

# Hydrogen-Rich Saline Attenuated Neuropathic Pain by Reducing Oxidative Stress

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**ABSTRACT: Background:** Reactive oxygen species (ROS) are often associated with persistent pains such as neuropathic and inflammatory pain. Hydrogen gas can reduce ROS and alleviate cerebral, myocardial, and hepatic ischemia/reperfusion injuries. In the present study, we aim to investigate whether hydrogen-rich saline can reduce neuropathic pain in a rat model of chronic constriction injury (CCI). **Methods:** Thirty SD rats were randomly divided into three groups: sham group was administered sodium chloride by intrathecal injection (n=10); control groups underwent CCI surgery and were administered sodium chloride by intrathecal injection (n=10); vehicle group underwent CCI surgery and was administered hydrogen-rich saline by intrathecal injection (n=10). Drugs were administered in the dose of 100ul/kg once a day at 0.5 hours before and 1-7 day after CCI surgery. The mechanical thresholds were tested at one day before and 3-14 day after CCI surgery. **Results:** We found that hydrogen-rich saline significantly elevated the mechanical thresholds of neuropathic pain compared to vehicle (physiologic saline) control in CCI rats (p<0.05); it also decreased the levels of myeloperoxidase, maleic dialdehyde, and protein carbonyl in spinal cord by 7 days post-chronic constriction injury(p<0.05). In addition, hydrogen-rich saline also suppressed the expression of p38-mitogen-activated protein kinase (p38MAPK) and brain-derived neurotrophic factor (BDNF) in the spinal cord by 7 days post-chronic constriction injury (p<0.01, p<0.01, respectively), but had no effect on P2X4R (p>0.05), an ATP receptor. **Conclusion:** Intrathecal injection of hydrogen-rich saline can decrease oxidative stress and the expression of p38MAPK and BDNF that may contribute to the elevated threshold of neuropathic pain in rat CCI model.

**RÉSUMÉ: Le salin riche en hydrogène atténue la douleur névropathique en réduisant le stress oxydatif. Contexte :** Les espèces réactives oxygénées (ROS) sont souvent associées à la douleur persistante comme la douleur névropathique et la douleur inflammatoire. Le gaz hydrogène peut réduire les ROS et soulager le dommage dû à l'ischémie/la reperfusion au niveau cérébral, myocardique et hépatique. Le but de cette étude était de déterminer si le salin riche en hydrogène peut réduire la douleur névropathique chez un modèle de lésion par constriction chronique (LCC) chez le rat. **Méthode :** Trente rats SD ont été assignés au hasard à trois groupes : un groupe a subi une intervention factice et a reçu du chlorure de sodium en injection intrathécale (n = 10) ; un groupe a subi une LCC et a reçu du chlorure de sodium en injection intrathécale (n = 10) ; un groupe a subi une LCC et a reçu du salin riche en hydrogène en injection intrathécale (n = 10). Les solutions ont été administrées à la dose de 100µl/KG une fois par jour 0,5 heure avant la LCC et du jour 1 au jour 7 après l'intervention. Les seuils mécaniques ont été évalués 1 jour avant et du jour 3 au jour 14 après la LCC. **Résultats :** Nous avons constaté que le salin riche en hydrogène augmentait significativement le seuil mécanique de la douleur névropathique par rapport au témoin (le salin physiologique) chez les rats ayant subi une LCC (p < 0,05). Il diminuait également les niveaux de myéloperoxydase, de dialdéhyde maléique et de protéines carbonylées dans la moelle épinière 7 jours après la LCC (p < 0,05). De plus, le salin riche en hydrogène supprimait également l'expression de la protéine kinase activée par le mitogène p38 (MAPK p38) et le facteur neurotrophe dérivé du cerveau (BDNF) dans la moelle épinière au 7e jour après la LCC (p < 0,01 et p < 0,01 respectivement), mais n'avait aucun effet sur P2X4R (p > 0,05), un récepteur de l'ATP. **Conclusion :** L'injection intrathécale de salin riche en hydrogène peut diminuer le stress oxydatif et l'expression de MAPK p38 et de BDNT, ce qui pourrait contribuer à l'élévation du seuil de la douleur névropathique chez le modèle de rat ayant subi une LCC.

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Reactive oxygen species (ROS) are involved in the development of pains in various conditions, including neuropathic and inflammatory pain<sup>1-5</sup>. Park et al reported increased ROS in the spinal cord of rats after peripheral nerve injury<sup>6</sup>. Others found that treatment with ROS scavengers phenyl Ntert-butyl nitron (PBN), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), 5,5-dimethylpyrrolidine-N-oxide (DMPO) or vitamin E could temporarily reverse the allodynia developed after nerve ligation in rats<sup>2,7,8</sup>. The function mechanisms of ROS scavenger, like that of PBN-induced analgesic effect, are reported to be mediated mainly via spinal mechanisms<sup>2</sup>.

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Recently, hydrogen gas has been reported as a potent free radical scavenger which can selectively reduce the hydroxyl radical, one of the most cytotoxic ROS. Ohsawa et al<sup>9</sup> demonstrated that hydrogen gas ameliorated ischemic brain injury by selectively reducing hydroxyl radicals. Moreover, hydrogen was also found to relieve oxidative stress-induced tissue injury in intestinal ischemia<sup>10,11</sup>, myocardial ischemia<sup>12</sup> and brain ischemia<sup>13</sup>. However, little is known about the potential role of hydrogen-rich saline in management of neuropathic pain. In the present study, we investigated the analgesic effect of hydrogen-rich saline in a rat model of chronic constriction injury (CCI)-induced neuropathic pain and discussed the possible underlying mechanisms.

## Experimental procedure

### *Animals and groups*

Adult male Sprague-Dawley rats, weighing 200-250 gram (g) (n=30), were used in the present study. The animals were housed under a 12/12hour (h) reversed light-dark cycle (dark cycle: 8:00 A.M.-8:00 P.M.) for at least one week before any experiments. All the animal protocols were approved by the Medical Ethical Committee of Second Military Medical University, Shanghai, China, in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH) (publication No. 96-01).

Animals were randomly divided into three groups with each group consisting of ten rats: (1) sham group with physiologic saline treatment (Sham group), (2) CCI animals treated with physiologic saline (CCI+saline group), and (3) CCI animals treated with hydrogen-rich saline (CCI+hydrogen group). Hydrogen-rich saline was administered through an intrathecal catheter at a daily dose of 100 $\mu$ l/kg during Day 1-7 after CCI in CCI+hydrogen group, and physiologic saline was injected in the same manner in CCI+saline group. The mechanical thresholds of rats were measured one day before and on Day 3-14 after CCI in all groups.

### *Intrathecal injection of hydrogen-rich saline*

Rats were anesthetized by peritoneal injection of 10% chloral hydrate (3 ml /kg) before intrathecal injection of drugs in each group. The paraspinal muscles were retracted and the posterior part of the 6th lumbar vertebrae was removed to expose the spinal meninges. A catheter (sterilized PE 10 tubing filled with sodium chloride) was inserted into the subarachnoid space through a small nick in the dura; the tip of the catheter was placed near the lumbar enlargement of the spinal cord. The remaining part of the catheter was fed subcutaneously to the atlan-pulvinar level and anchored to muscles by sutures; the tip of the catheter was exposed and sealed. One day after surgery 2% lidocaine (20  $\mu$ l) was injected through the catheter. If the catheters were accurately placed, the rats would develop anesthesia symptom 30s after injection as demonstrated by extremities paralysis or weakness.

### *Establishment of CCI model*

Three days after successful intrathecal catheterization, the rats were fasted for 12 h prior to surgery and then anaesthetized with 10% chloral hydrate (3 ml /kg, i.p.). After induction of

anesthesia, the fur around the mid-thigh was shaved and CCI was induced as described by Bennett and Xie<sup>14</sup>. The common sciatic nerve of the right hind limb was exposed at the middle of the thigh by blunt dissection through biceps femoris. About 7 mm nerve proximal to the sciatic trifurcation was isolated, on which four ligatures (4.0 silk) were tied loosely at an interval of 1 mm, with the length of the affected nerve being 4-5 mm. The desired degree of constriction was that it retarded but did not arrest the circulation of the superficial epineural vasculature. The incision was closed in layers. For estimation of free radical and antioxidant enzyme activities, additional animals receiving no ligation of the sciatic nerve served as sham-exposed controls.

Povidone iodine solution was applied externally and prophylactically penicillinum (Penicillin, Harbin Pharmaceutical Group Co., Ltd, China) was injected intraperitoneally (50 mg/kg body weight) for three consecutive days to prevent infection. The operated animals were caged individually, with food and water given arbitrarily.

### *Preparation of hydrogen-rich saline*

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4MPa) to a supersaturated level using an apparatus designed by the Department of Diving Medicine, Second Military Medical University. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume, and was sterilized by gamma radiation. Hydrogen-rich saline was freshly prepared every week to ensure a lowest concentration of 0.6 mmol/L. Gas chromatography was used to confirm the content of hydrogen in saline as described by Ohsawa et al<sup>9</sup>.

### *Measurement of mechanical threshold*

Mechanical thresholds of ipsilateral paw were assessed one day before and on Day 3-7 after CCI surgery. The animals were placed in a plastic chamber (10.0  $\times$  10.0  $\times$  20.0 cm) on the top of a mesh screen platform and habituated for at least 15 min. The thresholds were determined by the up-down method<sup>15,16</sup> using a set of von Frey monofilaments (Stoelting, USA) with bending forces of 0.6, 1, 1.4, 2, 4, 6, 8, 10 and 15 g. The filaments were presented to the midplantar surface as described by Chaplan et al<sup>16</sup>. An abrupt withdrawal of the foot during stimulation or immediately after stimulus removal was considered to be a positive response. The first stimulus was initiated with the 2 g filament. If there was a positive response, the next lower filament was used, and if not, the next higher filament was used. The test continued until we had recorded responses to six von Frey stimuli from the first change of response (either higher or lower than the first stimulus, depending on whether the first response was negative or positive). When positive or negative responses were still observed at the end of a stimulus session, values of 0.6 g or 15 g were assigned, respectively.

### *Morphology*

Five animals were sacrificed in each group on Day 7 after surgery. Rats were anesthetized with 10% chloral hydrate (3ml/kg, i.p.) and perfused with 100 ml isotonic ice-cold saline followed by 250 ml fixation fluid (4°C) through a cannula inserted into the ascending aorta. The fixative consisted of 4%

paraformaldehyde weight/volume (w/v) in 0.1M phosphate buffer (pH 7.4). A lumbar enlargement (10 mm) of the spinal cord was collected, post-fixed in the perfusion fixative overnight, and then cryoprotected for two days in 30% sucrose in 0.1 maleic polybutadiene (MPB). Paraffin sections of 16  $\mu$ m were made and mounted on gelatinized slides. Immunohistochemistry examinations for changes of P2X4R, p38-mitogen-activated protein kinase (p38MAPK) and brain-derived neurotrophic factor (BDNF) expression were performed in each group.

After paraffin sectioning, slides were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 15 minutes (min) at room temperature to inactivate endogenous enzymes, and then rinsed with distilled water three times, each time five min. The slides were immersed in 0.01M citrate buffer (pH 6.0) and microwave heated to boiling, cooled, then rinsed after in PBS (pH 7.2-7.6) three times. Normal Goat Serum Blocking solution was added at 37°C for 20 min; primary antibodies, including primary polyclonal mouse-anti-P2X4 antibody (1:100), primary polyclonal rabbit-anti-BDNF (1:400) antibody, and primary polyclonal rabbit-anti-p38mapk antibody (1:400) (Sigma–Aldrich, St. Louis, MO, USA), were added to a 1:100 dilution and refrigerated overnight at 4°C. The slides were incubated with Biotinylated goat anti-mouse or anti-rabbit IgG (Vector Labs, California, USA) at 37 °C for 20 min, then rinsed in PBS three times; Strept avidin-biotin complex (SABC) reagents were added at 37°C for 20 min. Diaminobenzidine (DAB) (DAB staining kit, Bluegene Biotech CO., LTD, Shanghai, China) was added for five min then rinsed with tap water. Cell counting was conducted in the spinal cord (lumbar enlargement) in ten randomly selected sections of each animal by a blinded experimenter. Digitized images were analyzed using the Image Pro-Plus image analysis system for quantitative analysis of the positive staining area. A density threshold was set above the background level to identify the positively stained structure. An area of 0.03 mm<sup>2</sup> was selected to measure the positive area in eight equidistant regions on each section.

#### Measurement of myeloperoxidase

Myeloperoxidase (MPO) is an enzyme involved in the production of free radicals, and its level can reflect the metabolism of free radicals. The spinal cord of rats was homogenized in 10 volumes of ice-cold 20 mmol/L potassium phosphate buffer (pH 7.4) containing 30 mmol/L KCl. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C; the pellet was then rehomogenized with an equivalent volume of 50 mmol/L acetic acid containing 5% hexadecyltrimethylammonium bromide (HETAB). Myeloperoxidase activity was assessed by measuring the H<sub>2</sub>O<sub>2</sub> dependent oxidation of o-dianizidine 2 HCl. One unit of enzyme activity was defined as the amount of the MPO that caused a change in absorbance of 1.0/min at 460 nm and 37°C.

#### Measurement of lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction as described by Ohkawa et al<sup>17</sup>. The method was used to obtain a spectrophotometric measurement of the color produced by reaction of TBA with malondialdehyde (MDA) at 532nm.

#### Measurement of protein carbonyl

Protein carbonyl was measured as an indicator of protein oxidation. Briefly, the homogenate of spinal cord was mixed with streptomycin, centrifuged, incubated with DNPH for 1h, and allowed to precipitate with trichloroacetic acid. The pellet was washed with ethanol-ethyl acetate to remove excessive DNPH and re-suspended in guanidine hydrochloride. The solution was then centrifuged and incubated at 37°C in a water bath for ten min. Finally, the absorbance was measured at 366 nm wavelength and the results were expressed as nmol/mg protein.

#### Quantitative Real Time RT-PCR (qRT-PCR) of P2X4R, p38 MAPK, and BDNF mRNA expression

Levels of P2X4R, p38 MAPK, and BDNF mRNA were determined by two-step RT-PCR following the manufacture's instruction. Briefly, in the first step, MMLV Reverse Transcriptase was used to synthesize a cDNA copy of input RNA. Then the resulting cDNA was subjected to qPCR that was performed with a Light Cycler using a SYBR Green Two-Step qPCR Kit. The amplified product was detected by the presence of an SYBR green fluorescent signal. The standard curve was designed with designed with b-actin cDNA. The resulted amplicon was quantified with the standard curve. The primers Primers for P2X4R, BDNF, P38MAPK and GAPDH were designed as follows: P2X4R (forward: 5'-ACAAATACTCAGGGTTGCC-3'; reverse: 5'-CTACCTCCATACGCTCAC-3'); BDNF(forward: 5'-CAGTGACAGGCGTTGAGA-3'; reverse: 5'-ATCCACCTTGGCGATTAC-3'); P38MAPK(forward: 5'-GCCTCACCCTCAGTAT-3'; reverse: 5'-GCAGTCTTCTCATTCCCTTG-3'); GAPDH(forward: 5'-TCCCTCAAGATTGTCAGCAA-3';reverse: 5'-AGATCCACAACGGATACATT-3') forward: 50-CGTGGGGAGCTGAGCGTGTG-30; reverse: 50-GCCCCTGCAGCCTTCCTTCG-30.

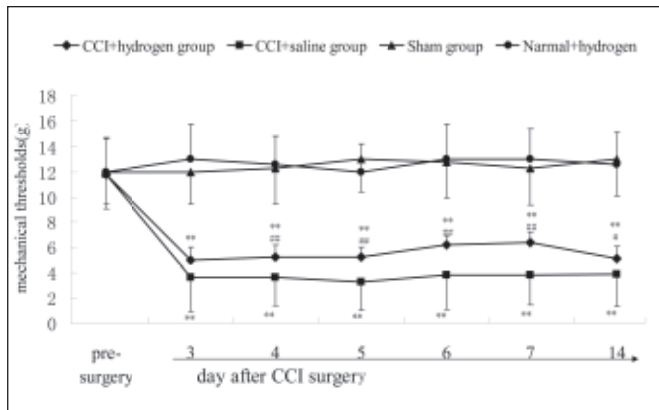
#### Statistical analysis

Data were presented as mean plus and minus standard error of the mean (SEM) and analyzed by statistical program SPSS 16.0. One way analysis of variance (ANOVA) was used to test the differences in the biochemical analysis and the expressions of P2X4R, BDNF and P38MAPK. A p value less than 0.05 was considered statistically significant. The paw withdrawal threshold values were analyzed with a two-way ANOVA with repeated measurements over time followed by Holm–Sidak post hoc test.

## RESULTS

#### Hydrogen-rich saline enhanced mechanical threshold of ipsilateral paw of CCI rats

The mean mechanical threshold of ipsilateral paw was 12.0 g in the sham-operated rats (Figure 1). During Day 3-14 after CCI, the mechanical thresholds of ipsilateral paw were dramatically decreased in both CCI+saline and CCI+hydrogen groups till the end of the study; however, that in the CCI+hydrogen group was significantly higher than that in CCI+saline group on Day 3-14 after CCI surgery ( $p < 0.01$ ,  $n = 10$ ). Furthermore, ten normal rats



**Figure 1:** Mechanical thresholds of ipsilateral paw in foot withdrawal test. Mechanical thresholds were assessed one day before and on day 3–14 after CCI surgery. The average mechanical threshold of the rat hind paw was 12.0 g in normal rats. On day 3 after CCI surgery, the mechanical thresholds decreased dramatically in CCI+saline and CCI+hydrogen groups and remained low till the end of the study. However, the mechanical threshold of hydrogen-rich saline group was significantly higher than that of the physiologic saline group on day 3–14 after CCI surgery. The mechanical thresholds had no significant change before and after administration of hydrogen-rich saline in normal rats. \* vs. Sham group,  $p < 0.05$ , \*\* vs. Sham group  $p < 0.01$ ; # vs. CCI+hydrogen group  $p < 0.05$ , ## vs. CCI+hydrogen group  $p < 0.01$ ;  $n = 10$ .

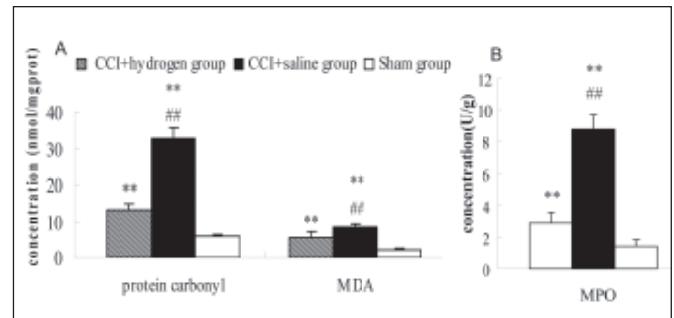
were also given intrathecal injection of hydrogen-rich saline (100 $\mu$ l/kg) for eight days, and their mechanical thresholds had no significant changes before and after injection.

### Hydrogen-rich saline decreased MPO, MDA and protein carbonyl levels in spinal cord

The total amounts of MPO, MDA and protein carbonyl in the spinal cord were measured seven days after CCI surgery. We found that the amounts of MPO, MDA and protein carbonyl were significantly higher in CCI+saline group than in the sham group ( $p < 0.01$ ,  $n = 5$ ), while hydrogen-rich saline significantly decreased MPO, MDA and protein carbonyl contents compared with CCI+saline group ( $p < 0.01$ ,  $n = 5$ ) (Figure 2).

### Immunohistochemical staining showed that hydrogen-rich saline decreased p38MAPK and BDNF expression in spinal cord

Immunohistochemical SABC staining demonstrated increased expression of P2X4R, p38MAPK and BDNF in the microglia of spinal cord in CCI+saline group seven days after CCI surgery (Figure 3). The positive areas were used to estimate the expressions of P2X4R, p38MAPK, and BDNF in the three groups. Compared with the sham group, the positive areas of P2X4R (CCI+saline vs. sham-operated:  $2061.04 \pm 127.55 \mu\text{m}^2$  vs.  $1096.98 \pm 136.42 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ; CCI+hydrogen vs. sham-operated:  $2130.30 \pm 109.90 \mu\text{m}^2$  vs.  $1096.98 \pm 136.42 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ); p38MAPK (CCI+saline vs. sham-operated:  $3062.17 \pm 126.74 \mu\text{m}^2$  vs.  $2092.47 \pm 246.84 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ) and BDNF (CCI+saline vs. sham-operated:  $2901.79 \pm 111.43 \mu\text{m}^2$  vs.  $1191.64 \pm 211.93 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ; CCI+hydrogen vs. sham-operated:  $1715.34 \pm 236.67 \mu\text{m}^2$  vs.  $1191.64 \pm 211.93 \mu\text{m}^2$ ,  $p < 0.01$ ,



**Figure 2:** Hydrogen-rich saline decreased MPO, MDA and protein carbonyl in spinal cord. A. Hydrogen-rich saline reduced MDA and protein carbonyl in the spinal cord on day 7 after CCI surgery compared to CCI+saline group ( $5.47 \pm 1.57$  vs.  $8.71 \pm 0.54$ ,  $13.27 \pm 1.58$  vs.  $32.56 \pm 3.18$ ,  $p < 0.01$ ,  $n = 5$ ). B. MPO was significantly increased in CCI+saline group compared with that in the sham group on day 7 after CCI surgery. Treatment with hydrogen-rich saline decreased MPO in the spinal cord ( $2.91 \pm 0.65$  vs.  $8.77 \pm 0.90$ ,  $p < 0.01$ ,  $n = 5$ ). \* vs. Sham group  $p < 0.05$ , \*\* vs. Sham group  $p < 0.01$ ; # vs. CCI+hydrogen group  $p < 0.05$ , ## vs. CCI+hydrogen group  $p < 0.01$ ,  $n = 5$ .

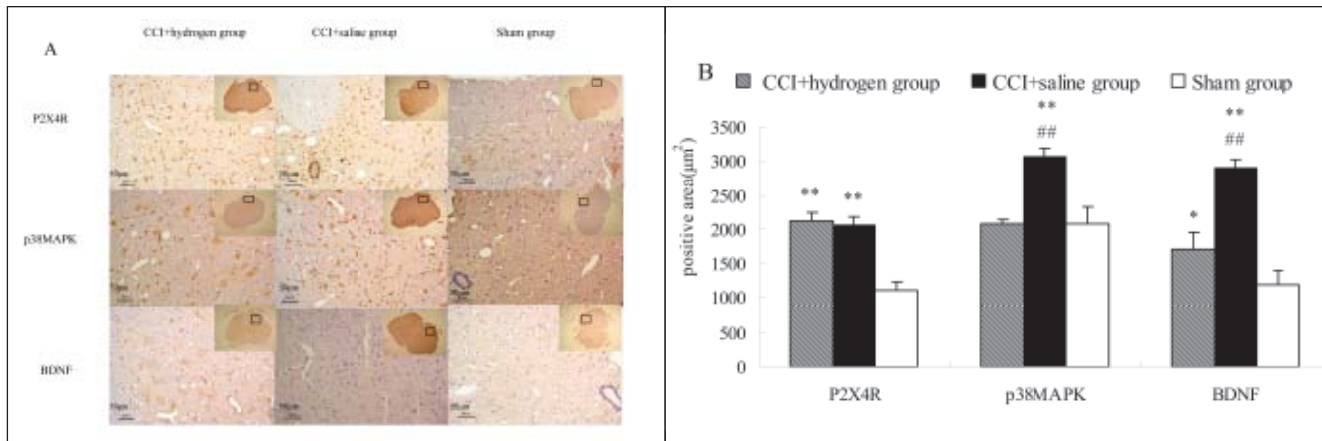
$n = 5$ ) were significantly increased in CCI+saline and CCI+hydrogen groups. Hydrogen-rich saline significantly decreased the positive areas of p38MAPK (CCI+saline vs. CCI+hydrogen:  $3062.17 \pm 126.74 \mu\text{m}^2$  vs.  $2073.91 \pm 76.06 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ) and BDNF (CCI+saline vs. CCI+hydrogen:  $2901.79 \pm 111.43 \mu\text{m}^2$  vs.  $2901.79 \pm 111.43 \mu\text{m}^2$ ,  $p < 0.01$ ,  $n = 5$ ) in the spinal cord compared with CCI+saline group, but it showed no significant effect on the expression of P2X4R.

### qRT-PCR showed hydrogen-rich saline decreased p38MAPK and BDNF mRNA levels in spinal cord

qRT-PCR results showed that the expressions of P2X4R, BDNF and p38MAPK mRNA were significantly higher in CCI+saline group than in the sham group on Day 7 after CCI surgery (Figure 4). In our study, we used b-actin cDNA as an external reference. Hydrogen-rich saline significantly decreased the expressions of BDNF and p38MAPK mRNA in the spinal cord compared with CCI+saline group ( $0.39 \pm 0.13\%$  vs.  $1.69 \pm 0.24\%$ ,  $0.72 \pm 0.28\%$  vs.  $1.37 \pm 0.16\%$ , respectively,  $p < 0.01$ ,  $n = 5$ ), but it had no significant influence on P2X4R expression ( $1.13 \pm 0.10\%$  vs.  $1.28 \pm 0.11\%$ ,  $p > 0.05$ ,  $n = 5$ ).

### DISCUSSION

In the present study we examined the effect of hydrogen-rich saline on CCI-induced neuropathic pain and discussed the possible mechanisms. We found that intrathecal injection of hydrogen-rich saline reduced CCI-induced allodynia as demonstrated by increased mechanical threshold on Day 3–7 after surgery, although the analgesic effects of hydrogen-rich saline were mild and did not completely prevent allodynia. Our study is the first to find that hydrogen-rich saline, as a radical scavenger, has attenuating effect on neuropathic pain. Reactive oxygen species includes free radicals, such as superoxide anion, nitric oxide and hydroxyl radical, and other molecular species. Superoxide anion and nitric oxide can easily be converted to the non-radical oxidant peroxynitrite, which is also highly toxic<sup>18</sup>.

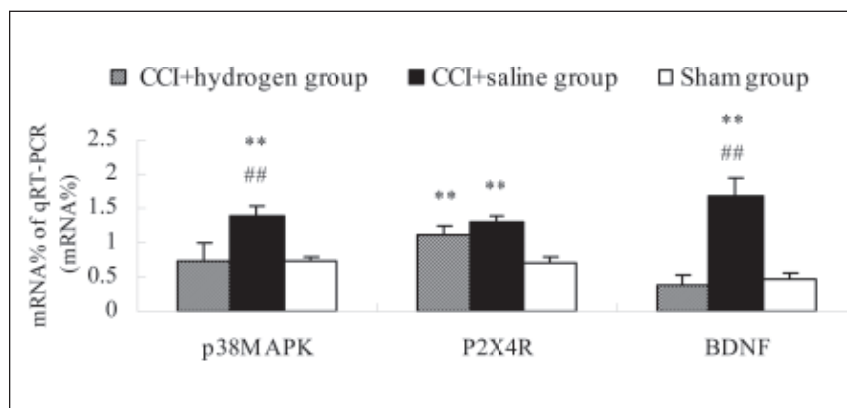


**Figure 3:** Positive staining areas of P2X4R, BDNF and p38 MAPK in spinal cord on day 7 after CCI surgery. A. Representative slides of P2X4R, BDNF and p38 MAPK in the spinal cord on day 7 after CCI surgery in three groups (Magnification= $\times 250$ ). B. The positive staining areas of P2X4R, BDNF and p38 MAPK were measured using the Image Pro-Plus image analysis system. Strong immunohistochemical reactivities for P2X4R, BDNF and p38 MAPK were found in the spinal cord after CCI surgery ( $p < 0.01$ , respectively,  $n = 5$ ). Hydrogen treatment resulted in reduced staining of BDNF and p38 MAPK compared with CCI+saline group ( $p < 0.01$ , respectively,  $n = 5$ ). (\*vs. Sham group  $p < 0.05$ , \*\*vs. Sham group  $p < 0.01$ ; ## vs. CCI+hydrogen group  $p < 0.01$ ,  $n = 5$ ). Scale bar = 50  $\mu\text{m}$ .

Hydrogen has been found to be a safe and effective antioxidant with minimal side effect<sup>19,20</sup>; it can effectively neutralize hydroxyl radical<sup>21</sup>. Furthermore, hydrogen has been reported to markedly decrease oxidative stress and protect cells and tissues in several animal models<sup>9-11,13,20</sup>. Malondialdehyde, the end product of membrane lipid peroxidation, and protein carbonyl, an indicator of protein oxidation, was used to measure the extent of oxidative stress in the present study. Myeloperoxidase is a well-known oxidative enzyme expressed by neutrophils, and it promotes generation of hypochlorous acid, which damages the nearby tissues. Myeloperoxidase activity is commonly used to evaluate the extent of neutrophil infiltration. In the present study

we found that hydrogen decreased MDA, MPO, and protein carbonyl levels, which is consistent with that reported previously<sup>20</sup>, indicating that hydrogen possess anti-oxidative activities.

Previous studies also found that systemic treatment with antioxidants reduced hyperalgesia in CCI model of neuropathic pain<sup>4,22,23</sup>. Intraperitoneal or intrathecal injection of free radical scavengers improved the mechanical thresholds 72 h after CCI surgery<sup>1,2,7,18</sup>. Our findings in this study indicate that hydrogen-rich saline may provide an alternative treatment for neuropathic pain.



**Figure 4:** Hydrogen-rich saline treatment decreased expression of p38MAPK and BDNF mRNA in spinal cord of CCI rats. ( $n = 5$ ). Quantitative Real Time PCR analyses of P2X4R, p38MAPK and BDNF mRNA levels in the spinal cord on day 7 after CCI surgery. CCI surgery significantly increased the expression of P2X4R, p38MAPK and BDNF in spinal cord on day 7 after the surgery. Hydrogen-rich saline treatment significantly decreased the expressions of p38MAPK and BDNF, but not P2X4R in the spinal cord.. \*vs. Sham group  $p < 0.05$ , \*\*vs. Sham group  $p < 0.01$ ; # vs. CCI+hydrogen group  $p < 0.05$ , ## vs. CCI+hydrogen group  $p < 0.01$ ,  $n = 5$ .

P2X4R expression increases in the microglia of the ipsilateral spinal dorsal horn after peripheral nerve injury, but not in neurons or astrocytes; increased P2X4R expression is accompanied by increase of pain hypersensitivity<sup>24,25</sup>. Pharmacological blockade of P2X4Rs acutely reverses the established pain hypersensitivity after nerve injury, and development of pain hypersensitivity can be attenuated by suppressing P2X4R expression with antisense RNA. Several studies reported that ATP receptor purinergic receptors 4 (P2X4R) and p38-mitogen-activated protein kinase (p38MAPK) were significantly increased after microglia activation as a result of peripheral nerve injury<sup>24,26</sup>. Reactive oxygen species produced after injury-induced p38MAPK and extracellular regulated protein kinases (ERK) activation in microglia, and mediated mechanical allodynia and thermal hyperalgesia<sup>27</sup>.

P2X4R also mediates synthesis and release of BDNF in the microglia in a calcium- and p38MAPK-dependent manner<sup>28</sup>. Neuronal expression of BDNF was found to be up-regulated through ERK and p38-MAPK-signaling pathways<sup>29-31</sup>. Furthermore, ATP-mediated BDNF production by activated microglia has been linked to the development of nerve injury-associated allodynia<sup>32</sup>. Activation of these pathways in the spinal microglia has been shown to contribute to the development of neuropathic pain in nerve-injured rats<sup>33,34</sup>.

The role of ROS in development of neuropathic pain remains unclear. Recent studies have indicated that ROS enhanced N-methyl-D-aspartic acid (NMDA)-receptor phosphorylation in animal models of pain, which is an essential step in central sensitization caused by peripheral nerve injury<sup>1,2,5,7</sup>. In addition, BDNF was known to induce phosphorylation of NR1 subunit (PNR1) of the NMDA receptors, which were expressed in the dorsal horn neurons in the spinal cord<sup>35</sup>.

We noticed that the expressions of p38MAPK and BDNF mRNA in the spinal cord were markedly up-regulated compared to the sham group seven days after CCI surgery, which is consistent with previous reports, indicating that hydrogen-rich saline can relieve neuropathic pain by reducing p38MAPK and BDNF expression in the spinal cord. Reduction of BDNF may inhibit phosphorylation of NMDA (PNR1) in the spinal cord and decrease the neuropathic pain mediated by BDNF-NMDA (PNR1) pathways<sup>35</sup>.

In conclusion, hydrogen-rich saline can improve the mechanical threshold of neuropathic pain in rat CCI model; this analgesic effect of hydrogen-rich saline might be associated with the reduction of oxidative stress and the subsequent decrease of p38MAPK and BDNF expression. It is therefore suggested that hydrogen-rich saline might be a potential therapy for pain control, especially when considering its convenient application and safety.

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