**Name of journal:** **World Journal of Gastroenterology**

**ESPS Manuscript NO: 5356**

**Columns: BRIEF ARTICLES**

**Protective role of hydrogen-rich water on aspirin-induced gastric mucosal damage in rats**

Zhang *et al*. Hydrogen pretects against aspirin-induced gastric injury

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**Received:** September 1, 2013 **Revised:** October 24, 2013

**Accepted:** December 5, 2013

**Published online:**

**Abstract**

**AIM:** to investigate the role of the hydrogen-rich water (HRW) in the prevention of aspirin-induced gastric mucosal injury.

**METHODS:** Forty male rats were allocated into 4 groups: normal control (carboxy methyl cellulose 0.05%), aspirin (asp, 400 mg/kg) disposing, HRW alone (randomly drinking for 14 d beforehand), and HRW plus aspirin. The protective efficacy was tested by determining the gastric mucosal damage score; malondialdehyde (MDA), superoxide dismutase (SOD), myeloperoxidase (MPO), interleukin (IL)-6, tumor necrosis factor (TNF)-α in gastric tissues were evaluated. The levels of IL-1β and TNF-α in the serum were also detected. Meanwhile, histopathology of gastric tissue and tissue localization of cyclooxygenase 2 (COX-2) were performed using Hematoxylin and Eosin staining and immunohistochemistry, respectively.

**RESULTS:** Pretreatment with HRW obviously reduced aspirin-induced gastric damage scores (4.04 ± 0.492 *vs* 2.10 ± 0.437, *P* < 0.05). The oxidative stress levels of MDA and MPO in the gastric tissue increased significantly in the aspirin-treated group compared with the HRW group (2.43 ± 0.145 *vs* 1.79 ± 0.116 nmol/mg prot, *P* < 0.05 and 2.53 ± 0.238 *vs* 1.40 ± 0.208 U/g tissue, *P* < 0.05 respectively). Disposing of rats with HRW could lead to obvious elevation in the level of SOD in the gastric tissue (37.94 ± 8.44 *vs* 59.55 ± 9.02 nmol/mg prot, *P* < 0.05). Pretreatment with HRW significantly decreased the elevation of IL-6, TNF-α in the gastric tissue (46.65 ± 5.50 *vs* 32.15 ± 4.83 pg/mg, *P* < 0.05 and 1305.08 ± 101.23 *vs* 855.96 ± 93.22 pg/mg, *P* < 0.05), and IL-1β, TNF-α in the serum (505.38 ± 32.97 *vs* 343.37 ± 25.09 pg/ml, *P* < 0.05 and 264.53 ± 28.63 *vs* 114.96 ± 21.79 pg/ml, *P* < 0.05) compared to treatment with aspirin alone. We also found that HRW could significantly decrease the COX-2 expression in the gastric tissue (Staining score: 8.4 ± 2.1 *vs* 2.9 ± 1.5, *P* < 0.05).

**CONCLUSION:** Hydrogen-rich water pretreatment alleviated the aspirin-induced gastric lesions by inhibition of the oxidative stress, inflammatory reaction and reducing of COX-2 in the gastric tissues.

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**Key words:** Hydrogen; Aspirin; Gastric lesion; Oxidative stress; Cytokines; Cyclooxygenase 2

**Core tip:** Aspirin, currently one of the most widely used medicines, is limited by the major adverse side effect of gastric injury. Hydrogen is a new medical approach which has been proved to have powerful anti-oxidant and anti-inflammatory effects. We launched a research to study the protective role of hydrogen-rich water on aspirin-induced gastric mucosal damage in rats. We found hydrogen could alleviate the aspirin-induced gastric lesions by by inhibition of the oxidative stress, inflammatory reaction and reducing of cyclooxygenase 2 in the gastric tissues. This results provide a potential therapy for the adverse effects of aspirin.

Zhang JZ, Wu QF, Wan Y, Song SD, Xu J, Xu XS, Chang HL, Tai MH, Dong YF, Liu C. Protective role of hydrogen-rich water on aspirin-induced gastric mucosal damage in rats. *World J Gastroenterol* 2013;

**Available from:** URL: http://www.wjgnet.com/1007-9327/

**DOI:** http://dx.doi.org/10.3748/wjg.

**INTRODUCTION**

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most common prescription medicines nowadays, which are widely used in many diseases as the anti-inflammatory and analgesic agents[[1](#_ENREF_1)]. Especially aspirin, because of its multiple functions, it is widely used in cardiovascular disorders and even in the treatment of cancer[[2](#_ENREF_2),[3](#_ENREF_3)]. However, the adverse effects are common even under the safety-low dose, which are particularly in the digestive tract, such as the dyspeptic symptoms, gastrointestinal erosions, peptic ulcers, overt bleeding or perforation[[4](#_ENREF_4),[5](#_ENREF_5)]. Hence, scientists attemp to invent a risk-free dose of aspirin, or coat and buffer aspirin to mitigate the injury. However, an efficient way has not been found[[6](#_ENREF_6)].

To date, researches have revealed that a lot of factors, such as endogenous prostaglandin (PG), neutrophil-dependent microvascular injury, oxygen-derived free radicals, inflammatory cytokines[[7-10](#_ENREF_7)], which are associated with the aspirin-induced gastric mucosal damages. For instance, TNF-α is a proinflammatory cytokine and can augment the neutrophil-derived superoxide generation, leading to oxygen radical-mediated tissue damage. Pretreatment of TNF-α inhibitors suppresses the gastric mucosal injury[[11](#_ENREF_11),[12](#_ENREF_12)]. Moreover, the tissue enzymatic activity, such as dismutase (SOD), myeloperoxidase (MPO) and malondialdehyde (MDA) are also considered to be related with aspirin-induced gastric mucosal injury[[13-16](#_ENREF_13)]. Among the multiple mechanisms, the function of cyclooxygenase (COX) is one of the biggest concerns for the scientists. COX-1 plays a definite protective role in the NSAIDs induced gastric injury[[17](#_ENREF_17)]. Nevertheless, the COX-2 isoform plays a perplexing role in this pathological process. First, COX-2 isoform can be induced by damaging agents such as luminal acid and aspirin, and inhibition of COX-2 can alleviate gastric damage[[18-20](#_ENREF_18)]. It involves the maintenance of gastric mucosal integrity by preventing exogenous injury and by promoting gastric mucosal healing, which is the key indicator of the gastric mucosal injury[[21](#_ENREF_21)]. However, there are also some studies reporting that COX-2 inhibitors exacerbate injury to the gut and attenuate the tissue’s ability to respond to mild damaging agents[[22-24](#_ENREF_22)]. The possible mechanism is that COX-2 expression may be a compensatory response to increase the levels of gastroprotective PG during periods of gastric injury[[17](#_ENREF_17)].

Hydrogen therapy is a new medical approach which has gained much appreciation recently and has proved to have therapeutic efficacy in many diseases. Hydrogen has anti-oxidant, anti-inflammatory,anti-apoptotic, anti-allergy, and anti-cancer effects. Several methods, including inhalation, drinking hydrogen-rich water (HRW) and injection of hydrogen-saturated saline, have been invented and proved to be valid and reliable[[25](#_ENREF_25),[26](#_ENREF_26)]. Oral intake of HRW is an effective and convenient way to deliver the molecular hydrogen, which was more suitable for application. Some researches showed oral intake of HRW could protect cardiac allografts from inﬂammation-associated deterioration, kidney allografts from chronic rejection and so on[[27-29](#_ENREF_27)]. Although the research of hydrogen as a medical therapy has been extensively investigated, its effect on the aspirin-induced gastric mucosal injury has not been reported.

The main aim of our study was to assess the protective role of hydrogen-rich water on aspirin-induced gastric mucosal damage in rats mainly through measurement of oxidative stress indicators and cytokines, including levels of MDA, SOD, MPO, interleukin (IL)-6 and TNF-α in the gastric tissue, IL-1β and TNF-α in the serum, and expression of COX-2 in gastric mucosa.

**MATERIALS AND METHODS**

***Experimental animals and preparation of hydrogen-rich water***

The study was conducted using male Sprague Dawley rats (200-250 g) (Animal Feeding Center of Xi’an Jiaotong University medical school). All rats were housed (5 per cage) in clear, pathogen-free polycarbonate cages in the animal care facility, and were fed a standard animal diet and water *ad libitum* under controlled temperature conditions with 12 h light-dark cycles. They were cared in accordance with the Ethical Committee, Xi’an Jiaotong University medical school. The HRW was produced by Naturally Plus Japan International Co, Ltd which was stored under atmospheric pressure at 4 ℃ in an aluminum bag with no dead volume. The gas chromatography was used to confirm the content of hydrogen by the method described by Ohsawa *et al*[[25](#_ENREF_25)] (hydrogen concentration of the HRW we used in this study: 0.63-0.82 mmol/L).

***Study design***

Forty rats were divided into four groups randomly, each consisting of 10 animals, with different pre-treatments for 14 d, as follows: (1) normal control group rats received carboxymethyl cellulose (0.05%) (Tianjin Kemiou Chemical Reagent Co.) as the vehicle by daily gavage; (2) HRW control group rats received hydrogen-rich water randomly by daily gavage (replacement of HRW every 3 h, consumption of each rat was 80 ml/d); (3) aspirin group rats received carboxymethyl cellulose (0.05%) as the vehicle by daily gavage; and (4) HRW plus aspirin group rats received hydrogen-rich water randomly by daily gavage (80 ml/d per rat). On the 15th day, gastric lesions were induced by administration of aspirin (400 mg/kg) (Sigma Chemical Co.) in the aspirin and HRW plus aspirin groups. Normal control and HRW control groups were given saline (1 ml) following overnight fasting. After 8 h of aspirin treatment, rats were anesthetized under mild ether, and sacriﬁced *via* cervical decapitation. Gastric mucosa was harvested by gently scraping the mucosa off the underlying muscularis mucosa and serosal layers with a microscope slide and were frozen in liquid nitrogen and stored at -80 ℃ until assayed. The serum was separated by centrifugation at 3000 g for 15 min at -4 ℃ to obtain clear serum, aliquoted, and stored at -80 ℃ until assayed.

***Macroscopic analysis***

Stomachs were obtained after the execution and were opened along the greater curvature, and mucosae were rinsed with cold PBS to remove blood concomitant.

Gastric mucosal changes were evaluated by two researchers who were blinded to the treatment regimen. A scoring system to grade the degree of gastric mucosal was assigned using a scale of 0 to 6 described by Coleman *et al*[[30](#_ENREF_30)] (Table 1).

***Histological study***

Samples from the gastric mucosa were excised from the gastric glandular epithelium at a region located 2 mm below the limiting ridge that separates the forestomach from the glandular epithelium along the greater curvature of the stomach. They were fixed in 10% formalin solution and embedded in paraffin after completion of the routine follow-up. Serial sections of 5-μm thickness were obtained and stained with hematoxylin/eosin (HE) to evaluate gastric morphology.The results were examined in a blinded fashion by two researchers. The mucosa was considered injured if one or more of the following criteria were present: discontinuous surface, dilated gland, hemorrhage, or damage to superﬁcial cells[[31](#_ENREF_31)].

***Gastric mucosal enzymatic activity assay***

The gastric mucosa tissue was homogenized, and tissue MPO, MDA, SOD activity was measured using the activity assay kits from NanJing JianCheng Bioengineering Institute according to the manufacturer’s instructions.

***Gastric mucosal and serum cytokine assay***

Gastric tissue samples and blood samples were homogenized and the supernatant was used for the determination of cytokines. The levels of cytokines (IL-6 and TNF-α) in the gastric tissue samples and IL-1β and TNF-α in the blood samples were evaluated using the ELISA kit reagent from Dakewe Biotech Co., according to the manufacturer's instructions. A standard curve was run on each assay plate using recombinants of the respective cytokines in serial dilutions.

***Immunohistochemistry***

To establish the immunolocalization of COX-2, a mouse polyclonal antibody (Beijing Biosynthesis Biotechnology Co., LTD) was used at a working dilution of 1:20. The antibody was applied directly to sections, and slides were incubated overnight at 4 °C in a humidiﬁed chamber. Immune complexes were subsequently treated with the secondary antibody (containing anti-rabbit and anti-mouse immunoglobulins) and detected *via* application of streptavidin peroxidase treatment for 20 min at room temperature. After rinsing sections with three changes of PBS, immunoreactivity was visualized with diamine benzidine (DAB)-hydrogen peroxide (20 min). Sections were gently rinsed in distilled water, counterstained with H and E, and photomicrographs taken under a microscope (Olympus Optical Co., Tokyo, Japan). The results of immunohistochemistrywere made by two researchers under blinded conditions. The intensity of immunohistochemical staining was scored as 0 (negative), 1 (weak), 2 (moderate strong) or 3 (strong). The extent of staining was assessed based on the percentage of positive tumor cells: 0 (negative), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). The ﬁnal staining score for each sample was the mean of the sum of the intensity and extent scores from five ﬁelds. The expression was considered as low if the ﬁnal score was 1–5 and as high if the ﬁnal score was 6–12.

***Statistical analysis***

All data were presented as mean ± SD for 10 rats in each group. To compare data among all groups of animals, one-way analysis of variance (one-way ANOVA) and Duncan comparisons were employed. All statistical tests were performed using SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, United States). Differences were considered statistically significant at *P* < 0.05.

**RESULTS**

***Histopathological changes***

Histopathological examination revealed aspirin-induced severe congestion and multiple hemorrhagic erosions in the stomach tissue, particularly in mucus-secreting cells, characterized by gastric pit damage and vacuolization of the glandular portion. Pretreatment with hydrogen-rich water considerably attenuated, but did not completely prevent the severity of these histopathological changes, while some erosion in sub-glandular and epithelial necks was evident (Figure 1A). In the aspirin group, the mean gastric mucosal damage score was 4.04 ± 0.492, while hydrogen-rich water pretreatment could reduce the damage to 2.10 ± 0.437 (*P* < 0.05) (Figure 1B).

***Effects of hydrogen-rich water on MDA, MPO, SOD and cytokine levels in aspirin-induced gastric mucosal injury***

The oxidative stress parameters including MDA and MPO in the gastric mucosa increased significantly in the aspirin treated group compared with the HRW pretreatment group (2.43 ± 0.145 *vs* 1.79 ± 0.116 nmol/mg prot, *P* < 0.05 and 2.53 ± 0.238 *vs* 1.40 ± 0.208 U/g tissue, *P* < 0.05). And the protective indicator SOD could increase significantly from 37.94 ± 8.44 nmol/mg prot to 59.55 ± 9.02 nmol/mg prot by the use of HRW (*P* < 0.05). Pretreatment with HRW could also significantly decrease the elevation of IL-6, TNF-α in the gastric tissue (46.65 ± 5.50 *vs* 32.15 ± 4.83 pg/mg, *P* < 0.05 and 1305.08 ± 101.23 *vs* 855.96 ± 93.22 pg/mg, *P* < 0.05) (Figure 2).

***Effects of hydrogen-rich water on cytokine levels in the peripheral blood***

The level of IL-1β and TNF-α in the peripheral blood were markedly increased in the aspirin treated group compared with the normal control and HRW control groups (*P* < 0.01). The increase in TNF-α and IL-1β concentration in the gastric mucosa elicited by aspirin were significantly suppressed by HRW pretreatment (505.38 ± 32.97 *vs* 343.37 ± 25.09 pg/ml, *P* < 0.05 and 264.53 ± 28.63 *vs* 114.96 ± 21.79 pg/ml, *P* < 0.05, respectively) (Figure 3).

***immune histochemical analysis of COX-2 in gastric***

Immunohistochemical analysis was performed to ascertain the localization of COX-2 in gastric mucosa tissue. Rats treated with aspirin displayed signiﬁcant immunoreactivity for COX-2 around the glandular regions, mucoid cells and neck gland cells of gastric tissue (Staining score: 8.4 ± 2.1). In contrast, the group of rats pretreated with hydrogen-rich water presented few COX-2 immunoreactivities (Staining score: 2.9 ± 1.5) (*P* < 0.05) (Figure 4).

**DISCUSSION**

Aspirin is widely used as an anti-inflammatory and analgesic drug, but it often induces gastrointestinal adverse effects, which limits the clinical uses severely, and research on the approaches to reduce the aspirin-induced gastric mucosal damages is urgent[[32-34](#_ENREF_32)]. Here, we examined the effect of HRW on the aspirin-induced gastric mucosal injury. Although hydrogen is the lightest gas in nature, it has an enormous capacity of anti-oxidation and anti- inflammatory effects. In fact, there are few studies investigating the preventive or therapeutic effects of gas on gastric mucosal damages. In one study, hydrogen sulfide was found to protect against aspirin-induced gastric injury *via* reducing oxidative stress[[35](#_ENREF_35)]. In another study, Liu *et al*[[36](#_ENREF_36)] found that hydrogen treatment successfully ameliorated stress-associated gastric ulceration *via* its anti-oxidant, anti-inﬂammatory and anti-apoptotic effects. Our research shows that hydrogen can reduce aspirin-induced gastric injury by reducing MDA, MPO, IL-6, TNF-α levels and increasing SOD activity in gastric tissues. Simultaneously, hydrogen could decrease the elevation of IL-1β and TNF-α in the serum compared to treatment with aspirin alone. We also find that hydrogen can decreased COX-2 expression in the gastric tissue, which may be the key mechanism of hydrogen action because of its importance in the aspirin-induced gastric injury. The present study demonstrated that hydrogen may prevent against aspirin-induced gastric mucosal injury, mainly depend on the modulation of anti-inﬂammatory cytokines, antioxidative stress and activation of COX-2.

In the past few years, the research of hydrogen therapy attracted wide attention from many scientists and physicians[[26](#_ENREF_26),[37](#_ENREF_37)]. Hydrogen molecules have proven to act as an important physiological regulatory factor to cells and organs by antioxidant, anti-inflammatory, anti-apoptotic and other protective effects, which can be applied in the treatment of various diseases[[26](#_ENREF_26)]. Compared with other research on hydrogen therapy, a distinctive experimental design in our research was the delivery of hydrogen molecule. We designed the random oral intake of HRW to deliver the hydrogen molecule, which was much closer to human physiological status. This special and convenient delivery method implies that the hydrogen-rich water can be used as a kind of drink, which can immensely expand its applications.

According to previous research, redox imbalance plays a major pathogenic role in aspirin gastropathy[[5](#_ENREF_5)].The over oxidative stress in the gastric mucosal can increase the production of oxygen radical or decrease the capability of antioxidant defenses[[38](#_ENREF_38)]. We found in the present study that hydrogen can significantly reduce the Asp-induced elevation of MDA and MPO, the most typical markers of free radical species-related injury. In addition, hydrogen also reversed the Asp-reduced elevation of SOD, which is responsible for converting superoxide radicals to molecular oxygen and hydrogen peroxide within cytoplasm and mitochondria[[39](#_ENREF_39)]. The cytokines, such as TNF-α ,IL-1β and IL-6, induced by lymphocytes and macrophages that inﬁltrate the gastric mucosa, are associated with the tissue injury[[40](#_ENREF_40)]. They are responsible for the neutrophil adherence in the microcirculation of gastric mucosa, and the release from activated macrophages with parallel accumulation of neutrophils within the gastric pits[[41](#_ENREF_41)]. In the present study, we observed that pretreatment with hydrogen resulted in signiﬁcantly decreased TNF-α, IL-1β and IL-6 production which could prevent subsequent neutrophils inﬁltration and alleviate injury.

On a deeper level, we focused on COX-2, which was the rate-limiting enzyme to regulate the synthesis of PG. This was a key factor in response to stress. It was reported that Asp can rapidly up-regulate COX-2 mRNA expression in rat gastric mucosa, probably as a compensatory response to inhibition of COX-2 activity and gastrin PG synthesis[[42](#_ENREF_42),[43](#_ENREF_43)]. COX-2 products play a relevant role for the maintenance of gastric mucosal integrity by preventing exogenous injury to the stomach and accelerating gastric mucosal healing[[44](#_ENREF_44)]. Interestingly, HRW pre-treatment counteracted the increased expression of COX-2 induced by aspirin treatment, which promote mucosal healing. According to the description in the introduction section that the COX-2 plays a controversial role in the aspirin induced gastric injury, we speculate that the COX-2 can act as the double-edged sword. First, COX-2 is induced by cytokines and then promotes the release of cytokines to exacerbate inflammation[[20](#_ENREF_20)]; meanwhile, as a compensatory mechanism, the expression of the COX-2 can accelerate the prostaglandin production to protect the gastric mucosa[[21](#_ENREF_21)]. Thus, when the noxious stimuli are strong enough to cover the protective role of the COX-2, the expression of the COX-2 is a harmful protein in the pathological process of aspirin-induced gastric injury. In the study, 400 mg/kg of aspirin is a high dosage for the rats and the high expression of COX-2 is induced by the cytokines which has covered its protective role. The hydrogen can suppress its expression to protect the gastric mucosa.Although the detailed molecular mechanism between hydrogen and COX-2 is not clear, COX-2 as a definite target of hydrogen can be confirmed. A clue of “Hydrogen → mitigating oxidative stress → alleviating inflammatory reaction → suppressing COX-2 expression → protecting aspirin-induced gastric injury” can be used to summarize the research.

In conclusion, the present study shows that: (1) hydrogen, a new medical therapy, is able to prevent aspirin-induced injury to the rat gastric mucosa through a mechanism, which is in part contributed by the anti-oxidant and anti-inflamatory activities; (2) aspirin causes up-regulation of COX-2 expression in the gastric mucosa; and (3) hydrogen signiﬁcantly counteracts aspirin-induced up-regulation of COX-2 expression, probably as a consequence of the reduction in the extent of aspirin-induced injury. So, hydrogen therapy might be safe and effective in the prevention of the stomach injury because of the NSAIDs application. Future clinical studies are necessary to determine whether this method will be as interesting as preventive or therapeutic agents as they are in this pre-clinical study.

**Acknowledgments**

We thank UNIVA Guangzhou Trading Co., Ltd for their assistance in providing the hydrogen-rich water.

**COMMENTS**

***Background***

Aspirin is widely used as an anti-inflammatory and analgesic drug, but it often induces gastrointestinal adverse effects which include dyspeptic symptoms, gastrointestinal erosions, peptic ulcers, overt bleeding or perforation. Hence, scientists tried to invent the risk-free dose of aspirin or coat and buffer aspirin to mitigate the injury, but an efficient way has not been found, yet. The imperfections limit the clinical uses of aspirin severely, and research on the approaches to reduce the aspirin-induced gastric mucosal damages is urgent.

***Research frontiers***

Hydrogen therapy is new medical approach which has gained much appreciation recently. It has been shown that hydrogen has anti-oxidant, anti-inflammatory, anti-apoptotic, anti-allergy, and anti-cancer effects. Several methods invented to deliver hydrogen, including inhalation, drinking hydrogen-rich water (HRW) and injection with hydrogen-saturated saline, have proved to be valid and reliable. In the area of prevention of aspirin-induced gastric injury, the research hotspot is to search for more effective and convenient methods which can be accepted by people more easily. Meanwhile, the mechanism of a new medicine is also another hotspot.

***Innovations and breakthroughs***

Compared with other research on hydrogen therapy, a distinctive experimental design in our research was the delivery of hydrogen molecule. We designed the random oral intake of HRW to deliver hydrogen molecule, which was much closer to human physiological status. This special and convenient delivery method implies that the hydrogen-rich water can be used as a kind of drink, which can immensely expand its applications. Simultaneously, we found that the hydrogen did have protective role in the aspirin-induced gastric injury, which is in part contributed by the anti-oxidant and anti-inflammation activities. Hydrogen can signiﬁcantly counteract aspirin-induced up-regulation of cyclooxygenase 2 (COX-2) expression, probably as a consequence of the reduction in the extent of aspirin-induced injury.

***Applications***

Hydrogen therapy might be a safe and effective prevention of the stomach injury derived from non-steroidal anti-inflammatory drugs application. This special and convenient delivery method implies that the hydrogen-rich water can be used as a kind of drink, which can immensely expand its applications.

***Terminology***

Hydrogen is the lightest gas in the nature, but it has huge anti-oxidant, anti-inflammatory, anti-apoptotic, anti-allergy, and anti-cancer effects. In the past few years, the research of hydrogen therapy attracted wide attention from many scientists and physicians. It has been proven to be effective in treating many diseases. HRW is produced by pressing the hydrogen gas into the water by a specific device under high pressure.

***Peer review***

This study demonstrates the protective effects of hydrogen therapy (hydrogen rich water) on acute aspirin gastropathy in rats, which are probably dependent on the modulation of COX-2, cytokines and antioxidant activities. This is an interesting paper, with a new clinical application of a new therapy, in an experimental model of gastropathy. The results are well presented and provide sufficient experimental evidence to support the conclusions.

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**P-Reviewer:** Auricchio S, Decorti G, Han X, Hassan M **S-Editor:** Ma YJ **L-Editor:** **E-Editor:**

**Table 1 Scale for grading gastric mucosal damage**[[30](#_ENREF_30)]

|  |  |
| --- | --- |
| Grade | Appearance of the gastric mucaosa |
| 0 | Normal |
| 1 | Slight edema and congestion |
| 2 | Edema, congestion, and bleeding |
| 3 | One or two spot erosions |
| 4 | One or two linear erosions |
| 5 | Many small and a few large erosions |
| 6 | Extensive srosions over entire mucosa |

**Figure legend**

**Figure 1 Histopathological examination of stomach sections.** A: Hematoxylin-eosin stained results showed severe degenerative changes in glandular region, epithelial folds and connective septa in aspirin-induced mucosal tissue (a, × 100 and b, × 200), while hydrogen-rich water (HRW) pretreatment displayed slight changes (c, × 100 and d, × 200). B. The gastric mucosal damage score in four groups. The mean scores are significantly higher in the aspirin group and HRW plus aspirin group [HRW + aspirin group (Asp)] when compared with the normal control group and HRW alone group (b*P* < 0.01). Pretreatment with HRW could significantly decrease the damage score in HRW + Asp group when compared with aspirin group (a*P* < 0.05).

**Figure 2 The levels of oxidative stress indicators and cytokines in all groups.** Malonaldehyde (MDA), myeloperoxidase (MPO), tumor necrosis factor (TNF)-α, interleukin (IL)-6 in the gastric mucosal tissue are significantly higher, and superoxide dismutase (SOD) levels is obviously lower in aspirin group and hydrogen-rich water (HRW) plus aspirin group when compared with the normal control group and/or HRW alone group (b*P* < 0.01, a*P* < 0.05 when compared with the control and HRW groups). Pretreatment with HRW could significantly decrease the MDA, MPO, TNF-α, IL-6 levels and increase the SOD activity in HRW + Asp group when compared with aspirin group (a*P* < 0.05).

**Figure 3 Serum tumor necrosis factor-alpha and interleukin-Iβ levels in all groups.** All data are expressed as mean ± SD. The mean tumor necrosis factor (TNF)-α and interleukin (IL)-Iβ levels are significantly higher in the aspirin group (Asp) and hydrogen-rich water (HRW) plus aspirin group (HRW + Asp) when compared with the normal control group and/or HRW alone group (b*P* < 0.01, a*P* < 0.05 when compared with the control and HRW groups). And pretreatment with HRW could significantly decrease serum TNF-α and IL-Iβ levels in HRW + Asp group when compared with aspirin group (a*P* < 0.05).

**Figure 4 Immunolocalization of cyclooxygenase 2 in gastric tissue.** Photomicrographs a, b show the results in the negative control group. Photomicrographs c, d show a scarce cyclooxygenase 2 (COX-2) immunoreactivity in sub-glandular region of hydrogen plus aspirin-treated rats. Photomicrographs e, f display an apparent COX-2 immunoreactivity in the glandular region and epithelial necks of stomach tissue of rats those received aspirin (a, c, e × 200 and b, d, f × 400).