Hydrogen-rich saline reduces the oxidative stress and relieves the severity of trauma-induced acute pancreatitis in rats

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BACKGROUND: Currently, little evidence exists to support whether the therapeutic approaches for treating ordinary acute pancreatitis (AP) are effective in trauma-induced pancreatitis. Hydrogen-rich (H_2) saline is an antioxidant treatment capable of ameliorating the severity of L-arginine-induced AP. In this study, we attempted to validate its protective role against traumatic pancreatitis (TP).

METHODS: A previously established experimental rat model of TP was generated by controlled delivery of high pressure air impact. The protective effects of H_2 saline against TP were evaluated in this model system by measuring survival rate and determining changes in histopathology, plasma enzymes, cytokines, and oxidative stress-associated molecules.

RESULTS: Intraperitoneal administration of H_2-rich saline produced a pronounced protection against TP in rats. Significant improvements were observed in survival rate and histopathological findings. In addition, plasma cytokines concentrations were reduced in H_2 saline-treated TP rats. Although no marked inhibitory effect on plasma amylase and lipase activities was observed, H_2 saline caused considerable suppression of pancreatic malondialdehyde level and recruitment of endogenous pancreatic antioxidants, such as glutathione and superoxide dismutase.

CONCLUSIONS: H_2-rich saline has beneficial effects on TP, presumably because of its detoxification activities against excessive reactive oxygen species. Our findings highlight the potential of H_2-rich saline as a therapeutic agent of trauma-induced AP. (*J Trauma Acute Care Surg*. 2012;72: 1555–1561. Copyright © 2012 by Lippincott Williams & Wilkins)

KEY WORDS: Hydrogen-rich saline; oxidative stress; antioxidant; traumatic pancreatitis.

Acute pancreatitis (AP) is a life-threatening inflammatory disease with a high rate of mortality, especially when complicated by systemic inflammatory response syndrome leading to multiple organ dysfunction syndrome.[1] Although several factors have been implicated in AP pathogenesis, including alcohol abuse, infections, tumors, and genetic abnormalities of the pancreas, gallstones are a predominant clinical finding in AP cases.[2] Trauma injury is another significant cause of AP and often occurs as a result of violent impact to the abdominal region, such as is incurred in motor vehicle collisions. Trauma-induced AP is often referred to as traumatic pancreatitis (TP).

Although the pathogenesis of AP has been extensively investigated, the relationship between pancreatic trauma and the consequent uncontrolled systemic inflammatory response has remained elusive. Increasing evidence from recent studies has implicated oxidative stress mechanisms and their related proinflammatory factors as mediators of the local and systemic complications associated with AP.[3–5] Enhanced oxygen-free radicals produced by oxidative stress pathways have been shown in both AP patients and experimental animal models to aggravate pancreatic tissue damage and promote leukocyte adherence and activation.[6] Because increases in reactive oxygen species (ROS) cause depletion of endogenous antioxidants, it has been proposed that AP-related inflammatory responses may be caused or promoted by pro-oxidative and antioxidative imbalance. This theory led to the development of a new therapeutic strategy for AP based on supplementation with exogenous antioxidants (e.g., *n*-acetylcysteine, selenium, vitamin c).

Hydrogen gas (H_2), a well-known molecule with the simplest molecular constitution, was recently found to selectively reduce cytotoxic ROS and exert therapeutic antioxidant activity.[7] H_2-rich saline, which is safer and more easily administered than H_2 gas, may be more suitable for clinical applications. Chen et al. described the first successful use of H_2-rich saline to treat L-arginine-induced AP using a rat model.[8] Since then, H_2-rich saline has gained much research attention to determine its potential for treating human cases of AP and other oxidative stress-related diseases.

It is known that the initial process of trauma-induced pancreatitis is similar to that of the other types of AP. In particular,

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unregulated activation of trypsinogen is induced by an unknown mechanism, which leads to autodigestion of the pancreas gland and subsequent edema and necrosis. However, the precise roles played by oxidative stress in TP pathogenesis remain unknown. In our previous work, an experimental rat TP model with acceptable reproducibility was developed based on the work of Dai et al.[9] Briefly, the rat pancreas was exposed by laparotomy and impacted by compressed air. A pressure-controlled impact device was used to produce compressed air at the required pressure, thereby facilitating precise control of injury extent. In our initial investigations, we compared the severity of TP in rats generated by 200 kPa impact force of compressed air to cerulein-induced AP. The amplitudes of change in plasma amylase activity and histopathological scores of TP were similar to those of cerulein-induced AP, but larger magnitudes in elevation of plasma cytokines and tissue malondialdehyde (MDA) levels were observed in the former (unpublished data). Thus, we extended our original analysis to determine whether the antioxidative effect of H_2-rich saline was beneficial in the trauma-induced rat model of AP.

Here, we describe our investigation into the therapeutic effects of H_2-rich saline on the rat model of experimental TP. Our secondary goal was to confirm the association between the protective effects of H_2 and its inhibition of oxidative stress.

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). All animals were housed at a constant temperature of 22°C ± 1°C and under 12-hour light-dark cycle conditions with free access to standard laboratory chow and water. Animals were acclimatized for 1 week before any experimentation. Average weight of the rats at the time of the experiment was 200 g ± 50 g. All procedures involving the rats were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Third Military Medical University.

Preparation of H_2-Rich Saline

H_2-rich saline was generously provided by Professor Xuejun Sun (the Second Military Medical University, Shanghai, China) and prepared as described previously.[10] In brief, H_2 gas (0.4 mPa) was dissolved in normal saline (NS) for 2 hours to achieve supersaturated level (>0.6 mmol/L). The saturated H_2 saline was then stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume and sterilized by gamma radiation before use.

TP-Induction Procedure

Compressed air at required pressures was delivered as impact force to the exposed pancreas by use of a custom-made biological impact machine-III, which was constructed by the Research Institute of Surgery at Daping Hospital (the Third Military Medical University, Chongqing, China). Before impact force delivery, the rat was anesthetized by intraperitoneal injection of 2% sodium pentobarbital (2.5 mL/kg). Five minutes later, the unconscious rat was immobilized in the supine position onto a wooden board and the incision area was prepared by shaving and disinfection. Next, the upper abdomen was opened by using a midline incision. The pancreas was located and carefully exposed and a thin plastic shim was placed underneath it to provide support during the subsequent single impact of 200 kPa compressed air. After the impact, the organ was carefully replaced and the abdomen was closed by first suturing the muscle layer and then the skin. The rat was allowed to recover naturally from the anesthetic before being returned to its cage with free access to water but no solid food for 24 hour.

Experimental Design

Eighty animals were randomly divided into four groups (n = 20 each): (1) sham operation control group, in which animals underwent only laparotomy; (2) TP group; (3) TP + NS, in which injured rats were treated with NS alone; and (4) TP + H_2-rich saline, in which TP rats received saturated H_2 saline alone. H_2 saline (0.6 mmol/L, 6 mL/kg) or NS (6 mL/kg) was administered by intraperitoneal injection at 15 minutes after the abdomen was closed.

To determine survival rates, rats from each group were observed daily for 1 week after surgery and treatment. Another 24 rats (6 in each group) were grouped and treated as mentioned above. They were killed at 12 hour after injury to collect samples for biochemical assays. Plasma samples were obtained from the harvested blood samples by centrifugation (1800*g* for 15 minutes at 4°C). Pancreatic tissues were removed for histopathological examination. A portion of the excised tissue was fixed in 10% neutral formalin, and the remaining sample was immediately submerged in ice-cold NS and homogenized.

Determination of Amylase and Lipase Activities and Cytokines Levels in Plasma

The amylase and lipase activities in plasma samples were evaluated by an enzyme-based colorimetric assay on a fully automated Hitachi 7170 biochemistry analyzer (Hitachi, Tokyo, Japan). Quantitative enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) were used to determine the plasma levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) according to the manufacturer's instructions. All experiments were conducted with duplicate samples.

Detection of Superoxide Dismutase Activity in Pancreas

The activity of superoxide dismutase (SOD) in pancreas was measured using a commercial assay kit purchased from Cayman Chemical Company (Ann Arbor, MI), following the manufacturer's instructions. This assay kit uses a tetrazolium salt for detection of superoxide anions generated by xanthine oxidase and hypoxanthine. These superoxide radicals oxidize hydroxylamine and lead to formation of nitrite, which reacts with naphthalene diamine and sulfanilic acid to produce a colored product. SOD in the sample reduces the overall superoxide anion concentration, thereby lowering the colorimetric signal and absorbance at 550 nm. One unit (U) of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The total tissue protein concentration was determined by a commercial kit (Nanjing Jiancheng Corp., Nanjing, China), and the activity of SOD was expressed as U/mg of protein.

Measurement of MDA and Glutathione Levels in Pancreas

Tissue MDA level, a marker of lipid peroxidation, was detected using a commercial MDA-586 assay kit (OxisResearch, Portland, OR). In brief, butylated hydroxytoluene was added to the homogenized pancreatic tissues and the mixture was centrifuged at 1500*g* for 15 minutes. The supernatant was collected and absorption at 586 nm was measured. The level of glutathione (GSH) in pancreas tissue was measured by the Tietze method.[11] This method uses Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. The MDA and GSH levels in tissue were normalized against total protein (pmol/mg).

Histologic Examination

Pancreatic tissues were fixed in formaldehyde and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin, using a standard staining procedure. Histopathological evaluation was performed under light microscope by an experienced laboratory pathologist who was blinded to the group identity for the samples. Edema, inflammatory infiltration, hemorrhage, and parenchymal necrosis were evaluated in accordance with the scoring system established by Rongione et al.[12]

Statistical Analysis

All experimental values are expressed as mean ± SD. Survival data were analyzed by the chi-squared exact test. Statistical evaluations of other data were performed by the Student's *t* test. A *p* value of less than 0.05 was considered significant.

RESULTS

H_2-Rich Saline Treatment Improved Survival Rates of Rats with TP

After surgery, animals suffering impact injuries were sluggish, conducted less grooming activities, and appeared less mobile than the noninjured rats. The first death in the TP group occurred at 12 hour after surgery. During the next 7 days, significantly more rats died in the TP group and the NS only treated group than in the sham-operated group ($p < 0.05$). In contrast, postinjury treatment with H_2-rich saline produced a significantly better survival rate, when compared with all other injury groups (Fig. 1).

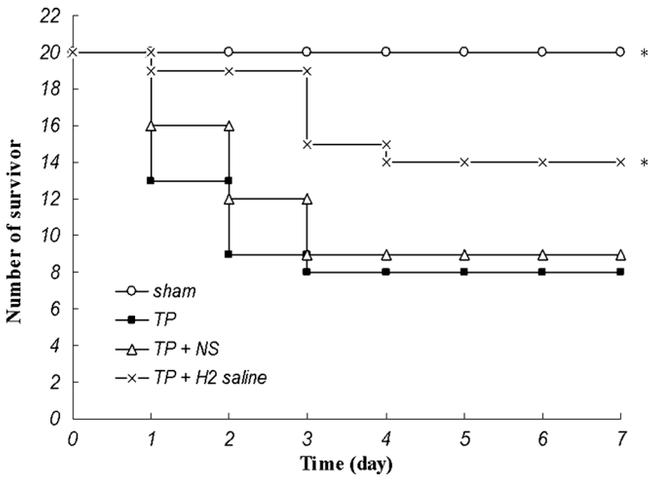

Figure 1. H_2-rich saline improved the survivals of rats suffering TP. Rats received a direct impact of compressed air (200 kPa) to cause the experimental TP. The survival rates of rats were elevated significantly after intraperitoneal administration of H_2-rich saline (0.6 mM, 6 mL/kg). *$p < 0.05$, compared with the TP group.

H_2-Rich Saline Treatment Attenuated Pancreatic Tissue Injury

The marked rise of serum amylase levels that was observed in all injured rats confirmed the effectiveness of this air pressure-based injury method to produce TP. However, no statistically significant differences were observed in the activities of plasma amylase or lipase between animals treated with H_2 saline and NS. This finding indicated that H_2 had no inhibitory effect on these two pancreatic enzymes ($p > 0.05$; Fig. 2).

Histopathological examination of the pancreas revealed that H_2 saline ameliorated TP in the treated rats. Conspicuous edema, marked interstitial leukocyte infiltration, intrapancreatic hemorrhage, and necrosis were observed in both the TP and TP + NS groups (Fig. 3). These histologic changes were significantly less extensive in the rats treated with H_2-rich saline, as evidenced by the histopathological scoring (Table 1).

H_2-Rich Saline Treatment Decreased Inflammatory Cytokine Levels in Plasma

Proinflammatory cytokines, such as TNF-α and IL-1β, are known to mediate amplification of the inflammatory process after pancreatic injury.[4] Remarkably high levels of plasma TNF-α and IL-1β were found in TP and NS only treated groups, when compared with sham-operated group ($p < 0.05$). In contrast, treatment with H_2-rich saline led to significantly decreased levels of plasma cytokines (Fig. 4).

H_2-Rich Saline Treatment Reduced Oxidative Stress during TP

Although enhanced oxidative stress was observed in all injured animals, as evidenced by elevated pancreatic tissue MDA levels, the elevation appeared to be significantly inhibited by H_2 saline treatment ($p < 0.05$; Fig. 5, A). Moreover, the pancre GSH levels measured in rats suffering from impact injuries were markedly reduced in comparison to that in the sham-operated controls. Treatment with H_2 saline notably attenuated this reduction ($p < 0.05$; Fig. 5, B). Furthermore, similar changes were observed for SOD activity. Pancreatic SOD activity was found to be significantly depleted in injured rats, presumably as a result of oxidative stress processes. In contrast, treatment with H_2 saline effectively improved the activity of SOD in pancreas ($p < 0.05$; Fig. 5, C).

DISCUSSION

AP can be induced by various factors, thereby stimulating different molecular pathways and leading to different physiologic complications. Thus, a single treatment modality or

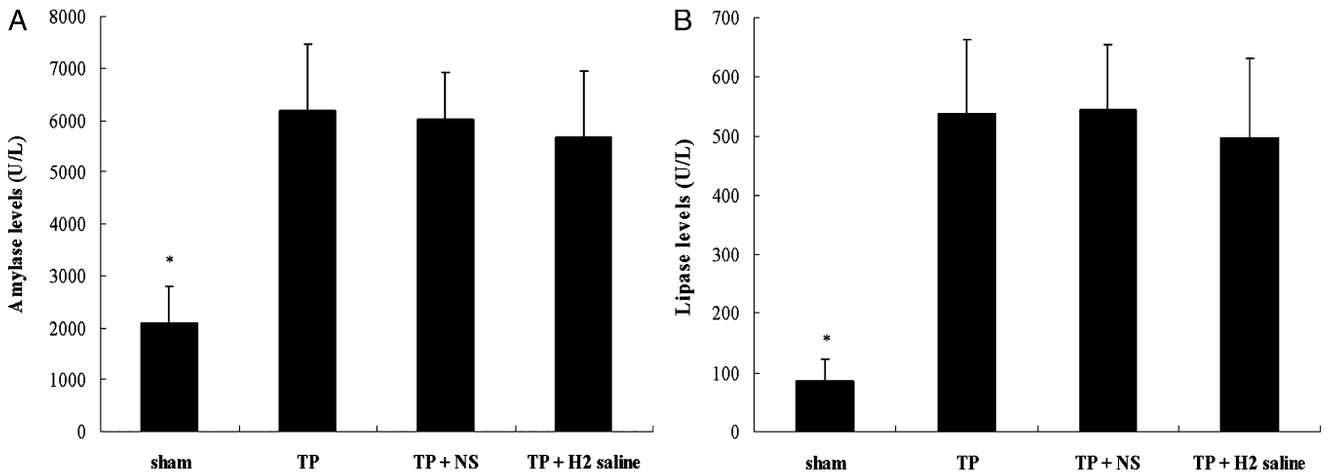

Figure 2. Effects of H_2-rich saline treatment on the plasma amylase and lipase activities in rats. The administration of H_2-rich saline (0.6 mM, 6 mL/kg) showed no statistical significant inhibition on plasma amylase (*A*) and lipase activities (*B*) in rats attacked by air impact. *$p < 0.05$, compared with the TP group.

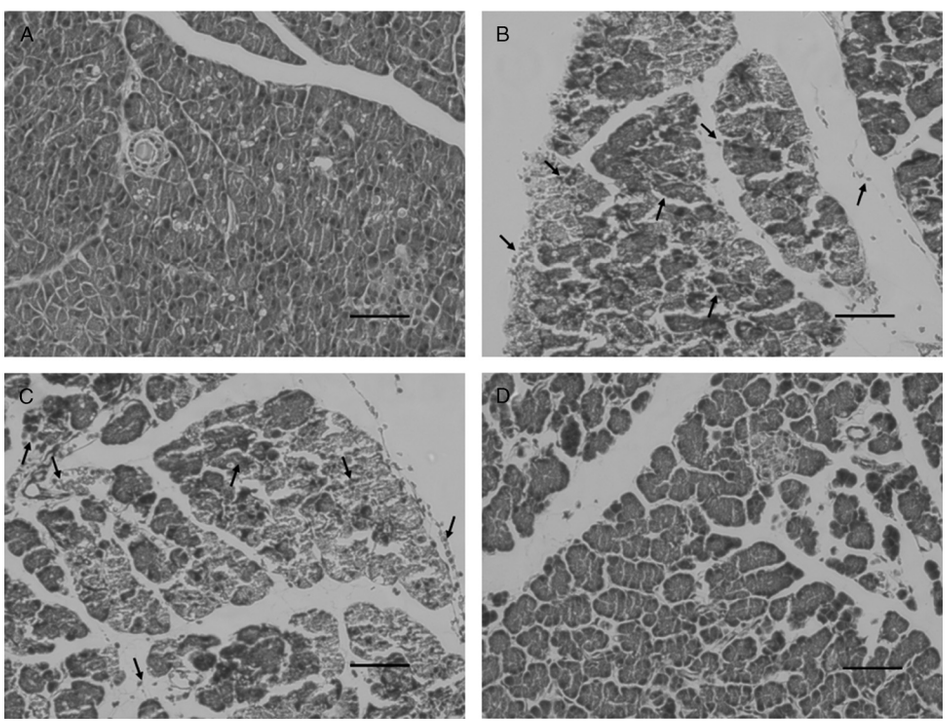

Figure 3. Pancreatic H&E stained sections from rats underwent various treatments. *A*, Sham operation group; *B*, TP model group; *C*, TP rats treated with only NS (6 mL/kg); and *D*, TP rats treated with H_2-rich saline (0.6 mM, 6 mL/kg). The typical normal architecture was found in sections from the sham-operated group. In contrast, the histopathologic appearances of increasing interspace of acinar cells, infiltration of inflammatory cells, intrapancreatic hemorrhage, and necrosis (indicated by *arrows*) were noted in TP model group or NS only treated group. Pancreas sections from rats treated with H_2-rich saline showed significantly reduced histological alterations. Scale bars = 50 μm.

agent, such as H_2-rich saline, may have different efficacies on the various forms of AP. Our objective in this study was to determine whether the previously established H_2 chemical agent was beneficial for the management of trauma-induced pancreatitis. A previous study had demonstrated that H_2 saline could relieve the severity of L-arginine-induced AP in an animal model,[8] and we tested its effects in a rat model of TP developed in our laboratory recently. H_2 was found to have a protective effect against

TABLE 1. Histopathological Scores of Pancreatic Injury

Groups	Edema (0–4)	Necrosis (0–4)	Hemorrhage (0–4)	Inflammatory Infiltration (0–4)	Histopathologic Scores
Sham	0	0	0	0	0
TP	1.7 ± 0.5	2.5 ± 0.8	2.2 ± 0.4	1.4 ± 0.5	7.7 ± 2.2
TP + NS	1.6 ± 0.7	2.5 ± 0.7	2.0 ± 0.9	1.5 ± 0.5	7.6 ± 2.8
TP + H_2 saline	1.2 ± 0.6	1.6 ± 0.2	1.3 ± 0.3	0.8 ± 0.3	4.9 ± 1.4*

Edema—absent (0); diffuse expansion of interlobar septa (1); 1+ diffuse expansion of interlobular septa (2); 2+ diffuse expansion of interacinar septa (3); 3+ diffuse expansion of intercellular septa (4); Necrosis—absent (0); 1 to 4 necrotic cells/high-power field (1); 5 to 10 necrotic cells/high-power field (2); 11 to 15 necrotic cells/high-power field (3); >16 necrotic cells/high-power field (4); Hemorrhage—absent (0); 1 to 2 points (1); 3 to 5 points (2); 6 points (3); > 8 points (4); Inflammatory infiltration—absent (0); around ductal margin (1); in parenchyma <50% of lobules (2); in parenchyma 51% to 75% of lobules (3); in parenchyma >75% of lobules (4). Values are means ± SE.

*$p < 0.05$, compared with TP group.

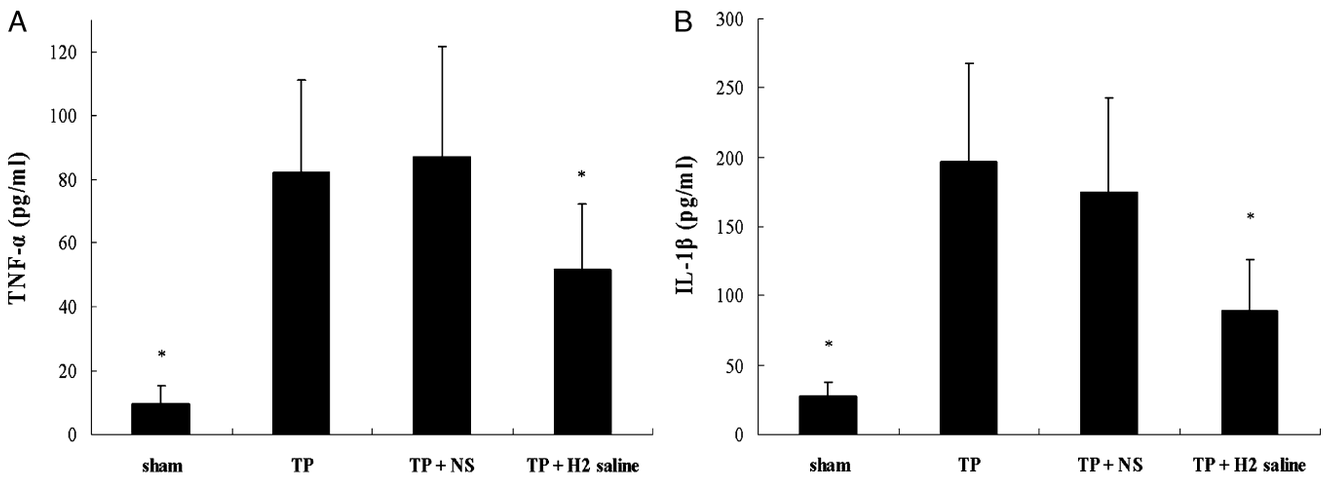

Figure 4. Inhibition of H_2-rich saline treatment on plasma cytokines levels in rats. The plasma TNF-α (*A*) and IL-1β (*B*) levels increased fiercely after TP. The administration of H_2-rich saline (0.6 mM, 6 mL/kg) led to significantly decreased levels of plasma TNF-α and IL-1β levels. *$p < 0.05$, compared with the TP group.

TP in these rats, as evidenced by improved survival rates and ameliorated histopathological findings. Moreover, treatment with H_2-rich saline significantly inhibited the TP-induced elevation of plasma cytokines and pancreatic MDA levels and was accompanied by considerable increases in pancreas levels of GSH and SOD.

In our previous study, wherein we developed a controllable animal model of trauma-induced AP, we applied a computer-controlled impact device to produce compressed air at required pressures. The exposed pancreas tissue of rats was directly injured by a single hit with compressed air under different magnitudes of impact, after which the severity of injury was evaluated. Unfortunately, when the air pressure was >200 kPa the TP model failed, because of the high-pressure air impact causing extreme injury and high mortality. Finally, we determined that 200 kPa could induce a moderate degree of pancreas injury with acceptable reproducibility. As a result, this newly established animal model was considered to closely simulate the pathogenesis of TP and represent a useful system in which to evaluate the therapeutic effects of potential treatments (unpublished data).

Although a growing number of reports have demonstrated that oxidative stress and its resultant production of ROS play prominent roles in the pathogenesis of inflammatory responses during AP, some evidence has also been presented against the therapeutic potential of antioxidants supplementation in AP. Siriwardena et al.[13] demonstrated that some antioxidants, including *n*-acetylcysteine, selenium, and vitamin c, failed to display significant efficiency on AP in a randomized, double blind, placebo-controlled trial. Milewski et al.[14] reported that antioxidant *n*-acetylcysteine was unable to provide substantial protection against pancreatitis. In addition, some antioxidants are known to exert strong reductive reactivity and react with other physiologic ROS, such as superoxide anion and H_2O_2, resulting in metabolic disturbances of the oxidation or reduction processes and consequent physiologic disorders.[15] Nearly, all the antioxidants identified and tested to date have shown deficiency in their ability to antagonize ·OH, which is one of the strongest oxidant species and has indiscriminate reactions to nucleic acids, lipids, and proteins. More importantly, mammalian species are lacking endogenous detoxification systems for ·OH radicals. Therefore, scavenging ·OH without influencing physiologic superoxide anions and H_2O_2 is a critical antioxidant process and is expected to have significant beneficial effects in treatment of AP or TP.

H_2-rich saline is an established, safe, convenient, and effective antioxidant that produces minimal side effects. Its protective effects against tissue injury are mediated by its ability

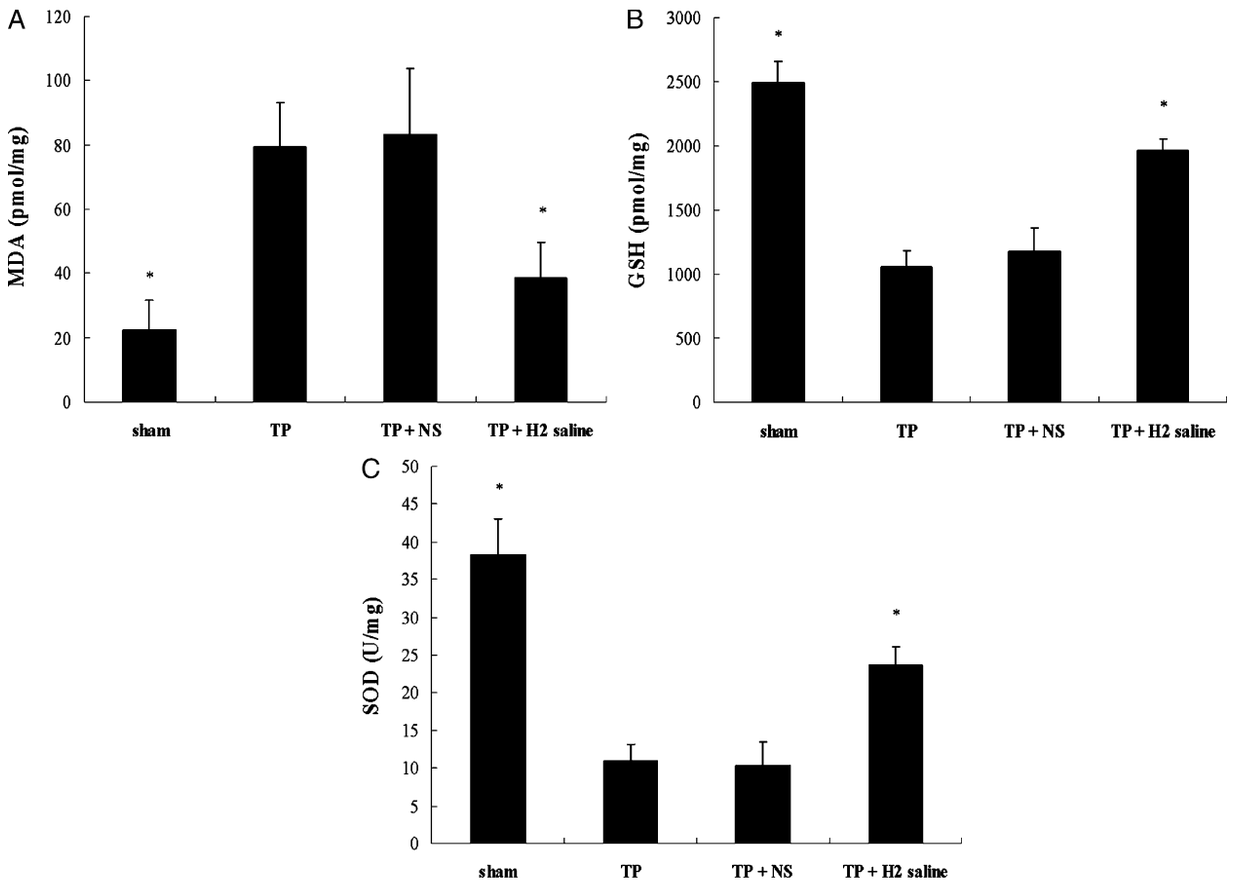

Figure 5. Pancreatic tissue levels of MDA, GSH, and SOD in rats underwent various treatments. As the results of oxidative stress after TP, MDA (*A*) level in pancreatic tissue presented a pronounced increase, and meanwhile the GSH (*B*) and SOD (*C*) in pancreas were nearly exhausted. The administration of H_2-rich saline (0.6 mM, 6 mL/kg) effectively improved the GSH and SOD levels in pancreas, with significantly reduced pancreatic MDA level. *$p < 0.05$, compared with the TP group.

to selectively scavenge ·OH and other detrimental ROS.[16] This property provides valuable insights into the potential underlying mechanisms by which H_2 may ameliorate trauma-induced pancreatitis. The exclusive reaction to ·OH suggests that H_2 may be a superior treatment of TP, in comparison with other known antioxidants.

This study demonstrated that the potential of H_2-rich saline to reduce the severity of trauma-induced pancreatitis was in agreement with recruitment of endogenous antioxidants (GSH and SOD) and suppression of pancreatic MDA level. In addition, once TP was initiated, H_2 saline failed to inhibit the overwhelming activation of digestive enzyme (as shown in Fig. 2). This finding suggested that, instead of acting as a pancreatic enzyme inhibitor, H_2 might exert its therapeutic effects on TP by inhibiting the excessive activation of oxidative stress or by correcting the pro- or anti-oxidative imbalance.

Taken together, the findings from our study indicate that the antioxidant H_2-rich saline is a promising therapy of trauma-induced AP. Further studies are necessary to reveal the detailed mechanisms underlying the therapeutic effects of H_2 and may reveal processes shared by other antioxidants that could be manipulable targets of future therapeutic strategies of TP.

AUTHORSHIP

J.R. designed this study, for which F.T. secured funding. J.R. performed experiments and Z.L. analyzed data. J.R. and Z.L. wrote the manuscript, which was revised by F.T. Q.W. provided experimental support. K.L. and C.W. contributed methodological advice. All authors read and approved the final manuscript.

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DISCLOSURE

The authors declare no conflict of interest.

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EDITORIAL CRITIQUE

Ren et al. have done an interesting study on the modulation of oxidative stress by hydrogen-rich saline in response to traumatic pancreatitis. This study is very timely because, although the role of hydrogen as an antioxidant in ischemia/reperfusion injury and several other pathologic conditions was demonstrated more than five years ago, research involving specific trauma-induced disorders are scarce.[1–3]

Endogenous hydrogen is produced by intestinal bacteria during fermentation of nondigestible forms of carbohydrates and act as an antioxidant in humans; it has been shown that hydrogen given exogenously can enhance that effect and act synergistically with other endogenous antioxidants.[4] As pointed out by the authors, hydroxyl radical (·OH) is the strongest reactive oxygen species, and class Mamalia lacks systems to counteract it. However, hydrogen inactivates ·OH forming water. Therefore, the use of hydrogen as means to reduce inflammatory response in trauma is tantalizing indeed. Nonetheless, there are critical questions that need to be addressed by future research, such as what hydrogen concentration will result in optimal (·OH) neutralization, are the means to assess systemic hydrogen concentration accurate, what is the best way to administer hydrogen (inhalation gas, in water [hydrogen-rich water], intravenously, intraperitoneally), what are the signaling pathways involved in hydrogen modulation of the inflammatory response in trauma? Furthermore, reactive oxygen species are not only harmful but also important for the immune system and have regulatory roles in several biological events.[5] Consequently, nonselective and uncontrolled neutralization of antioxidants can result in unwanted effects.

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