Original Article
Inhalation of hydrogen gas ameliorates glyoxylate-induced calcium oxalate deposition and renal oxidative stress in mice

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Abstract: The aim of this study is to evaluate the protective effect and underlying mechanism of hydrogen gas (H2) to glyoxylate induced renal calcium oxalate (CaOx) crystal deposition in mice. In present work, rodent renal CaOx crystal deposition model was introduced by intra-abdominal injection of glyoxylate (100 mg/kg/d) for 5 days. Two days before administration of glyoxylate, inhalation of H2 for 30 min per day was initiated and continued for 7 days. By the end of the study, the samples of 24 hours urine, serum and renal tissue were collected for biochemical and pathological assay. According to levels of urine calcium excretion, renal calcium deposition, a serum excretion of kidney injury molecule-1 (KIM-1) assay and a TUNEL assay, inhalation of H2 could successfully decrease the CaOx crystallizations and protect against renal injury. Crystal deposition in the kidneys is associated with oxidative stress, which was indicated by increased levels of renal malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) and decreased activities of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT). These effects were reversed by a high-dose H2 pretreatment. The renal expressions of osteopontin (OPN), CD44, monocyte chemoattractant protein-1 (MCP-1) and interleukin-10 (IL-10) were markedly increased in glyoxylate-treated mice, and H2 significantly attenuated the increase of OPN, CD44 and MCP-1 but upregulated the expression of IL-10. Our findings demonstrate that inhalation of H2 reduces renal crystallization, renal oxidative injury and inflammation and it may be a candidate agent with few adverse effects for prevention of nephrolithiasis.

Keywords: Hydrogen gas, calcium oxalate, kidney injury molecule-1, glyoxylate, oxidative stress

Introduction
Stone disease, also known as urolithiasis, is becoming a major public health burden worldwide [1, 2]. Nephrolithiasis, deposition of crystal in the kidneys and the formation of kidney stones, is a common urological disorder. Calcium oxalate (CaOx) is the most common type of nephrolithiasis; hyperoxaluria is the major risk factor [3]. Nephrolithiasis formation consists of several stages including supersaturation, nucleation, growth, aggregation, and retention within renal tubules. Although the specific mechanism is not clear, there is accumulating evidence that crystal deposition in the kidneys is associated with reactive oxygen species (ROS) production, oxidative stress development, inflammation and renal tubule injury [4, 5]. Anti-oxidative treatment has become an experimental strategy of anti-nephrolithiasis, such as vitamin E [6], vitamin C [7], and some traditional medicinal herbs [8, 9]. Nevertheless, no such substance could be translated into clinical practice because some side-effects were present [7]. Thus, developing an effective alternative with few adverse effects is an urgent need in this field.

Hydrogen is considered an inert gas and has been used in medical applications to prevent decompression sickness in deep divers [10]. In 2007, it was reported that an inhalation of H2 has antioxidant and anti-apoptotic properties that can protect the brain against ischemia/reperfusion injury by selectively neutralizing hydroxyl radicals [11]. This report aroused considerable interest worldwide. Thus far, the therapeutic effects of molecular hydrogen on vari-
ous diseases, including kidney diseases [12, 13], have been tested mostly regarding its anti-
odoxidation capability [14], but also its anti-
flammation [15] and anti-apoptosis [16] capabilities. Compared with traditional antioxi-
dants, hydrogen is a small molecule that can
easily dissipate throughout the body and cells, and it is sufficiently mild that it does not disturb
metabolic oxidation-reduction reactions or dis-
rupt ROS mediated cell signaling. Thus, it may
be a safe and effective antioxidant for
nephrolithiasis.

To date, most inhaled hydrogen gas research
has focused on a low dose (2%-4%), but the
effect of inhaling a high dose has rarely been
tested. In theory, high-dose hydrogen might have good effects and can be easily used to
explore the dose-dependent response of hydro-
gen with increasing dosage. In this study, we
hypothesized that pretreatment with a high
dose of hydrogen gas will attenuate renal crys-
tallization by anti-oxidation, anti-inflammation, and protection from renal tubule injury.

Materials and methods

Animals

Male C57BL/6 mice (8 weeks old) were given
water and food *ad libitum* and were kept for one
week under a controlled 12-hour light/dark
cycle at 20-25°C with the relative humidity set
at 55-65%. This study was approved by the
Animal Care and Use Committee of Second
Military Medical University.

Study groups

Twenty-four male C57BL/6 mice were randomly
divided into the following four groups of 6 mice
each: 1) Nitrogen-oxygen gas (NO) group: The
animals inhaled mixed gas consisting of 67% N₂
and 33% O₂ (vol/vol) for 30 min daily for 7 con-
secutive days in addition to daily intra-abdomi-
nal injections with a normal volume of saline
(20 ml/kg/day) from day 3. 2) Hydrogen-oxygen
gas (HO) group: The animals inhaled mixed gas
consisting of 67% H₂ and 33% O₂ (vol/vol) for
30 min daily for 7 consecutive days in addition
to daily intra-abdominal injections with a nor-
mal volume of saline (20 ml/kg/day) from day 3.
3) CaOx group: The animals inhaled mixed
gas consisting of 67% N₂ and 33% O₂ (vol/vol)
for 30 min daily for 7 days in addition to daily
intra-abdominal injections with glyoxylate (100
mg/kg/d, TCI, Tokyo, Japan) from day 3. 4) Hydrogen-oxygen gas pretreatment (HO plus
CaOx) group: The animals inhaled mixed gas
consisting of 67% H₂ and 33% O₂ (vol/vol) for
30 min daily for 7 days in addition to daily intra-
abdominal injections with glyoxylate from day
3.

All mice were placed in individual metabolic
cages with free access to water after the glyox-
ylate injection for 24 hours of urine collection.
After urine and blood collection, the kidneys
were harvested. The left kidney was frozen at
-80°C for future use, and the right kidney was
fixed in 4% paraformaldehyde for histologic
examination.

Gas administration

The mixed gas consisting of 67% H₂ and 33% O₂
was produced by the AMS-H-01 hydrogen oxy-
ogen nebulizer (Asclepius, Shanghai, China),
which was specifically designed to extract the
hydrogen and oxygen from water. The mice
were placed into a transparent closed box (20 ×
18 × 15 cm, length x width x height), into which
the mixed gas was introduced at a rate of 200
ml/min throughout the experiments. The box
was flushed with mixed gases for 30 min to
replace the air in the box. Mixed gas consisting
of 67% N₂ and 33% O₂ was administered at the
same rate. During each experiment, the con-
centration of hydrogen gas in the box was moni-
tored by Thermal trace GC ultra gas chromato-
graphy (Thermo Fisher, MA, USA).

Urine biochemistry

The ratios of calcium, phosphate, magnesium
and creatinine in urine were measured using a
BC-2800 Vet animal auto biochemistry analyz-
er (Shihai, Guangdong, China).

Serum excretion of Kidney injury molecule-1
(KIM-1)

We measured the level of serum excretion of
KIM-1 using an enzyme-linked immunosorben-
t (ELISA) kit (Uscn, Wuhan, China), according to
the manufacturer’s instructions.

Histological analysis

Paraffin-embedded sections (3 μm) of mice kid-
neys were stained with a Von Kossa staining
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Table 1. The ratios of the urine concentration of calcium (Ca), phosphate (P) and magnesium (Mg) to creatinine in NO, HO, CaOx and HO plus CaOx groups

<table>
<thead>
<tr>
<th></th>
<th>Ca/Creatinine</th>
<th>P/Creatinine</th>
<th>Mg/Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>0.33 ± 0.05</td>
<td>3.72 ± 0.47</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>HO</td>
<td>0.35 ± 0.09</td>
<td>4.18 ± 0.56</td>
<td>0.32 ± 0.12</td>
</tr>
<tr>
<td>CaOx</td>
<td>0.63 ± 0.15*</td>
<td>8.12 ± 1.17*</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>HO + CaOx</td>
<td>0.43 ± 0.10#</td>
<td>5.33 ± 1.20#</td>
<td>0.29 ± 0.07</td>
</tr>
</tbody>
</table>

*P < 0.05 versus NO group, #P < 0.05 versus CaOx group.

commercial kit (Showbio, Shanghai, China) to quantify crystallization. The area of crystal deposits in kidney tissue was counted in 20 randomly selected fields and photographed under an Eclipse 50i light microscope (Nikon, Japan) at ×400 magnification.

The level of calcium in kidney tissue

We measured the level of calcium in kidney tissue using a colorimetric assay kit (Nanjing Jiancheng, Nanjing, China), according to the manufacturer’s instructions.

Oxidative stress index in kidney tissue

A portion of the tissue preparation was made from kidney tissue that was homogenized with normal saline, and the preparation was centrifuged at 2500 rpm at 4°C for 10 min. The resultant supernatant was equilibrated with saline and used to determine the levels of proteins in the tissue homogenates. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), catalase (CAT), myeloperoxidase (MPO), and 8-hydroxydeoxyguanosine (8-OHdG) were determined using commercial kits (Nanjing Jiancheng, Nanjing, China). The determinations were performed strictly according to the manufacturer’s instructions.

Immunohistochemistry

Kidney tissues sections (3 µm) were subjected to immunohistochemistry. The sections were incubated with primary antibodies of osteopontin (OPN) (1:100) (Santa Cruz, CA, USA), CD44 (1:100) (Proteintech, IL, USA), monocyte chemoattractant protein-1 (MCP-1) (1:100) (Santa Cruz, CA, USA) and interleukin-10 (IL-10) (1:100) (Santa Cruz, CA, USA) at 4°C overnight. After rinsing with PBST, secondary antibodies (1:500) (Proteintech, IL, USA) were added to the sections, conjugated to horseradish peroxidase and incubated at 37°C for 30 min. The resultant sections were visualized using di-aminobenzidine kits (Maixin, Fuzhou, China) and counterstained with hematoxylin solution (Beyotime, Haimen, China).

TUNEL assay

TUNEL staining was performed using a FragEL™ DNA Fragmentation Detection Kit and Colorimetric-TdT Enzyme (Merck & Co Inc, NJ, USA). All stained sections were counted in 10 randomly selected fields and photographed under an Eclipse 50i light microscope (Nikon, Japan) at ×400 magnification.

Statistical analysis

Results were presented as mean ± SD. We used one-way factorial ANOVA to test for overall differences among the groups, followed by Student-Newman-Keuls test to compare separate groups. Nonparametric data were analyzed by the Kruskal-Wallis H-test. P < 0.05 denoted the presence of a statistically significant difference.

Results

Urine biochemistry

As shown in Table 1, the ratio of calcium and creatinine significantly increased in the CaOx group compared with the NO group (P < 0.05) and decreased in the HO plus CaOx group (P < 0.05). Additionally, the ratio of phosphate and creatinine increased in the CaOx group (P < 0.05) and decreased in the HO plus CaOx group (P < 0.05). Additionally, H₂ pretreatment alone showed no effect on the ratio of calcium, phosphate and creatinine.

The level of serum excretion of KIM-1

The level of serum excretion of KIM-1 increased significantly after the administration of glyoxylate, and the effect was reversed by H₂ pretreatment. Meanwhile, H₂ pretreatment alone showed no effect on the level of serum excretion of KIM-1 (Figure 1A).

TUNEL assay

To further evaluate the potential protective effect of H₂, renal cell apoptosis was assessed using TUNEL assay. The number of TUNEL-positive cells was dramatically increased by glyoxylate treatment, to 8.7%, but this increase was dramatically blunted by pretreatment with H₂, to 3.1% (Figure 1B, 1C).
**Von Kossa-stained kidney sections**

To examine the presence of CaOx crystal deposition in the kidney, Von Kossa-stained sections of kidney were examined under light microscopy. As shown in **Figure 2**, in histological examination of kidneys in the CaOx and HO plus CaOx groups, crystals were observed in the lumen and were primarily located at the border between the renal cortex and medulla (×40 magnification). Compared with the CaOx group, histological examination of kidneys in the HO plus CaOx group showed fewer crystal depositions in the kidney, and no crystals were observed in the NO or HO groups.

**The concentration of calcium in kidney tissue**

The concentration of calcium in renal tissue increased significantly (P < 0.05) after the mice were administered glyoxylate and decreased significantly (P < 0.05) in the HO plus CaOx group.
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Figure 2. Detection of renal calcium oxalate crystal deposition by Von Kossa staining and the concentration of renal calcium. (A Magnification x40) and (B Magnification x400) represent calcium oxalate crystal deposition, (C) Semi-quantitative analysis of calcium deposition by the area of positive staining from every 20 random views, (D) The concentration of renal calcium. *P < 0.05 versus NO group, #P < 0.05 versus CaOx group.

Group compared with the CaOx group (Figure 2D). H₂ pretreatment alone showed no effect on the renal calcium level.

ROS production and antioxidative enzyme

The levels or activities of SOD, MDA, GSH, CAT, MPO, and 8-OHdG in renal tissue were determined to assist the H₂ in protecting renal tissue from oxidative damage (Figure 3). The levels of MDA and 8-OHdG increased significantly (P < 0.05), and the activity of SOD and CAT and the GSH level decreased significantly (P < 0.05) in the CaOx group. In the HO plus CaOx group, a significant reduction of MDA and 8-OHdG levels (P < 0.05) was observed, and a significant increase in SOD and CAT activities and GSH levels (P < 0.05) was observed. However, the level of MPO did not change significantly.

Immunohistochemical analysis of the kidneys

Immunostaining was used to determine the expression of OPN, CD44, MCP-1 and IL-10 in the kidneys. OPN and CD44 expression was almost nonexistent in the NO group but was highly expressed in the renal tubules of the CaOx group (Figure 4). MCP-1 was also highly expressed in the adjacent interstitium of renal tubules in the CaOx group (Figure 4). Staining for OPN, CD44, and MCP-1 demonstrated a marked reduction in the kidneys of glyoxylate-induced mice pretreated with H₂. Compared with the NO group, the expression of IL-10 increased in the CaOx group, and a markedly increased expression was clearly observed in the HO plus CaOx group.

Discussion

Hyperoxaluria is considered a major risk factor of CaOx stone disease. According to a previous study [17], we used glyoxylate, an oxalate precursor, to induce intratubular CaOx deposition. Based on the changes in urine biochemistry, the renal calcium concentration and the area of calcium deposition (Von Kossa staining), we
found that \( \text{H}_2 \) pretreatment could reduce renal crystallization. Hyperoxaluria and CaOx crystal deposition are associated with renal tubular epithelial cell injury. KIM-1 is a sensitive marker of the presence of renal tubular damage [18]. Urinary excretion and the renal expression of KIM-1 have been reported in association with renal CaOx crystal deposition [9]. In this study, we found that the level of serum excretion of KIM-1 was markedly upregulated after the administration of glyoxylate and downregulated by pretreatment with \( \text{H}_2 \). Additionally, we examined renal apoptosis using TUNEL staining and found that kidneys in the CaOx group had more TUNEL positive cells compared with the NO group. \( \text{H}_2 \) pretreatment significantly decreased TUNEL positive cells in the HO plus CaOx group. These results indicated that \( \text{H}_2 \) could attenuate

**Figure 3.** The levels or activities of SOD, MDA, GSH, CAT, MPO, 8-OHdG, *'P < 0.05 versus NO group, &'P < 0.05 versus CaOx group.
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Simultaneously, H$_2$ pretreatment alone showed no effect on the ratios of calcium, phosphate and creatinine, the concentration of renal calcium or the level of serum excretion of KIM-1. It seems that H$_2$ has no side effects on crystal deposition and renal function.

Many studies have revealed that ROS overproduction and decreased antioxidants lead to oxidative stress and are involved with kidney stone formation [4, 5]. ROS are generally considered cytotoxic and can damage a variety of macromolecules, including lipids, proteins and DNA [19]. MDA is an end product of lipid peroxidation.
tion that is usually used as a measure of the lipid peroxidation status of the body. MPO is a catalyst for oxidants. 8-OHdG is a marker of oxidative damage of DNA. SOD, GSH and CAT are common antioxidant substances in the body. H₂ is considered a specific scavenger of hydroxyl radical and peroxynitrite that could decrease the levels of MDA, MPO and 8-OHdG and increase antioxidant enzyme activity [20]. In agreement with a previous study, the pretreatment with H₂ in this study was associated with the reduced production of MDA and 8-OHdG and with enhanced activities of antioxidant enzymes such as SOD, GSH and CAT in the renal tissue of mice; however, the MPO level did not change significantly.

Inflammation is accompanied with oxidative stress in crystal nephropathy, and crystallization modulators, such as OPN and CD44, play a significant role in inflammation and tissue repair [5]. OPN is implicated in crystal retention within the kidneys by affecting the adherence of CaOx crystals to renal epithelial cells and may be a chemoattractant for renal interstitium [21, 22]. CD44 serves as a cell surface receptor for both hyaluronan and OPN and as a crystal attachment promoter [5, 23]. The interaction with OPN and CD44, as expressed by injured/regenerating tubular cells, is a prerequisite for the retention of crystals in the kidney [23]. MCP-1, a chemokine with potent chemoattractive activity for monocytes/macrophages, is a mediator of local inflammatory responses to interstitial crystals [24]. The interaction of renal epithelial cells with CaOx monohydrate crystals has been shown to stimulate both the expression of OPN and MCP-1 [25] and an increase in the generation of ROS from nicotinamide adenine dinucleotide phosphate as a second messenger system [26]. In the present study, we found that the expression of OPN, CD44 and MCP-1 increased significantly in the CaOx group and that this effect was reversed by H₂ pretreatment. IL-10, initially denoted cytokine synthesis inhibitor factor plays a renoprotective role in several kidney models [27-29]. In lipopolysaccharide induced neuroinflammation mice, the expression of IL-10 was upregulated by the administration of hydrogen-enriched electrochemically reduced water, suggesting a strong anti-inflammatory response aiming at restoring the redox homeostasis and functional recovery [30]. Consistent with previous studies, the expression of IL-10 increased significantly in the CaOx group and was markedly upregulated in the HO plus CaOx group. These results indicated that H₂ pretreatment could upregulate the expression of IL-10 to protect renal tubular epithelial cell injury from inflammation. It seems that H₂ pretreatment could regulate the expression of crystallization modulators, suppress the pro-inflammatory cytokine and upregulate the anti-inflammatory factor in response to inflammation involved in CaOx crystal deposition.

In summary, our results support that H₂ reduced renal crystallization and protected renal tubular epithelial cells against glyoxylate-induced renal injury by suppressing oxidative stress and inhibiting inflammation. This study investigates the anti-nephrolithiasis effect of H₂. It may provide a novel clinical treatment of kidney stones. Future studies should provide more information concerning the effects of different times and doses of inhaled H₂ on kidney stones.

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Disclosure of conflict of interest

None.

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