20–22]. Although ingesting $H_2$-water is a convenient route of administration, the absorptive dose of $H_2$ is extremely limited because of low saturation (0.8 mmol/L at atmospheric pressure). In the present study, we showed for the first time that subcutaneous injection of $H_2$, which is locally stored in tissue and subsequently stably diffused, has significant effects on type 2 diabetes mellitus-associated disease features.

In preliminary experiments, we found that it took approximately 1 week to completely absorb 1 mL of subcutaneously injected $H_2$, and these observations were used to design the $H_2$ administration schedule. Based on the $H_2$ solubility coefficient

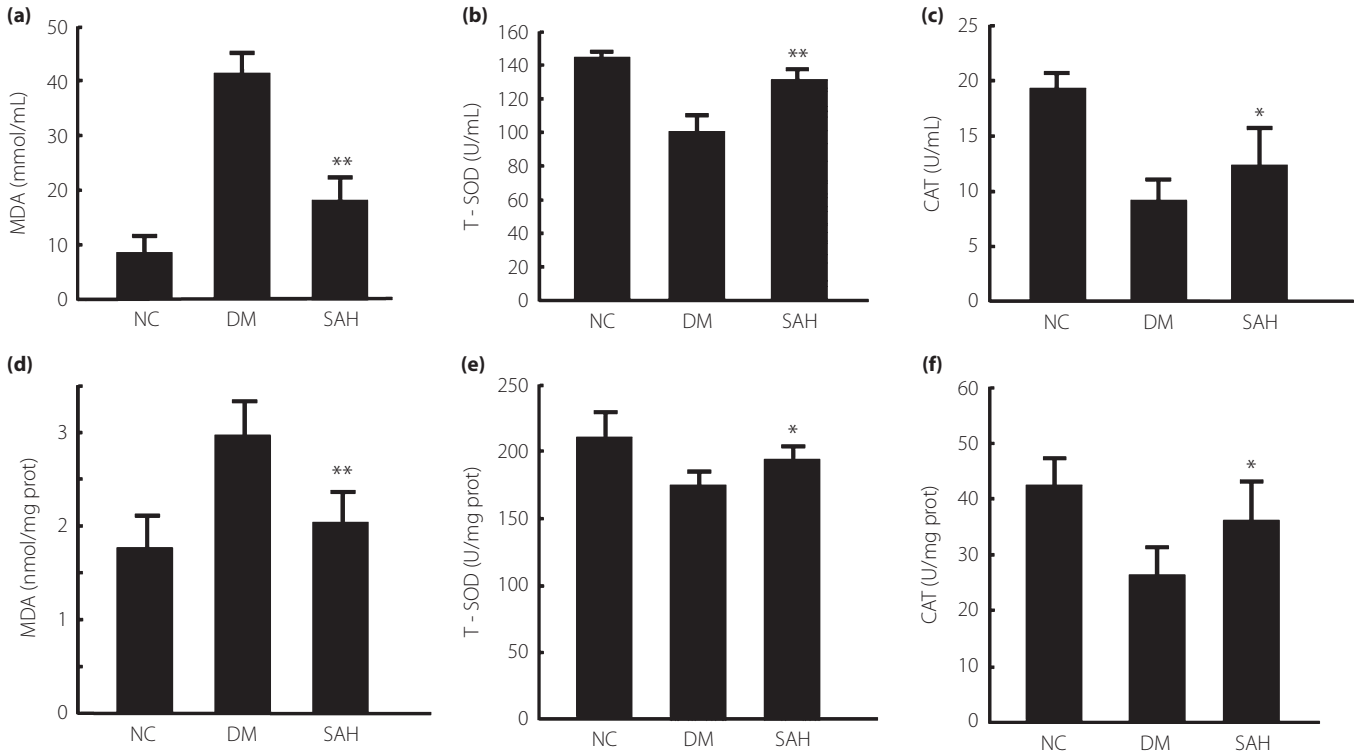


**Figure 3** | Levels of malondialdehyde (MDA), total superoxide dismutase (T-SOD) and catalase (CAT) activity in the plasma and kidney of mice with diabetes mellitus (DM) induced by a high-fat diet and a low dose of streptozotocin. Subcutaneous administration of hydrogen gas ($H_2$) to the mice (a) decreased the levels of MDA, and promoted the activities of (b) T-SOD and (c) CAT in plasma. After 4 weeks of $H_2$ treatment, the plasma samples from each group were collected to detect the levels of indicators for plasma oxidative stress. The oxidative stress was significantly reduced in the subcutaneous administration of $H_2$ group (SAH) group compared with the DM group. Subcutaneous administration of $H_2$ (d) reduced the content of MDA and promoted the activities of (e) T-SOD and (f) CAT in the kidney. Renal tissues were homogenized to examine T-SOD and CAT activity, and MDA content. bicinchoninic acid assay was used to determine protein levels in renal samples to normalize oxidative parameters. The data are expressed as mean ± SD ($n$ = 8–16). *$P$ < 0.05; **$P$ < 0.01. NC, normal control group.

(0.0182 at 20°C) and international standard atmosphere (101.3 kPa), 55 mL of water is required to dissolve 1 mL of $H_2$. At an ingestion rate of 5–7 mL/mouse/day, it would require 8 days to consume 1 mL of $H_2$. In the digestive tract, the absorption of $H_2$ into the body is even less efficient. Furthermore, injection of saturated-$H_2$ saline at a dose of 0.6 mL/mouse/day would require approximately 91 days to consume 1 mL of $H_2$. Although previously reported $H_2$ administration routes have been shown to have significant anti-oxidative effects[9,22,23], our studies suggest that subcutaneous $H_2$ injection is an efficient way to enhance $H_2$ absorption by tissues and thereby improve therapeutic efficacy.

Hyperlipemia is a typical clinical feature of metabolic syndrome induced by diabetes. Other studies showed that the plasma TG level of diabetic mice[12] and the plasma LDL, especially oxidized LDL, of type 2 diabetes patients, were suppressed by drinking $H_2$-water[20]. However, this improvement in hyperlipemia by drinking $H_2$-water was not observed in type 2 diabetic mice by Haruka[10]. The present experiments show that subcutaneous administration of $H_2$ for 4 weeks (1 mL/week) in diabetic mice significantly decreased the serum levels of LDL and TG. In particular, we showed the high-density lipoprotein content was increased by subcutaneous injection of $H_2$, which was not previously reported. The results show that subcutaneous administration is a more effective method for $H_2$ treatment.

Another important finding of the present study was that subcutaneous administration of $H_2$ ameliorated hyperglycemia. Furthermore, the data of GTT and ITT showed that glucose homeostasis and insulin sensitivity in diabetic mice were also significantly improved by subcutaneous $H_2$ treatment. However, these effects were not stably achieved through ingestion of $H_2$ water. Haruka *et al.*[10] reported that hyperglycemia attenuation from drinking $H_2$ water was effective only for type 1, but not type 2, diabetic mice.

Oxidative stress, involved in the pathogenesis of various diseases[23–29], is a process of imbalance between increased ROS and impaired anti-oxidant defenses to induce cellular injury. Most of the superoxide anion radical ($O_2^-$) is produced by electron leakage from the electron transport chain and the Krebs

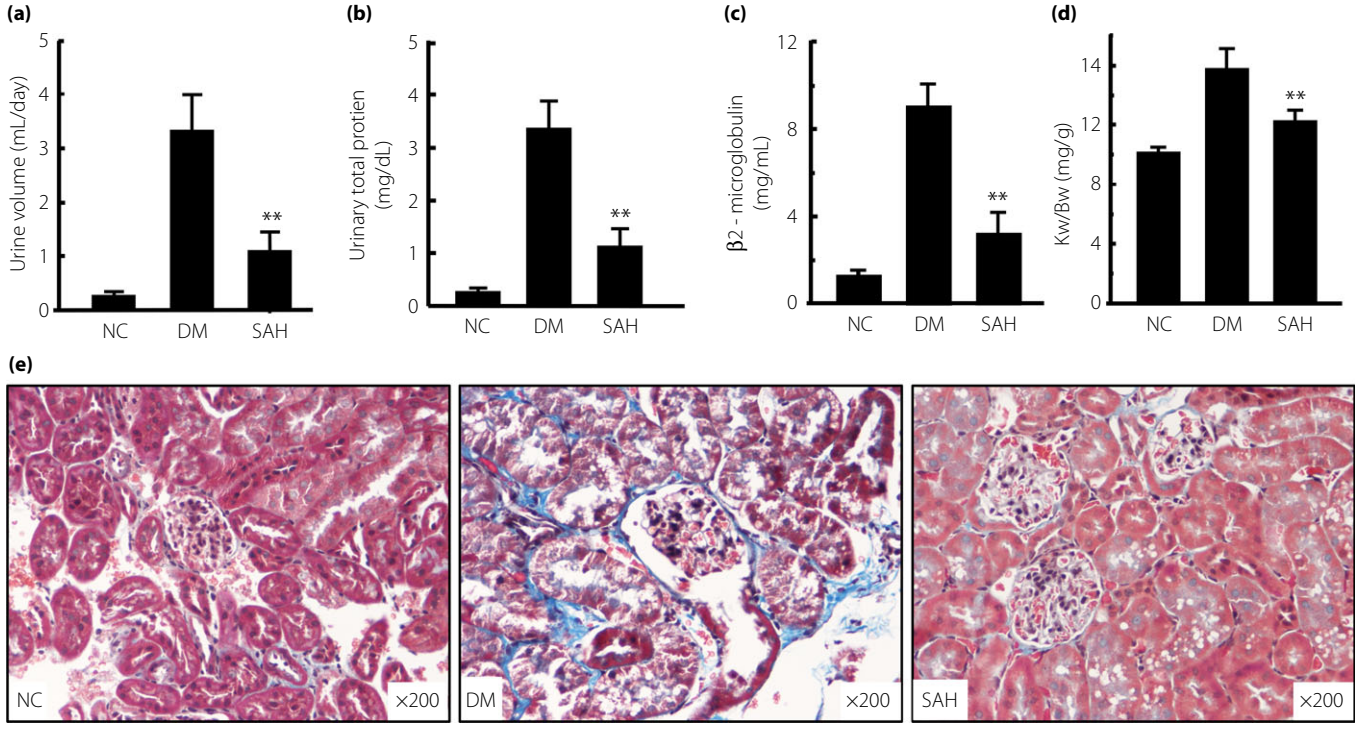


**Figure 4** | Subcutaneous administration of hydrogen gas ($H_2$) reduced 24-h urine volume, urinary total protein, β2-microglobulin, kidney weight/bodyweight ratio (Kw/Bw) and renal fibrosis resulting from diabetes. After 4 weeks of $H_2$ treatment, (a) 24-h urine volume, (b) urinary total protein, (c) β2-microglobulin and (d) Kw/Bw were detected, and (e) kidney tissues were fixed for Masson staining. The 24-h urine volume, urinary total protein, β2-microglobulin, Kw/Bw and renal fibrosis in the group with $H_2$ treatment were significantly reduced, compared with the diabetes mellitus (DM) group ($n$ = 16 for each group). The data are expressed as mean ± SD ($n$ = 8–16). **$P$ < 0.01. NC, normal control group; SAH, subcutaneous administration of $H_2$ group.

cycle. SOD converts $O_2^-$ into hydrogen peroxide ($H_2O_2$), which is detoxified into $H_2O$ by either glutathione peroxidase or CAT. Excessive $O_2^-$ reduces transition metal ions, such as $Fe^{3+}$ and $Cu^{2+}$, which in turn react with $H_2O_2$ to produce hydroxyl radicals (OH·) by the Fenton reaction. OH·, the strongest of the oxidant species without an especially targeted detoxification system, reacts easily with nucleic acids, lipids and proteins. Therefore, scavenging OH· is a critical anti-oxidant process. Oxidative stress is an important mechanism underlying diabetes mellitus[24,30,31], which impacts millions of people worldwide. Oxidative stress can cause pancreatic β-cell damage[32], and induce insulin resistance in fat cells and liver cells[33,34], thus affecting glycolipid metabolism. As an anti-oxidant gas, $H_2$ can reduce oxidative damage in multiple tissues and organs. Previous studies have shown that $H_2$ can improve diabetes and diabetes-associated complications[1,35]. Therefore, we believe that $H_2$ improves glucose and lipid metabolism, likely through reducing oxidative damage in the liver, adipose tissue and pancreatic β-cells.

$H_2$ is a promising scavenger of reactive oxygen species, and shows remarkable protective effects in various diseases[5,7]. Ohsawa *et al*[5] showed that $H_2$ is a novel anti-oxidative gas molecule to selectively address OH·. In the present study, we examined oxidative stress-related parameters including T-SOD, CAT and MDA levels in plasma, and found that subcutaneous injection of $H_2$ strikingly alleviated the oxidative stress of diabetes, which is consistent with previous studies[35]. Here, we also showed that subcutaneous injection of $H_2$ improved diabetic nephropathy (DN) through anti-oxidative stress. DN is one of the most common complications of diabetes. We examined multiple parameters to evaluate anti-DN effects of subcutaneous injection of $H_2$ including 24-h urine volume, urinary total protein and urine β2-microglobulin, which is a sensitive diagnostic feature for DN[36]. DN was successfully induced by the high-fat diet and low-dose STZ in the present study, and was strikingly improved by subcutaneous injection of $H_2$. Fibrosis, a typical pathological change of DN[37], observed in diabetic mice by Masson staining of kidney tissue sections, was consistently alleviated by subcutaneous injection of $H_2$. Oxidative stress is a critical factor in diabetic kidney damages[30], and $H_2$ was previously reported to prevent chronic allograft nephropathy through anti-oxidation properties[38]. The present study showed that DN was effectively inhibited by subcutaneous injection of $H_2$.

In conclusion, the present study showed for the first time that subcutaneous injection of $H_2$ confers beneficial effects on

lipid and glucose metabolism, and DN in diabetic mice by providing protection against oxidative stress. These observations support the potential clinical application of this novel route of $H_2$ administration for the treatment of type 2 diabetes mellitus.

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## DISCLOSURE

The authors declare no conflict of interest.

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