Original Paper

Hydrogen Treatment Protects Mice Against Chronic Pancreatitis by Restoring Regulatory T Cells Loss

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Key Words

Pancreatitis • Hydrogen • Immunoregulation • Treg

Abstract

Background/Aims: Chronic pancreatitis is an inflammatory disease of the pancreas characterized by progressive tissue destruction and fibrogenesis. The development of chronic pancreatitis is associated with immune cell dysregulation. Currently, the specific and effective treatment of chronic pancreatitis remains absent. **Methods:** By using an L-arginine induced chronic pancreatitis mouse model, we tested the therapeutic potential of hydrogen, a strong hydroxyl radicals scavenger, in the chronic pancreatitis model. Tissue inflammation, damage and fibrosis were analyzed on HE, TUNEL, MPO, and sirius staining. Pancreas levels of MDA content, SOD activity, TNF- α , IL-10 cytokine expression and serum amylase and lipase activity were determined by ELISA and absorbance assay. Apoptosis, T cells subtype proportion and intracellular level of reactive oxygen species (ROS) were analyzed by flow cytometry. Tregs adoptive transfer and CD25 neutralization were used to validate the role of Tregs in chronic pancreatitis. **Results:** We found that hydrogen treatment significantly improved multiple symptoms of chronic pancreatitis. The number of Tregs was reduced in chronic pancreatitis mice, while hydrogen treatment restored the Treg loss by L-arginine administrations. Depletion of Tregs abolished the protective effect of hydrogen treatment in chronic pancreatitis. In vitro study showed that hydrogen blocked ROS generation in Tregs and promoted Tregs survival. **Conclusion:** Hydrogen treatment showed reliable benefits in controlling the severity of chronic pancreatitis. Our study supported that hydrogen could be used as a novel treatment in chronic pancreatitis patient in the future.

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Introduction

Chronic pancreatitis is characterized by sustained inflammation in the pancreas, which leads to the irreversible change of the normal structure and function of normal pancreas.

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Prominent fibrosis is a major histological feature of chronic pancreatitis [1]. Chronic pancreatitis results from both environmental (such as alcohol abuse) and genetic factors (such as mutation in a trypsin-controlling gene) [2]. Pancreatic fibrogenesis is a typical response to repeatedly acinar injury. This process involves a complex of orchestration of cytokines, chemokines and growth factors derived from immune cells and other cells. Although the exact mechanisms for the pathogenesis of chronic pancreatitis are not completely understood, the role of dysregulation of immune cells has attracted extensive attentions [3, 4]. Inhibition of inflammatory response by drug treatment or inflammatory cell ablation has been confirmed effective in treating pancreatitis [5, 6].

T cells play a central role in the adaptive immune response. The role of T cells in the development of both acute and chronic pancreatitis has been well documented [4, 7]. T cells, especially CD4⁺ T cells contributed to the progress of acute pancreatitis, evidenced by depletion of CD4⁺ T cells but not CD8⁺ T cells significantly reduced the severity of acute pancreatitis [8]. In chronic pancreatitis, significant increase in CD4⁺ and CD8⁺ T-cell infiltration was observed in pancreas of patients with chronic pancreatitis [9]. Moreover, strong IL-10 based T cell response against pancreatitis-associated antigen was found in chronic pancreatitis patients [10]. Further study confirmed that pancreatitis-specific IL-10 responses were mediated by IL-10⁺IFN- γ FoxP3⁺ regulatory T cells (Tregs). The expansion of these Tregs helped to suppress conventional T cell response in chronic pancreatitis patients [10].

Hydrogen gas (H₂), a well-known molecule with the simplest structure, has been demonstrated recently to have selectively reduced reactive oxygen species (ROS) and anti-inflammatory properties [11, 12]. Instead of H₂, hydrogen-rich saline, which is safer and easier for administration, may be more suitable for clinical applications. Beneficial effects of molecular hydrogen have been observed essentially in ischemia-reperfusion (I/R) injury [13, 14], type 2 diabetes [15], Alzheimer's disease, Parkinson's disease [16], acute pancreatitis [17], and other oxidative stress-related diseases. However, there are few researches to explore the protective mechanisms of hydrogen for chronic pancreatitis. As immune cells, especially T cell played critical role in the pathogenesis of chronic pancreatitis, whether hydrogen influenced immune cells remains elusive.

Here we used a well-established mouse model of chronic pancreatitis to evaluate the effects of hydrogen on chronic pancreatitis and explore the possible mechanisms involved. We will focus on the regulatory effects of hydrogen on immune cells in chronic pancreatitis.

Materials and Methods

Animals and experiment design

Male C57BL/6J mice (18-20 g) were supplied by the Animal Research Center at Second Military Medical University. All surgical procedures and care administered to the animals were approved by the institutional ethic committee, and this study also complied with the criteria in Guide for the Care and Use of Laboratory Animals.

45 mice were randomly assigned to 3 groups (each group had 15 mice): control, chronic pancreatitis, and chronic pancreatitis+2% hydrogen. Chronic pancreatitis was induced by intraperitoneal injection of 10 mg/kg L-arginine (Sigma-Aldrich, St. Louis, MO, USA) for 28 days. The mice in the hydrogen group received 2% hydrogen by Oxyhydrogen atomizing machine (Shanghai Asclepius Meditech Ltd, China) for 1 h after operation, respectively. At the indicated time points, the surviving mice in each group were randomly sacrificed, and blood and pancreatic samples were collected. Blood samples were centrifuged and then sera were collected and stored at -20°C. Pancreatic samples were stored at -80°C for subsequent measurement.

Histological analysis

Pancreas from mice was fixed in 4% paraformaldehyde, dehydrated for 12 h, embedded in paraffin wax, cut into 3 μ m-thick slices, and then examined via light microscopy (Olympus, Tokyo, Japan) for hematoxylin-eosin (HE), Sirius stain, TUNEL, and MPO immunohistochemistry, respectively.

ELISA

Cytokines in the medium was measured using ELISA kit (eBioscience, San Diego, CA). Levels of IL-10, TNF- α and TGF- β were assessed by ELISA using monoclonal antibodies and the procedure recommended by the supplier.

Measurement of superoxide dismutase (SOD) and malondialdehyde (MDA) in pancreas

Measuring the activity of SOD and MDA in pancreas tissues has been described in previous report [18]. SOD activity as well as MDA contents of the pancreas were tested by chromatometry kit (Invitrogen, Camarillo, CA, USA) and enzymatic-spectrophotometric kit (Jian Cheng Biotech Ltd, China), respectively. The SOD activity value measurement was used to assess polymorphonuclear leukocyte infiltration, and the levels of MDA were interpreted as an indicator of lipid peroxidation.

Amylase and lipase activity assay

Amylase activity was measured by starch-iodine method. 10 μ L 1% starch solution (w/v) and 5 μ L sample were incubated at 37°C for 30 min. After incubation, 30 μ L HCl (1 M) was added. An aliquot (150 μ L) of iodine reagent (5 mM I₂ and 5 mM KI) was added and the reaction mixture was centrifuged at 12,000 $\times$ g for 1 min. Absorbance at 600 nm of the reaction mixture was measured.

Lipase activity was measured using p-nitrophenylmyristate as substrate. 50 μ L ammonium bicarbonate solution (100 mM), 5 μ L sample, 100 μ L 1% Triton-X 100, and 8 μ L p-nitrophenylmyristate (10 mM) were incubated at room temperature for 15 min. The reaction mixture was measured at 405 nm for absorbance changes.

Flow cytometry analysis of T cell subtype

Analysis of T cell subtype was performed by flow cytometry (BD Biosciences, San Jose, CA). The cells were collected and stained with PE-CD3, FITC-CD4 and APC-CD8 (eBioscience), respectively, to detect the percentage of CD3⁺T, CD4⁺T, and CD8⁺T cells. FITC-CD4, PE-Foxp3, and APC-CD25 were used to stain Treg.

Treg culture and adoptive transfer experiments

Treg cells were purified using MOUSE CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturers' instructions. Then Treg cells were cultured in complete medium (RPMI 1640 containing 10 % FBS and 1 % Pen/Strep all from Gibco, CA, USA). For adoptive transfer experiments, 1 $\times$ 10⁶ cells cultured Treg cells were transferred into each recipient mouse via tail intravenous injection.

Neutralization of CD25

Anti-mouse CD25 functional grade purified was purchased from eBioscience. Rat IgG2b K (catalog 16-4031, eBioscience) was used as an isotype-matched control. In chronic pancreatitis model, male C57 mice were intraperitoneal injected with anti-CD25 (1 mg/kg) on day 1, 8, 15, and 22.

Hydrogen-Rich saline Preparation

Hydrogen-Rich saline was prepared by an equipment provided by the Department of Diving Medicine (Second Military Medical University) as previously described [19]. Hydrogen-Rich saline was freshly prepared every day to ensure that the hydrogen concentration of more than 1 mM was maintained.

Cell apoptosis assay

The apoptotic cells were quantified using the annexin V and PI double staining kit (BD Biosciences). Briefly, cells were collected, washed with PBS, and resuspended in 200 μ L binding buffer containing 5 μ L annexin V (10 μ g/mL) and 10 μ L PI (20 μ g/mL) for 30 min. Then samples were immediately analyzed using flow cytometry.

ROS production measurement

ROS levels were detected using a flow cytometer and a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Cells were harvested and washed with PBS and suspended in 10 μ M 5(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Invitrogen) at 37 °C for 20 min. The cells were then washed twice with PBS and subjected to flow cytometry analysis.

Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). The data were analyzed by one-way ANOVA analysis of variance followed by Dunnett's test. Statistical differences were considered statistically significant when $P < 0.05$.

Results

Hydrogen treatment reduced chronic pancreatitis damage

The daily L-arginine administration for 28 days produced evidence of chronic pancreatitis in Model group mice, including increased pancreas size/weight, water content of pancreas, abnormal architecture, increased TUNEL+ acinar cells, massive MPO+ neutrophil infiltration, elevated serum amylase/lipase levels and pancreatic fibrosis (Figs. 1A~C, Figs. 2A~B and Fig. 3C). Compared with Model group mice, daily treatment of 2% hydrogen significantly reduced the severity of chronic pancreatitis. 2% hydrogen inhibited the increase of pancreas weight and water content caused by L-arginine (Figs. 1A~C and Fig. 3C). Also, pathological score, number of TUNEL+ acinar cells and neutrophil infiltration were greatly improved with 2% hydrogen treatment (Figs. 2A~B). We also observed the lower serum amylase/lipase levels in 2% hydrogen treated mice compared with Model group (Fig. 3C). Importantly, pancreatic fibrosis was partly reversed by 2% hydrogen (Fig. 2A).

As oxidative stress and cytokine profile change played critical roles in the pathogenesis of pancreatitis [20, 21], we checked pancreas SOD activity and MDA levels. As expected, reduced SOD activity and increased MDA levels were observed in Model group mice compared with Control group. 2% hydrogen treatment restored decreased SOD activity and reduced MDA levels in pancreas which suggested 2% hydrogen significantly improved oxidative stress induced by L-arginine (Fig. 3A). In addition, 2% hydrogen blocked the increased pro-inflammatory cytokine TNF- α production but enhanced anti-inflammatory cytokine IL-10 expression (Fig. 3B). This interesting finding led us to further investigate how hydrogen impact the immune cells in chronic pancreatitis model.

Treg cells were increased after hydrogen treatment in chronic pancreatitis mice

As the main source of cytokines, T cells, especially CD4⁺ T cells were reported to be critical in the development of pancreatitis [8, 10]. We checked CD4⁺ and CD8⁺ ratio in both peripheral blood mononuclear cells (PBMCs) and spleen mononuclear cells (MNCs). As shown in Figs. 4A and B, similar CD4⁺/CD8⁺ ratios were observed in Control, Model and Model+2% hydrogen groups. However, a significant decrease of percentage of CD25⁺Foxp3⁺ Tregs in both PBMCs and spleen MNCs were found in chronic pancreatitis Model group (Figs. 4C and D). Interestingly, the treatment of 2% hydrogen reversed the loss of CD25⁺Foxp3⁺ Tregs

Fig. 1. Hydrogen mitigated edema in chronic pancreatitis mice. Time schedule (A), pancreas photographs (B), pancreas weight and water content (C) were designed and measured at 24 h after operation. * $P < 0.05$ and ** $P < 0.01$ compared with model group.

Fig. 2. Hydrogen protected against chronic pancreatitis in mice by histological examination. (A) Tissue inflammation, damage and fibrosis were analyzed on HE (magnification $\times 200$), TUNEL, MPO (magnification $\times 400$), and Sirius staining (magnification $\times 100$). (B) Quantification of (A) $**P < 0.01$ compared with model group.

Fig. 3. Hydrogen inhibited oxidative stress, inflammatory cytokines, and the levels of amylase and lipase in chronic pancreatitis mice. Pancreas levels of MDA content, SOD activity (A), TNF- α , IL-10 (B) cytokine expression and serum amylase and lipase activity (C) were determined by ELISA and absorbance assay. $**P < 0.01$ compared with model group.

chronic pancreatitis (Figs. 4C and D). These data implied that Treg cells might be implicated in the development of chronic pancreatitis.

Fig. 4. Effects of hydrogen on the differentiation of spleen and peripheral blood T cell subtypes by flow cytometry. Cells were separated and incubated with different antibodies for 30 min. The proportion of spleen CD4 and CD8 (A), Treg (C) and peripheral blood CD4 and CD8 (B), Treg (D) was analyzed by flow cytometry. ** $P < 0.01$ compared with model group.

The protective effect of hydrogen on chronic pancreatitis was mediated by Tregs

To validate the role of Tregs in chronic pancreatitis, purified Tregs were adoptively transferred into mice with chronic pancreatitis (Figs. 5A and B). Compared with Model group, mice received Tregs transfer showed significantly reduced pancreas size/weight and water content (Figs. 5C and D). Also, improved pathological score, number of TUNEL+ acinar cells, MPO+ neutrophil infiltration were found in Tregs transfered group (Figs. 6A and B). In addition, reduced serum amylase/lipase levels and pancreatic fibrosis were observed in Tregs group compared with Model group (Fig. 6A and 7C). Tregs transfer shown similar capability in reducing the severity of chronic pancreatitis with severity of chronic pancreatitis with 2% hydrogen treatment.

To verify whether the protective effect of 2% hydrogen treatment on chronic pancreatitis was mediated by elevated Tregs, we used mouse CD25 neutralizing antibody to deplete Tregs in 2% hydrogen treated chronic pancreatitis mice. As shown in Figs. 5C and D, depletion of Tregs almost completely abolished the protective effect of 2% hydrogen treatment

Fig. 5. Adoptive transfer of Treg cells ameliorated edema of chronic pancreatitis. Time schedule (A), purity of Treg (B) pancreas photographs (C), pancreas weight and water content (D) were designed and measured at 24 h after operation. * $P < 0.05$ compared with model group.

on chronic pancreatitis, evidenced by increased pancreas size/weight, water content of pancreas and deteriorated pancreas pathological score, increased TUNEL+ acinar cells and MPO+ neutrophil in pancreas (Figs. 5C and D, Figs. 6A and B). These data supported that the increased Tregs was responsible for the beneficial effect of 2% hydrogen treatment on chronic pancreatitis.

Moreover, we checked the oxidative stress and cytokine production on chronic pancreatitis mice with Tregs transfer, 2% hydrogen treatment and 2% hydrogen treatment with CD25 neutralizing antibody. Consistent with the results mentioned above, Tregs transfer reduced oxidative stress and TNF- α level but increased IL-10 production (Figs. 7A and B). In contrast, depletion of Tregs by CD25 neutralizing antibody abolished reduced oxidative stress and TNF- α level by 2% hydrogen treatment, also, increased IL-10 levels went back with Tregs depletion (Figs. 7A and B).

Hydrogen-Rich saline inhibited H_2O_2 induced Treg apoptosis, quantity and ROS level

We next explored the mechanism how hydrogen restored Treg loss in chronic pancreatitis. To mimic the oxidative stress environment in chronic pancreatitis mice, we treated Tregs with H_2O_2 , which caused increased apoptosis of Tregs and reduced Treg percentage in spleen MNCs (Figs. 8A and B). Hydrogen-Rich saline prevented the apoptosis and restored Treg loss induced by H_2O_2 (Figs. 8A and B). The protective effects of hydrogen may be attributed to reduced ROS production by hydrogen (Fig. 8C). In addition, we showed that hydrogen can also restore impaired production of anti-inflammatory cytokine IL-10 and TGF- β caused by H_2O_2 (Fig. 8D).

Discussion

In our study, we found that 2% hydrogen treatment significantly protected mice from L-arginine induced chronic pancreatitis. The mechanism of the protective effect of hydrogen treatment could be explained by increased Tregs in hydrogen treated chronic pancreatitis mice. Tregs depletion by CD25 neutralizing antibody abolished the protective

Fig. 6. Adoptive transfer of Treg cells protected chronic pancreatitis mice in histopathology. Tissue inflammation and global cell metaplasia analyzed on HE (magnification $\times 200$), TUNEL, MPO (magnification $\times 400$), and Sirius staining (magnification $\times 100$). * $P < 0.05$ and ** $P < 0.01$ compared with model group.

Fig. 7. Adoptive transfer of Treg cells ameliorated oxidative stress, inflammatory cytokines, and the levels of amylase and lipase in chronic pancreatitis mice. Pancreas levels of MDA content, SOD activity (A), TNF- α , IL-10 (B) cytokine expression and amylase and lipase activity (C) were determined by ELISA and absorbance assay. ** $P < 0.01$ compared with model group.

effect of hydrogen treatment. We also showed that *in vitro* exposure to Hydrogen-Rich saline prevented H₂O₂ induced Treg apoptosis by inhibiting ROS generation.

Fig. 8. Hydrogen-Rich saline inhibited H₂O₂ induced Treg apoptosis, quantity and ROS level. Treg apoptosis (A), quantity (B) and intracellular level of ROS (C) were analyzed by flow cytometry. The expression of IL-10 and TGFβ (D) were determined by ELISA. *P<0.05 and **P<0.01 compared with H₂O₂ group.

Chronic pancreatitis is characterized by immune cell infiltration and replacement of the destroyed acinar cells by fibrotic cells. Therefore, dysregulation of immune cells, such as monocytes/macrophages, mast cells and T cells was critical in the pathogenesis of chronic pancreatitis [7]. Among these immune cells, T cells, which were significantly increased in the chronic pancreatitis lesions, were believed to play very important role in killing acinar cells [9]. Tregs functioned as a critical immune cells to maintain tolerance and prevent autoimmune diseases [22]. Multiple mechanisms have been described for how Tregs regulated immune response [23], among them, production of anti-inflammatory cytokine IL-10 was essential in suppressing T cell over activation [24]. In chronic autoimmune pancreatitis patients, the number of Tregs was associated with concentrations of IL-10 in pancreatic tissue lesion [25]. A more recent report confirmed that Tregs could specifically suppress cytotoxic T cell response [10], which was very important in pancreas tissue destruction of chronic pancreatitis patients [9]. The suppressive effects of Tregs on cytotoxic T cells were IL-10 dependent [10, 26]. Similarly, in our study, we found that decreased Tregs in chronic pancreatitis mice (Figs. 4C and D). In contrast, adoptive transfer of Tregs attenuated the severity of chronic pancreatitis, which further supported the protective role of Tregs in chronic pancreatitis (Figs. 5C and D). Moreover, the treatment of hydrogen restored Treg number loss and increased IL-10 levels (Figs. 5C and D, Fig. 7B), which was believed to suppress the over activation of cytotoxic T cells and reduce the severity of chronic pancreatitis. As the report from Miyoshi et.al, naive Tregs significantly decreased in peripheral blood from patients with autoimmune pancreatitis, although CD4+CD25^{high} Tregs were significantly increased [27]. The finding means increased memory Tregs in the periphery and local tissues may be inhibitory immune response against inflammation in the patients with autoimmune pancreatitis. In animal studies, circulating Tregs decreased after L-arginine injection, and it might be a performance at the early stage of disease. And animal models might be different to patients.

Molecular hydrogen is a strong scavenger of the hydroxyl radical. Hydrogen was reported to selectively reduce the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protected cells [28]. The therapeutic potential of hydrogen was tested as early as 1970s [29]. More than 30 years later, an important study confirmed the beneficial effects of hydrogen on brain injury by reducing cytotoxic ROS and protecting tissue from oxidative damage [28]. Hydrogen treatments, including inhalation of hydrogen gas and Hydrogen-Rich saline administration, have been tested in many animal models and clinical trials [11, 30, 31]. In general, hydrogen has been described effective in treating a variety range of diseases such as post-cardiac syndrome, Parkinson's disease, acute cerebral ischemia, metabolic syndrome, rheumatoid arthritis, hemodialysis and postpsoriasis [11, 31, 32]. For pancreas disease, previous studies suggested hydrogen was protective in experimental acute pancreatitis by ameliorating oxidative stress [17, 33]. Consistently, in chronic pancreatitis model, we observed reduced oxidative stress and pancreas damage, more importantly, reduced pancreas fibrosis was observed with hydrogen treatment. We also showed the hydrogen can also regulate immune response by promoting Treg survival (Figs. 8A and B). In addition, our study provided novel mechanisms to explain the anti-inflammatory effects of hydrogen in several previous reports [34-36].

Conclusion

We found hydrogen treatment effectively attenuated the acinar cell death and improved pancreas fibrosis in L-arginine induced chronic pancreatitis in mice. Hydrogen treatment restored Treg loss in chronic pancreatitis mice and ablation of Tregs abolished the protective effect of hydrogen. Hydrogen promoted the survival of Tregs by blocking ROS production. Therefore, hydrogen can be considered as a very attractive therapeutic method for chronic pancreatitis patients.

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Disclosure Statement

The authors declare no potential conflicts of interest.

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