Hydrogen-rich saline ameliorates the severity of L-arginine-induced acute pancreatitis in rats

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Abstract

Molecular hydrogen, which reacts with the hydroxyl radical, has been considered as a novel antioxidant. Here, we evaluated the protective effects of hydrogen-rich saline on the L-arginine (L-Arg)-induced acute pancreatitis (AP). AP was induced in Sprague–Dawley rats by giving two intraperitoneal injections of L-Arg, each at concentrations of 250 mg/100 g body weight, with an interval of 1 h. Hydrogen-rich saline (>0.6 mM, 6 ml/kg) or saline (6 ml/kg) was administered, respectively, via tail vein 15 min after each L-Arg administration. Severity of AP was assessed by analysis of serum amylase activity, pancreatic water content and histology. Samples of pancreas were taken for measuring malondialdehyde and myeloperoxidase. Apoptosis in pancreatic acinar cell was determined with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling technique (TUNEL). Expression of proliferating cell nuclear antigen (PCNA) and nuclear factor kappa B (NF-kB) were detected with immunohistochemistry. Hydrogen-rich saline treatment significantly attenuated the severity of L-Arg-induced AP by ameliorating the increased serum amylase activity, inhibiting neutrophil infiltration, lipid oxidation and pancreatic tissue edema. Moreover, hydrogen-rich saline treatment could promote acinar cell proliferation, inhibit apoptosis and NF-kB activation. These results indicate that hydrogen treatment has a protective effect against AP, and the effect is possibly due to its ability to inhibit oxidative stress, apoptosis, NF-kB activation and to promote acinar cell proliferation.

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Introduction

Acute pancreatitis (AP) is a common disease with variable severity ranging from a mild and self-limiting condition to a severe form, the latter still being associated with an overall mortality rate of 10–25% [1]. The pathogenesis of AP remains unclear although there is good evidence that autodigestion of the gland is the cause of the disease. A still unknown trigger converts digestive proenzymes into their active forms within the acinar cell, leading to cell membrane disruption, edema, interstitial hemorrhage, necrosis, and to activation of other proenzymes, accompanied by an inflammatory response with infiltrating leukocytes that contributes to the progression of both, the local pancreatic destruction and the subsequent systemic manifestations. This can result in complete destruction of the parenchyma leading to severe systemic complications which mainly cause a fatal outcome.

The severity and outcome of AP might be determined by the events that occur subsequently to acinar cell injury, including the activation of transcription factors such as nuclear factor kappa B (NF-kB), the recruitment and activation of inflammatory cells, and the generation and release of cytokines and other inflammation mediators [2,3]. In the development of AP oxygen free radicals (OFRs) and their derivatives play an important role as the molecular trigger in constituting lesions in the pancreas. Damaged acinar cells as well as activated neutrophils and macrophages had been shown to produce large amount of OFRs in AP [4].

Hydrogen (H2), which could react with hydroxyl radical to produce water, has been considered as a novel antioxidant and has been demonstrated recently to have high protective properties in human, animal, and in vivo and in vitro studies, including the protective effect on lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance [5], transplantation induced intestinal graft injury [6], chronic liver inflammation [7], arteriosclerosis [8], myocardial ischemia–reperfusion (I/R) injury [9], acute oxidative stress and focal brain I/R injury [10], and intestinal I/R injury [11,12]. A recent study provided evidence that inhaled H2 gas markedly protect the brain against I/R injury and...
stroke in cell-free systems by selectively reducing the hydroxyl radical, the most cytotoxic one of reactive oxygen species (ROS), which is commonly produced under various pathological conditions including in AP [10]. In addition, our previous works have shown that hydrogen-rich saline has a protective effect on intestinal I/R against intestinal contractile dysfunction and damage, and this protective effect is largely due to its ability to limit the neutrophil infiltration, I/R-induced lipid oxidation, apoptosis, and to promote epithelial cell proliferation [11,12]. This raises the possibility that the hydrogen-rich saline might lead to protection against damage in AP. To test this, we measured a variety of parameters related to pancreatic damage in l-arginine (l-Arg)-induced AP in rats after administration of hydrogen-rich saline, and demonstrated that hydrogen-rich saline treatment can significantly attenuate the severity of l-Arg-induced AP by inhibiting neutrophil infiltration, lipid oxidation and pancreatic tissue edema. Most importantly, hydrogen-rich saline treatment could promote acinar cell proliferation, and inhibit apoptosis and NF-kB activation.

**Materials and methods**

*Animal and protocol.* This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996) and was approved by the local ethics committee for animal research. Six-week-old male Sprague-Dawley rats, weighing 180–200 g, fed ad libitum with standard laboratory chow, were randomly divided into normal, l-Arg + saline and l-Arg + hydrogen-rich saline groups, including eight rats in each group.

AP was induced in rats by two intraperitoneal injections of 250 mg/100 g body weight of l-Arg as a 20% solution in 0.15 mol/L saline, at an interval of 1 h between injections [13]. Rats in normal group received an equal volume of 0.15 mol/L NaCl intraperitoneal injection twice alone, which served as control of Arg + saline group. In l-Arg + saline group, rats underwent saline infusion (6 mL/kg) via tail vein 15 min after each of l-Arg injection. Rats in l-Arg + hydrogen-rich saline group administered with hydrogen-rich saline (6 mL/kg) via tail vein as the saline infused in the l-Arg + saline group. All rats were then sacrificed at 24 h after the last l-Arg injection, and blood from angular vein as well as pancreatic tissue harvested for study.

The hydrogen-rich saline was prepared as we previously described [14]. Briefly, hydrogen was dissolved in normal saline for 2 h under high pressure (0.4 MPa) to the supersaturated level using a self-designed hydrogen-rich water-producing apparatus. The saturated hydrogen saline was stored under atmospheric pressure at 4 °C in an aluminum bag without dead volume. Hydrogen-rich saline was freshly prepared every week to ensure a constant concentration more than 0.6 mM.

**Determination of pancreas edema.** The extent of pancreatic edema was assayed by measuring tissue water content on freshly obtained sample of pancreas [15].

**Amylase estimation.** Serum amylase activity was measured by an enzyme-based colorimetric assay on a P800 Modular Roche Diagnostics apparatus (Roche Diagnostics, Mannheim, Germany).

**Histopathologic observation.** The paraffin-embedded pancreatic specimens were sectioned at 5 μm, and were stained with hematoxylin and eosin (HE) for microscopic examination, by an experienced histopathologist blinded to the identity of the samples. Acinar cell injury/necrosis was quantified by morphometry as previously described [16]. Ten randomly selected microscopic fields (125×) were examined for each sample, and the extent of acinar cell injury/necrosis was expressed as the percent of the total acinar tissue. The criteria for injury/necrosis were the following (1) the presence of acinar cell ghosts or (2) vacuolization and swelling of acinar cells and destruction of the architecture of whole or parts of the acini, both of which had to be associated with an inflammatory reaction.

**Immunohistochemistry for proliferating cell nuclear antigen (PCNA) and NF-kB in pancreas.** The expression of PCNA and P65 were detected by immunohistochemistry to determine the proliferating cells and expression of the P65 subunit of NF-kB in pancreatic gland. Briefly, the specimens were dewaxed and incubated with 3% H2O2 in methanol at 37 °C for 10 min to quench endogenous peroxidase activity. After blocked at room temperature for 30 min, the sections were incubated with monoclonal antibody against PCNA (1:100 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and P65 (1:50 dilution, Cell Signaling Technology Inc., Danvers, MA) overnight at 4 °C. The labeled antigen was visualized with an Envision™ detection kit (Gene Tech, Shanghai, China) followed by the diaminobenzidine reaction. Finally, the specimens were counterstained by immersion in hematoxylin. P65-containing cells were identified by the presence of a dark reddish-brown chromogen. The nuclear-positive staining cells were observed under microscope (400×). Twenty randomly selected microscopic fields were examined blindly by a pathologist for the ratio of positive cells.

**Acinar cell apoptosis.** The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling method (TUNEL) was used for histological identification of pancreatic acinar cell apoptosis. Briefly, the slides were digested with proteinase K, incubated with terminal deoxynucleotidyltransferase enzyme, and subsequently incubated with antidigoxigenin peroxidase, using the in situ apoptosis detection kit (R&D Systems, Minneapolis, MN, USA) according to the protocol. Peroxidase was applied for color development under microscopic control. Apoptotic cells were identified as those with a brown-stained nucleus. TUNEL-positive pancreatic acinar cells (%) were quantified on image analysis software (Image J, NIH, USA) by investigators blinded to the samples, in which the apoptotic index was defined as the proportion of TUNEL-positive cells to the total number of cells in 20 non-overlapping serial scopes taken from each slide, beginning from a random start, at 400× magnification.

**Pancratic malondialdehyde (MDA) content.** Pancreatic MDA content was measured to evaluate the severity of pancreatic peroxidation injury by thiobarbituric acid colorimetric method using MDA assay kit (Nanjing Jiancheng Corp., Nanjing, China). The harvested pancreas was homogenized with 10 volumes of 20 mmol/L potassium phosphate buffer (pH 7.4) containing 30 mmol/L KCl and then centrifuged at 1500 g for 15 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm for MDA content, as MDA reacted with thiobarbituric acid and formed a pink, maximum absorbent complex at 532 nm wavelength [17]. The MDA concentration was calculated from the standard curve and expressed as nmol/mg protein.

**Pancratic myeloperoxidase (MPO) activity.** The pancreatic tissue was homogenized immediately on ice in 5 vol of normal saline. The MPO activity was measured using MPO assay kit (Nanjing Jiancheng Corp., Nanjing, China), following the manufacturer's recommendations. One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37 °C, and MPO activity of tissue was expressed as unit per gram (U/g).

**Statistical analysis.** Values were presented as mean ± SD. Statistical analysis was done using the SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA) to establish whether the difference was statistically significant. Between groups, variance was determined using the Student–Newman–Keuls post hoc test. P value less than 0.05 was considered statistically significant.
Results

Hydrogen-rich saline treatment ameliorated the damage to L-Arg-induced AP

L-Arg administration to rats in the L-Arg + saline group resulted in AP as characterized by severe pancreatic interstitial edema, vacuolization, prominent neutrophil infiltration, and acinar cell injury and necrosis in the pancreas, without any morphological change in the Langerhans islets as others described previously (Fig. 1) [18]. The serum amylase activity and pancreatic water content in the L-Arg + saline group were significantly elevated at 24 h after induction relative to normal group (P < 0.01, Fig. 2A and B). In addition, the L-Arg IP administration in the L-Arg + saline group led to significantly enhanced apoptosis and NF-κB activation in acinar cell, and neutrophil infiltration and lipid oxidation in pancreatic tissue as judged by TUNEL assay, immunohistochemistry, and MDA and MPO assessment (P < 0.05, Figs. 2–4).

Histological examination of pancreas sections confirmed an ameliorated effect on AP after hydrogen-rich saline administration, as demonstrated with a marked reduction of the extent and severity of the histological signs of the pancreas injury (i.e., the extent of acinar cell injury/necrosis) when compared to the untreated sections (Fig. 1). The pancreatic water content as well as serum amylase activity in rats after hydrogen-rich saline administration were significantly attenuated compared to rats treated with L-Arg + saline alone (P < 0.05, Fig. 2A and B).

The effect of hydrogen-rich saline treatment on cell proliferation and apoptosis

Acute endogenous generation of ROS in live pancreatic acinar cells has been suggested to constitute a natural pathway by which acinar cells promote programmed cell death [19]. In the development of AP in diverse animal models, a high degree of ROS generation and apoptosis were observed in pancreatic acinar cells [20,21], and the possibility has been raised that the development of apoptotic cell death caused by ROS may reflect the severity of pancreatitis, which might have substantial clinical value [22].

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**Fig. 1.** Representative photomicrographs of the rat pancreatic sections from the normal, L-Arg + saline and L-Arg + hydrogen groups in L-Arg-induced AP. The histological features in normal group were typical of a normal architecture. On the contrary, pancreas sections from the L-Arg + saline group demonstrate tissue damage characterized by interstitial edema, acinar cell necrosis, and prominent neutrophil infiltration. Pancreas section from rats that had received hydrogen-rich saline at the dose of 6 ml/kg (>0.6 mM) showed significantly less histological alterations. Sections were stained with HE (100× upper panel, 200× lower panel).

**Fig. 2.** Effects of hydrogen-rich saline treatment on the serum amylase activity and pancreatic water content. The administration of hydrogen-rich saline at the dose of 6 ml/kg (>0.6 mM) significantly reduced the increase of serum amylase activity and pancreatic water content in L-Arg-induced AP. Data were expressed as mean ± SD (n = 8). *P < 0.05 vs. L-Arg + saline group. **P < 0.01 vs. normal group.
However, the apoptotic index in L-Arg + saline group displayed markedly increased compared with normal group (P < 0.01, Fig. 3C). In comparison to the L-Arg + saline group, there were fewer TUNEL-positive cells and the apoptotic index decreased significantly in rats treated with hydrogen-rich saline (P < 0.01, Fig. 3C).

PCNA is a significant cell-cycle regulated nuclear protein for DNA-polymerase, and the PCNA-labeled nuclei had been shown to identify cells in the late G1 and early S phases of the cell cycle, as well as cells undergoing DNA repair [12]. As shown in Fig. 3A, there was weakly nuclear stained in the normal and L-Arg + saline groups. However, when compared with the L-Arg + saline group, the PCNA expression of acinar cells in the L-Arg + hydrogen-rich saline group was markedly increased (Fig. 3B, P < 0.01), suggesting that hydrogen-rich saline treatment could promote pancreatic acinar cell regeneration and proliferation.

Hydrogen-rich saline treatment altered activation of NF-κB in L-Arg-induced AP

Prevention or inhibition of NF-κB activation has been shown to be positively associated with a reduction in the severity of AP [23]. NF-κB is a key transcription factor regulating the immunity and inflammation in AP [24]. Accumulating evidence suggests that nuclear translocation of the NF-κB is associated with upregulated P-selectin, ICAM-1 and adhesion molecules in acinar and endothelial cells [25], which led to an enormous increase of adherent and transmigrated polymorphonuclear neutrophils and has been implicated as central players in the development and progression of AP. In particular, altered NF-κB DNA binding activity had been shown to be highly associated with the protective effects of antioxidants in L-Arg-induced AP, such as N-acetylcysteine and pyrrolidine.
dithiocarbamate [23]. On the other hand, the ability of hydrogen-rich saline administration for inhibition of the NF-κB activation had been demonstrated recently by our research group in lung injury induced by intestinal I/R injury [26]. To confirm our data, we immunohistochemically investigated effects of hydrogen-rich saline administration on the NF-κB activation in pancreatic tissue in the L-Arg-model. As shown in Fig. 3A, there was little NF-κB expression in the normal pancreatic tissue, whereas the expression of NF-κB was significantly elevated in the L-Arg + saline group. In contrast, hydrogen-rich saline treatment significantly decreased NF-κB expression, and there was much less nuclear-positive staining acinar cells in the L-Arg + hydrogen-rich saline group as compared with that in the L-Arg + saline group (Fig. 3D). These results indicate that the hydrogen-rich saline treatment can markedly elevate the NF-κB activation in L-Arg-induced AP.

Since NF-κB is believed to be a key factor in the development of AP as it regulates the synthesis of tumor necrosis factor-α, interleukin-1 and -6, inducible nitric oxide synthase, cyclooxigenase-2 and many other molecules, the ameliorated effect of hydrogen-rich saline on AP may be explained by the reduced NF-κB activation and consequentially reduced expression of cytokines and other molecules. Additionally, once activated, neutrophil oxidative burst (ROS production) in turn led to further activation of pancreatic NF-κB [27].

**Discussion**

This study shows that hydrogen-rich saline treatment can effectively reduce the severity of L-Arg-induced AP in rats. The protective effect against AP is supported by the results from ameliorated histological findings, as well as its abilities to inhibit L-Arg-induced apoptosis, NF-κB activation and to promote acinar cell proliferation. In addition, hydrogen-rich saline treatment has been shown to significantly ameliorate the increased serum amylose activity and pancreatic tissue edema, accompanied by reduction of MDA level and MPO activity in the pancreatic tissues.

Molecular hydrogen (H₂) is recognized as possessing a number of antioxidative effects. It has been demonstrated that hydrogen (H₂) could selectively react with exclusively detrimental ROS, such as hydroxyl radical and peroxynitrite, exerting protective effects. However, H₂ did not react with other physiological ROS, such as superoxide anion and H₂O₂, which possess physiological roles [10]. Therefore, unlike the other antioxidant supplements with strong reductive reactivity, hydrogen is mild enough without disturbing metabolic oxidation–reduction reactions or disrupting ROS involved in cell signaling [31]. Additionally, as the molecular hydrogen is electrically neutral and much smaller than the oxygen molecule, it easily penetrates membranes and enters cells and organelles such as nucleus and mitochondria. This is particularly important, as the latter is the primary site of ROS generation and is notoriously difficult to target [32], and hydrogen could diffuse freely within the body, without any side effects [7].

To the best of our knowledge, this is the first study to demonstrate a protective effect of hydrogen-rich saline on L-Arg-induced AP. This protective effect is possibly due to its ability to inhibit...
oxidative stress, apoptosis, NF-κB activation and to promote acinar cell proliferation.

References