Hydrogen-rich saline reducing the expression of BDNF in the spinal cord in neuropathic pain

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Recent studies indicated that reactive oxygen species (ROS) were involved in persistent pain, including neuropathic and inflammatory pain. Hydrogen gas was reported to reduce reactive oxygen species and alleviate cerebral, myocardial and hepatic ischemia/reperfusion (I/R) injuries. However, it is unknown whether hydrogen-rich saline was effective on neuropathic pain. In the present study, we used the chronic constriction injury (CCI)-induced neuropathic pain model of rats to investigate the role of successive administration with hydrogen-rich saline in the generation or development of neuropathic pain. Hydrogen-rich saline was administered by intrathecal injection in the dose of 100ul/kg once a day before and 1-7 day after CCI surgery. Results of the present study showed that mechanical thresholds of hydrogen-rich saline treatment was significantly higher compared to physiologic saline treatment at 4th and 5th day after CCI-surgery. We also examined the myeloperoxidase (MPO), maleic dialdehyde (MDA) and protein carbonyl, and the results indicated that hydrogen-rich saline could effectively eliminate radical at 14th day after CCI surgery in spinal cord. With immunohistochemical staining of SABC method, we demonstrated that the expression of p38MAPK and brain-derived neurotrophic factor (BDNF) but not P2X4R, was（were） significantly decreased by administration with hydrogen-rich saline in

spinal cord. This effect was verified by RT-PCR at a transcriptional level, as indicated by the decreased expression of p38MAPK and BDNF- mRNA with the treatment of hydrogen-rich saline in spinal cord. In conclusion, our experiment suggested that intrathecal injection of hydrogen-rich saline could significantly decrease the expression of p38MAPK and BDNF. Hydrogen-rich saline might delay the development of pain behaviors by reducing the expression of BDNF in neuropathic pain.

KEY WORDS: hydrogen-rich saline, brain-derived neurotrophic factor, p38MAPK, reactive oxygen species, chronic constriction injury.

Recent studies suggested that reactive oxygen species (ROS) were critically involved in the generation of pain in various painful conditions, including neuropathic and inflammatory pain. An increase in ROS production has been proposed to contribute to many neurodegenerative diseases, such as Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis [1-4] Emerging evidence indicates that ROS is also involved in the chronic pain, including neuropathic and inflammatory pain [5-9]. For example, ROS production increased in the spinal cord of rats after peripheral nerve injury[10]. Treatment with the ROS scavengers phenyl Ntert-butylnitrone (PBN), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), 5,5-dimethylpyrroline-N- oxide (DMPO) or vitamin E[7, 11] temporarily could significantly reverse the allodynia that developed after nerve ligation in rats. Since PBN was also very effective in producing analgesia by intrathecal injection, it was concluded that PBN-induced analgesia was mediated mainly through spinal mechanisms[7]. The above evidence suggested that oxidative stress might be an important factor for chronic pain, and ROS scavengers could reverse the allodynia significantly by short delivery time.

The potential for hydrogen gas (H2) to scavenge free radicals has been proposed by several studies. Recently, it has been suggested that hydrogen (H2), a potent free radical scavenger, selectively reduced the hydroxyl radical, the most cytotoxic of reactive oxygen species. Ohsawa et al.[12] demonstrated that hydrogen gas (1–4% concentration) ameliorated ischemic injury in the setting of experimentalc focal ischemia in adult rats by selectively reducing cytotoxic reactive oxygen species,such as OH. Furthermore, other studies also found hydrogen could reduce oxidative stress in experimental intestinal ischemia[13, 14], myocardial ischemia[15] and brain ischemia[16].

However, little is known of the role of hydrogen-rich saline treatment in neuropathic pain. In the present study, we investigate the analgesic effects of successive administration with hydrogen-rich saline in the chronic constriction injury(CCI) -induced neuropathic pain model in the rat and the possible underlying cellular mechanisms.

All the protocols were approved by the Second Military Medical University, China, in accordance with the Guide for Care and Use of Laboratory Animals published by the USNIH (publication No. 96–01).Adult male Sprague-Dawley rats weighing 200-250 g were used All animals were housed under a 12/12h reversed light-dark cycle (dark cycle: 8:00A.M.-8:00P.M.) for at least one week before beginning any experiments.

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4MPa) to a supersaturated level using hydrogen- rich water-producing apparatus produced by our department. The saturated hydrogen saline was stored under atmospheric pressure at 4℃ in an aluminum bag

with no dead volume. Hydrogen-rich saline was sterilized by gamma radiation. Hydrogen-rich saline was freshly prepared every week, which ensured that a concentration of more than 0.6 mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al[12].

For intrathecal injection of drugs, before made CCI model, a catheter should be implanted into spinal. The rat was anesthetized with 10% chloral hydrate (3 ml /kg) peritoneal injection i.p, the paraspinal muscles were retracted, and the posterior part of the 6 th lumbar vertebrae were removed to expose the spinal meninges. A catheter(sterilized PE 10 tubing fill with Sodium Chloride) was inserted into the subarachnoid space through a small nick in dura and the tip was placed near the lumbar enlargement of the spinal cord. The remaining part of the catheter was fed subcutaneously to atlan-pulvinar level and anchored to muscles by sutures and the tip was exposed and sealed. The day after surgery, a total of 20 ul of 2% lidocaine was injected through the catheter, if the catheter was put the exactitude place, the rat would appearance subarachnoid space block anesthesia symptom 30s after injection described as extremities paralysis and powerless. These kits were selected to undergo neuropathic surgery.

Selected rats were kept off feed for 12 h prior to surgery. The rats were anaesthetized with 10% chloral hydrate (3 ml /kg) i.p. After induction of anaesthesia, the hair around the mid-thigh were clipped and then shaved. CCI was induced as described by Bennett and Xie [17]. The common sciatic nerve of the right hind limb was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. Proximal to the sciatics trifurcation, about 7 mm of the nerve was freed of the adhering tissue and four ligatures (4.0 silk) were tied loosely around it with about 1 mm spacing, with the length of the affected nerve being 4-5 mm long. The desired degree of constriction was such that it could retard, but not arrest circulation through the superficial epineural vasculature. The incision was closed in layers. For estimation of free radical and antioxidant enzyme activities, separate animals without ligating the sciatic nerve were used as sham-exposed controls.

After suturing the skin, povidone iodine solution was applied externally by cotton swab and prophylactically penicillinum (Penicillin, Harbin Pharmaceutical Group Co., Ltd, China) was injected by intraperitoneally at a dose of 50 mg/kg body weight for three consecutive days to prevent any infection. The operated animals were caged individually, food and water were given arbitrarily.

Animals were randomly divided into three groups consisting of 12 rats each randomly before CCI surgery: (1) sham-operated group with physiologic saline treatment (Shame group); (2)CCI group which underwent CCI surgery and treatment with physiologic saline (CCI+physiologic group);(3) hydrogen-rich saline treatment group (CCI+hydrogen group). Hydrogen-rich saline was administered through the catheter in the dose of 100ul/kg once a day before and 1-7 day after CCI surgery, and physiologic saline was injected with the same dosage at the same time. Mechanical thresholds were measured before and 4-14 day after neuropathic surgery in all groups. All animals were sacrificed at 14 days after surgery.

Mechanical thresholds were assessed at 1 hour and 3-15 days after CCI surgery. For each test, the animal was placed in a plastic chamber (10.0 × 10.0 × 20.0 cm) on top of a mesh screen platform and habituated for at least 15 minutes. Thresholds were determined by the up-down method [18, 19] 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[19]. An abrupt withdrawal of the foot during stimulation or immediately after stimulus removal was considered to be a positive response. The first stimulus was always 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 applied. This testing pattern was 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 0.6g or 15g were assigned, respectively.

At 14 days after surgery, half of all groups were sacrificed. At the time of sacrifice, rats were anesthetized with 10% chloral hydrate (3ml/kg, i.p.) and perfused through a cannula inserted in the ascending aorta with 100 ml isotonic ice-cold saline followed by 250 ml fixation fluid (4℃) during 6 min. The fixative consisted of 4% paraformaldehyde (w/v) in 0.1M phosphate buffer (PH 7.4). A 10 mm of the lumbar enlargement of the spinal cord.was removed and post-fixed in the perfusion fixative overnight, and then cryoprotected for 2 days in 30% sucrose in 0.1 M PB. Frozen sections were cut at 16 μm on a cryostat and mounted on gelatinized slides. We stained sections with hematoxylin and eosin (H&E) for histopathologic changes. Immunostaining changes of P2X4R, P38MAPK and BDNF was examined in all groups.

After paraffin section to water, 3% H_20_2 were incubated 15min at room temperature to inactivate endogenous enzymes, rinse in distilled water for 3x5 min. The slice immersed in 0.01M citrate buffer arrested (PH 6.0), microwave power heated to boiling, after an interval of 5 minutes, repeat 2 times, rinse in PBS (P H7.2-7.6) for 3x5 min after naturally cooled; dropping the Normal Goat Serum Blocking solution, 37℃ 20 minutes, tilting to go, do not wash; dropping a 1:100 dilution of Primary Antibody, 40℃ refrigerator overnight, rewarming at room temperature for 30min, PBS solution washing three times, each time 5min; incubate sections in Biotinylated goat anti-mouse IgG for 20 minutes at 37℃, Rinse in PBS for 3x5 min; dropping reagents SABC, 37℃ 20 minutes, Rinse in PBS for 3x5 min; DAB color, time 5 minutes, tap water rinse; ematoxylin-stained nuclei l min, 0.1% hydrochloric acid differentiation, lithium carbonate back to blue, Rinse in running tap water for 30min; gradient alcohol dehydration, xylene transparent, 60℃ constant temperature oven-roasted piece 30min. Cell counts were obtained in the spinal cord (lumbar enlargement) in 10 randomly selected sections from each animal by an experimenter who did not know the origin of the tissue. Cells were analyzed using the Image Pro-Plus image analysis system.

Since myeloperoxidase (MPO) was an abundant enzyme involved in the production of free radicals, the quantity of MPO could reflect the metabolizing of free radicals. For this purpose, the spinal cord was homogenized in 10 volume of ice-cold 20 mmol/L pltassium phosphate buffer (PH 7.4) containing 30 mmol/L KCL. The homogenate was centrifuged at 12000 rpm for 10 min at 4℃. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L acetic acid containing 5% hexadecyltrimethylammonium bromide (HETAB). MPO activity was assessed by measuring the H_2O_2 dependent oxidation of 0-dianizidine 2 HCl. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance of 1.0/min at 460 nm and 37℃.

Lipid peroxidation caused by ROS was measured with the thiobarbituric acid (TBA) reaction. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with MDA at 532nm. The method defined by Ohkawa et al.was used[20].

Protein carbonyl as an indicator protein oxidation was also measured. Briefly, the spinal cord homogenate was mixed with steptomycin followed by centrifugation and then it was incubated with DNPH for 1h followed by precipitation with TCA. The pellet was washed with ethanol-ethyl acetate to remove excess DNPH and resupended in guanidine hydrochloride. The solution was centrifuged and incubated at 37℃ in a water bath for 10min. Finally, the absorbance was measured at 366 nm wavelength and the results were expressed as nmol/mg protein.

For the remaining animals, after deep anesthesia, a 10 mm section of the spinal cord section at the same lever was dissected. The samples were cleaned thoroughly of blood, and the meninges were removed carefully. The samples were then immediately frozen and stored at -80℃.

Levels of P2X4R, BDNF and P38MAPK were determined by RT-PCR following the manufacture's instruction. Spinal cord tissue was homogenized in 1 ml Trizol solution(GIBCO BRC, USA) and centrifuged at 15,000×g for 15min at 4℃. The supernatant was decanted and used for RT-PCR. We also used GAPDH (forward: 5'-TCCCTCAAGATTGTCAGCAA-3'; reverse: 5'-AGATCCACAACGGATACATT-3') as a external reference control, densities were calculate, the data were expressed by the ratio of density of P2X4R, BDNF and P38MAPK to the density of GAPDH.

Data are presented as mean plus or minus standard error of the mean (SEM) and were analyzed using the statistical program SPSS 11.2. Statistical analyses were performed using one-way and/or two-way analysis of variance (ANOVA) with one repeated factor (time), followed by Holm–Sidak post hoc test.

To test mechanical responses, 12 rats were used for each group. The paw withdrawal threshold were tested to examine the time course of changes in mechanical threshold in hydrogen-rich saline treated rats. Threshold values obtained were analyzed with a two-way ANOVA with repeated measures over time followed by Holm–Sidak post hoc test.

The average mechanical threshold of the rat hind paw was 12.17 g, in normal rats (Fig.1). At 4 days after the CCI surgery, the mechanical thresholds of both CCI+physiologic group and CCI+hydrogen group decreased dramatically and continued to the end of the study. However, mechanical thresholds of hydrogen-rich saline treatment were significantly higher compared to physiologic saline treatment in 4th day to 6th day after CCI-surgery.

MPO, MDA and protein carbonyl was commonly used to measure the reactive oxygen species. The total amount of MPO, MDA and protein carbonyl in the spinal cord was measured 14 days after CCI surgery. Fig 2 illustrated that MPO, MDA and protein carbonyl was significantly increased in CCI+physiologic group compared with the sham-operated group (7.18 ± 0.95 vs 3.22 ± 0.83; 8.67 ± 1.34 vs 6.02 ± 1.65; 30.67 ± 5.69 vs 13.50 ± 2.96, $p < 0.01$), while hydrogen-rich saline treatment could efficiently decrease MPO, MDA and protein carbonyl in the spinal cord compared with CCI+physiologic group.

The results of inmmuohistochemical SABC staining (Fig. 3) indicated that in the CCI+physiologic group, the expression of P2X4R, p38MAPK and BDNF from microglia in the spinal cord were increased at 15th day after CCI surgery, administration with hydrogen-rich saline in CCI+hydrogen group could obviously decrease the expression of p38MAPK and BDNF in the spinal cord, yet there was no difference of the expression of P2X4R between CCI+physiologic group and CCI+hydrogen group.

Similar phenomena were observed in the results of RT-PCR. At 14 days after CCI surgery, the expression of P2X4R, BDNF and p38MAPK in the spinal cord in CCI+physiologic group was

strongly upregulated of the ipsilateral to the lesion compared to Shame group. However, administration with hydrogen-rich saline significantly decreased the expression of BDNF and p38MAPK in the spinal cord (0.35 ± 0.15 vs 1.59 ± 0.51, $p<0.01$;0.82 ± 0.10 vs 1.19 ± 0.18, $p<0.01$). However, the expression of P2X4R was no difference between CCI+hydrogen group and CCI+physiologic group (1.08 ± 0.14 vs 0.98 ± 0.19, $P>0.05$).

In the present study, the effect of hydrogen-rich saline on CCI-induced neuropathic pain behavior and the possible underlying mechanisms were examined. This is the first study demonstrating successive administration of hydrogen-rich saline reduced CCI-induced allodynia by intrathecal injection, as revealed by the increased mechanical threshold at 4 and 5 day, but the analgesic effects of hydrogen-rich saline are minor, indicating a limited role of hydrogen-rich saline in the development and generation of neuropathic pain.

ROS includes both 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 nonradical oxidant peroxynitrite, which is also highly toxic. Hydrogen has been found to be an safe and effective antioxidant with minimal side effect by several laboratories. It could effectively neutralizes OH and other strong oxidant species invitro. Furthermore, H2 was reported to markedly decrease oxidative stress and protected cells and tissues in several animal models. In the present study, we found that MDA , MPO and protein carbonyl levels in the CCI+hydrogen group were lower than the CCI+physiologic group and the difference was statistically significant.

Previous studies has reported that in the chronic constriction injury model of neuropathic pain, systemic treatment with an antioxidant [5, 21, 22] reduced hyperalgesia. Injected with free radical scavenger could effectly improve mechanical thresholds 72h post nerve surgery either intraperitoneal injection or intrathecal injection [7, 9, 11, 23]. Recent studies indicated that ROS were involved in enhancement of NMDA-receptor phosphorylation in animal models of pain, which was an essential step in central sensitization, and played an important role of inducing center sensitization due to peripheral nerve injury[7-9, 11]. BDNF was known to induce phosphorylation of the NR1 subunit (PNR1) of the NMDA receptors expressed in dorsal horn neurons [24]. Our data indicates that at 14 days after CCI surgery, expression of BDNF in the spinal cord of CCI+physiologic group was strongly upregulated of the ipsilateral to the lesion compared to Shame group, while hydrogen-rich saline treatment significantly decreased BDNF expression in the spinal cord.

ATP-mediated BDNF release from activated microglia has been linked to the development of allodynia associated with nerve injury[25]Several studies also reported P2X4R and p38MAPK significantly increased after the activated of microglia as a result of peripheral nerve injury[26, 27]. It has reported P2X4R mediated synthesis and release of BDNF in microglia was dependent on calcium and p38MAPK activation[28]. Previous indicated that superoxide anion and nitric oxide initiate the p38 signal pathway leading to activation of both mitochondrial and extramitochondrial apoptotic pathways in culture models of Parkinson's disease[29].Our date showed the expression p38MAPK in ipsilateral to the lesion was higher in CCI+physiologic group compared to CCI+hydrogen group, and the difference was statistically significant. Nevertheless, there were no difference between CCI+physiologic group and CCI+hydrogen group of the expression of P2X4R.(模型与正常组有区别吗？)

In conclusion, hydrogen- rich saline may delay the development of pain behaviors by reducing the expression of BDNF in neuropathic pain. And the most possible potential mechanisms was

that hydrogen-rich saline might reduce the release of BDNF from activated microglia which could induce phosphorylation of PNR1 expressed in the spinal cord and contribute to neuropathic pain. We though the primary target point of hydrogen-rich saline was p38MAPK. However, further studies were needed to reveal the exact mechanisms by how hydrogen- rich saline reduce the expression of BDNF in spinal cord in animal models.

Acknowledgement: This study was supported by both grant from the Shanghai Natural Science Foundation of China(Grant 08ZR14050000 to Dr.Hong-Bin Yuan) and the National Natural Science Foundation of China (Grant 30971199 to Dr Xue-Jun Sun).

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