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Original Article

Effect of hydrogen-rich water on the angiogenesis in lesion boundary brain tissue of traumatic brain injury-challenged rats

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Abstract: Traumatic brain injury (TBI) is a major cause of mortality and morbidity worldwide and leads serious long-term disability. To determine the effect of hydrogen-rich water on the angiogenesis in the lesion boundary brain tissue of TBI-challenged rats, 54 adult male Sprague-Dawley (SD) rats were used and randomly divided into three groups: sham-operated, TBI (Traumatic brain injury) and TBI+HW (Traumatic brain injury + hydrogen-rich water). After inducing TBI, neurological severity scores (NSS), hematoxylin-eosin staining, immunohistochemical analysis, western blot analysis and reverse transcription polymerase chain reaction (RT-PCR) were performed. As a result, at 3rd d and 7th d after injury, NSS of TBI+HW rats significantly decreased ($P<0.05$); at 24 h and 3rd d after injury, pathological changes in lesion boundary brain tissue of TBI rats were characterized by obvious hemorrhagic necrosis, severe brain edema, loose neural substrates, and the pathological changes were more obvious at 3rd d. In comparison with the TBI group, edema volume was lower in TBI+HW rats. At 7th d after injury, newborn blood capillary hyperplasia in TBI+HW was significantly higher than that in TBI ($P<0.01$). At each time-point, the expression of HIF-1α and VEGF protein and mRNA in TBI+HW significantly increased than that in TBI ($P<0.01$/$P<0.05$) and both significantly higher than that in the sham group ($P<0.01$). Hydrogen-rich water promotes angiogenesis and improves nerve function via up-regulating the expression of HIF-1α and VEGF, which may offer a promising opportunity to improve clinical effects during brain functional recovery.

Keywords: Hydrogen-rich water, traumatic brain injury, angiogenesis, neurogenesis, HIF-1α, VEGF

Introduction

Traumatic brain injury (TBI) is a major cause of mortality and morbidity worldwide, and is the leading cause of serious long-term disability [1, 2]. Although extensive improvements in medical interventions have been carried out, unfortunately, almost all neuroprotective clinical trials for TBI have shown no benefits and there are still no clinical neuroprotective agents available for patients with TBI [3, 4]. Therefore, there is an urgent need to develop treatments to promote the repair and regeneration of injured brain tissue and improve the recovery of brain function. TBI-challenged rats showed distinct brain injuries characterized by the enhancement of blood-brain barrier (BBB) permeability, the increase of lesion volume and brain edema as well as the behavioral symptoms (neurological dysfunction) [5]. Recent findings suggest that to enhance angiogenesis and neurogenesis after brain trauma may be a promising opportunity to improve clinical effects during brain functional recovery [6, 7]. Brain injury causes necrosis and apoptosis of nerve cells, meantime, induces nervous tissue repair via angiogenesis (the growth of new blood vessels) and neurogenesis (the generation of new neurons) [3, 8]. After brain injury, brain tissue can generate a variety of promoting angiogenesis factors, contributing to form new blood vessels in the lesion boundary zone. The new blood vessels can restore the blood supply of brain tissue after trauma, reduce ischemia and hypoxia for the traumatic brain tissue, at the same time provide nutrients, so as to protect surviving nerve tissue around trauma, and promote the regeneration of the nerve cells [9]. Some papers

have proved that endogenous neural stem cells (NSCs) located in the central nervous system (CNS) of adult mammals (including humans) have the ability of self-renewal, active proliferation, migration to certain parts and differentiate into neurons, astrocytes and oligodendrocytes [10]. Moreover, after mobilization or transplantation of EPCs (endothelial progenitor cells) into ischemic tissues, EPCs proliferate and differentiate into endothelial cells to reduce blood vessel disorders by improving vascular intima restoration and new capillary network establishment as well as improvement of blood supply for ischemic tissue [11]. The underlying mechanisms of this improved functional recovery by angiogenesis not only include increased oxygen and nutrient supply to the injured tissue, but also involve neurogenesis and synaptogenesis [3, 9].

Hydrogen is a strong reducing gas with antioxidant effect, which can reduce cerebral ischemia-reperfusion injury [12]. Besides, some papers have reported that hydrogen can be anti-inflammatory, anti-apoptosis, and protect against TBI [13]. Moreover, materials related hydrogen including hydrogen sulfide and hydrogen-rich saline have been proved to improve brain function recovery after TBI [14, 15].

Hydrogen-rich water used in this study is physiological saline saturated with hydrogen, after detecting indicators related to the promotion of angiogenesis through TBI rat model, the influence of hydrogen-rich water on angiogenesis in lesion boundary brain tissue was explored.

Materials and methods

Animals

A total of 54 adult male SD (Sprague-Dawley) rats (provided by Laboratory Animal Center of the Third Military Medical University), weighing from 250 to 300 g were randomly divided into three groups: sham-operated, TBI (Traumatic brain injury), and TBI+HW (Traumatic brain injury + hydrogen-rich water). All animals were raised under the same conditions with the temperature of 20-24°C and supplied with rodent animal feed under national standards. All animal protocols were approved by the Institutional Animal Care and Use Committee at Soochow University.

Traumatic brain injury model and sample collection

The modified Feency's method was used to induce TBI. All rats were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg), and fixed on the brain stereotaxic system with a prone position (Shenzhen RWD Life Science Co., Ltd.). Then surgery was performed under aseptic conditions. After exposing the skull by a midline incision on the scalped, a hole with a diameter of 5.0 mm × 5.0 mm was drilled on the right parietal bone. Then rats were subjected to TBI in the right hemisphere of the brain using a weight-drop device, which is a 40 g-weight was falling free from 40 cm height onto a 4 mm diameter firing pin resting on the dura. For the sham group, only craniotomy was performed on rats without hemisphere impact. Rats in TBI+HW group were given hydrogen-rich water (5 ml/kg) by intraperitoneal (i.p.) injection after TBI and offered one time every day in the next days, and rats in the sham group and TBI group were given a same amount of saline at the same time.

At 24 hour, 3rd day and 7th day after Traumatic brain injury, 6 rats were selected randomly from each group to carry out Neurological Severity Scores (NSS), then all were beheaded after deep anesthesia and lesion boundary brain tissue of injured hemisphere (extending 3 mm to the incision) was collected and saved at -80°C for immunohistochemical analysis, Western blotting, RT-PCR, respectively.

Neurological severity scores (NSS)

At 24 h, 3rd day and 7th day after TBI, neurobehavioral test was performed with an 18-point system [16] to evaluate animals' neurologic deficits. The test carried out from four aspects, including movement (stretch of head and all four limbs, crawling on the ground), feeling (pain sense, warm sense, and body proprioception), balance and reflection (auricle reflection, corneal reflection, startle reflex, epilepsy, myoclonus, dystonia). As a result, the more scores, the more deficits.

Tissue preparation

Part of the collected brain tissue was fixed in 10% paraformaldehyde, embedded in paraffin and cut into five-micrometer-thick coronal sec-

Table 1. NSS of different group rats in different time-points after injury

Group	Animal number	NSS (scores)		
		24 h	3rd d	7th d
TBI	6	14.00±1.41	11.56±1.94	8.17±0.98
TBI+HW	6	12.33±0.82	8.67±0.52	7.33±0.52
t value		2.5	3.518	1.838
p value		0.418	0.038	0.04

tions. Then slices were used for hematoxylin and eosin (HE) staining and immunohistochemical analysis.

Hematoxylin-eosin staining and immunohistochemical analysis

Some paraffin sections were stained with hematoxylin and eosin to determine pathological changes of the brain tissue. Some were dewaxed and incubated with primary antibody (CD34 monoclonal antibody, Abcam, US) at 4°C overnight, then probed with corresponding second antibody (IgG-HRP, Abcam, US) for 25 min at 37°C. Subsequently, the mixture was washed three times and immunostain with DAB color kit (ZSGB-BIO, China). At last, CD34 cells and newborn capillaries were observed.

Western blot analysis

The saved brain tissue was cut into pieces and lysed after freezing and thawing, then protein was extracted and the concentration was determined by BCA assay. Then the protein (40 pg) from different samples was separated on a 12.5% sodium dodecyl sulfate-polyacrylaminde gels (SDS-PAGE), respectively. Proteins were then electrophoresed and transferred to a polyvinylidene fluoride (PVDF) membrane.

After incubation with primary antibody (mouse monoclonal antibody for HIF-1α, 1:5000, Cell Signaling Technology, US; rabbit monoclonal antibody for VEGF, 1:5000, Abcam, US) at 4°C overnight, the membrane was probed with corresponding second antibody (goat anti-mouse IgG (H+L) for HIF-1α, LI-COR, US.; donkey anti-rabbit IgG (H+L) for VEGF, LI-COR, US) for 2 hrs at room temperature, then washed with TPBS. At last, immunoblot presenting the expression of HIF-1α and VEGF was visualized by the Odyssey two-color infrared fluorescence imaging system (LI-COR, US) with the internal control of GAPDH.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the saved brain samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. After DNase digestion, RNA quantification and purity were assessed at 260/280 nm. RNA integrity and purity were measured with denaturing agarose gel electrophoresis. Then a reverse transcriptase reaction kit (ABI Biosystems, Foster City CA, USA) was used to reverse-transcribe RNA samples. The primers were designed as follows: HIF-1α, forward primer, 5'-AGCAATTCTCCAAGCCCTCC-3', reverse primer, 5'-TTCATCAGTGGTGGCAGTTG-3', VEGF, forward primer, 5'-TCATCAGCCAGGGAGTCTGT-3', reverse primer, 5'-GAGCAACCTCTCCAAACCGT-3', GAPDH, forward primer, 5'-GTTACCAGGGCTGCCTTCTC-3', reverse primer, 5'-GATGGTGATGGGTTTCCCGT-3'.

The PCRs were performed in triplicate using 20 μL reaction system, amplification conditions were 1 cycle of 95°C for 10 min (initial denaturation), followed by 42 cycles of 95°C for 15 s (denaturing), 60°C for 60 s (annealing) and 72°C for 30 s (extension), and finally 1 cycle of 72°C for 5 min (final extension). The relative expression value was calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software package (SPSS Inc, Chicago, IL, USA). Data was expressed as mean ± standard deviation ($\bar{x}\pm s$) from three independent experiment groups. Statistical differences between the groups were compared using Student's t-test and LSD-test or Tamhane-test. A P value of less than 0.05 was considered statistically significant.

Results

Neurological severity scores

For sham group rats, the NSS were 0 in all time-points. After injury, rats in TBI group and TBI+HW group showed a significant neurological damage, and the damage in TBI group rats was more serious than that in TBI+HW group rats. Compared with TBI animals, hydrogen-treated rats presented with reduced neurobehavioral score at the 3rd day and the 7th

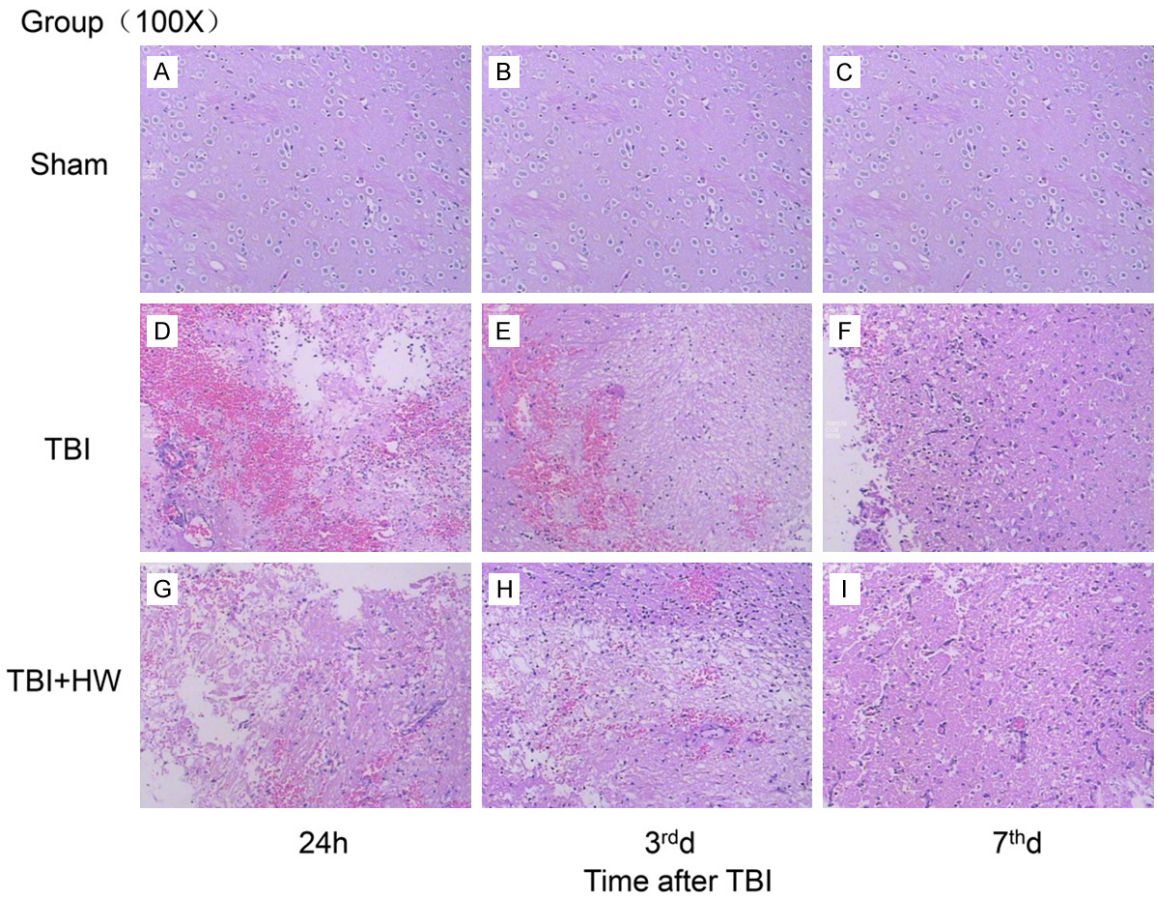

Figure 1. Pathological changes in lesion boundary brain tissue of Sham, TBI and TBI+HW rats at different time-points. A, D, G. Pathological changes of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 24 h after TBI, respectively. B, E, H. Pathological changes of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 3rd d after TBI, respectively. C, F, I. Pathological changes of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 7th d after TBI, respectively.

day ($P<0.05$), but not at 24 h ($P>0.05$) (**Table 1**).

Pathological changes

After TBI, many pathological changes were presented in rat brain of TBI and TBI+HW groups (**Figure 1**). At 24 h and 3rd d, we can see obvious hemorrhagic necrosis, severe brain edema, wider gap among blood vessels, increased intercellular space, loose neural substrates, a large number of nerve cells degeneration and necrosis, obvious inflammatory cell infiltration, the pathological changes were more obvious at 3rd d. Compared with the sham group, the necrotic area and the edema zone were smaller at the same time-point. At 7th d, compared with the sham group, slighter brain edema and a lot of newborn blood capillary hyperplasia were shown in TBI and TBI+HW. Organizational structure was evident in the sham group with no brain swelling and inflammatory cell infiltration.

Immunohistochemical analysis

CD34 cells were observed in vascular endothelium staining yellow brown (**Figure 2**). Rat brain tissue around the trauma which was with most capillary hyperplasia was taken into the visual field of microscope with 400 times magnification. Five visual fields were selected randomly to count hyperplasia capillary. The results showed that there was little hyperplasia capillaries in the sham group, and in both TBI group and TBI+HW group at 24 h after TBI ($P>0.05$); Amount of hyperplasia capillaries was increased at 3rd d and further increased at 7th d. Compared with TBI animals, hydrogen-treated rats presented with improved hyperplasia capillaries at 3rd day ($P<0.05$) and 7th day ($P<0.01$), but not at 24 hrs ($P>0.05$) (**Table 2**; **Figure 2**).

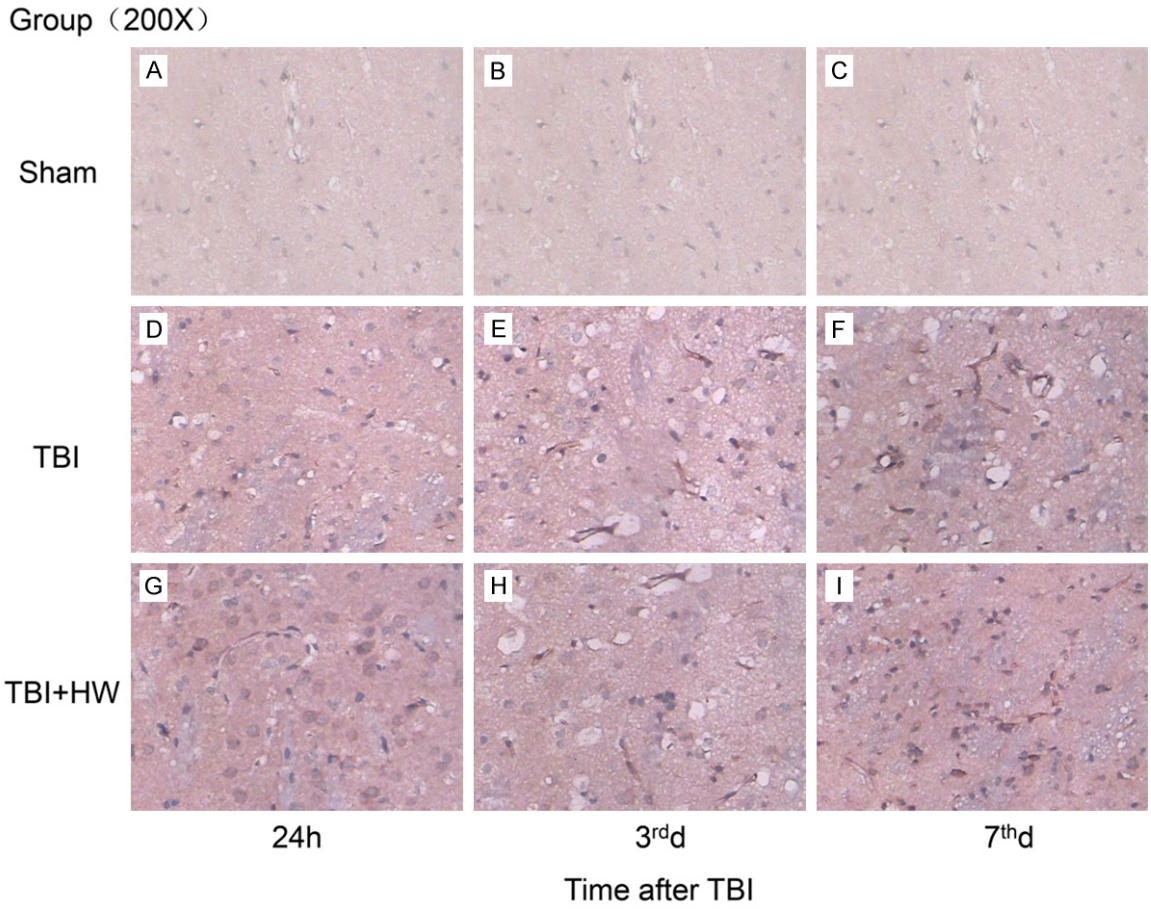

Figure 2. CD34 expression and newborn blood capillary hyperplasia in lesion boundary brain tissue of Sham, TBI and TBI+HW rats at different time-points. A, D, G. CD34 expression and newborn blood capillary hyperplasia of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 24 h after TBI, respectively. B, E, H. CD34 expression and newborn blood capillary hyperplasia of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 3rd d after TBI, respectively. C, F, I. CD34 expression and newborn blood capillary hyperplasia of lesion boundary brain tissue of Sham, TBI and TBI+HW rats at 7th d after TBI, respectively.

Table 2. Capillary number in rat brain tissue around trauma of different groups at three time-points

Group	Animal	Newborn capillaries/ microscope (400 ×)		
		24 h	3rd d	7th d
Sham	6	0.6±0.11	0.7±0.1	0.7±0.13
TBI	6	3.7±0.47[a]	8.6±1.2[a]	17.0±2.6[a]
TBI+HW	6	4.0±0.38[a]	10.6±1.9[a,b]	23.2±3.16[a,c]

Compare to sham, [a]P<0.01; Compare to TBI, [b]P<0.05, [c]P<0.01.

Western blotting

The results showed that after 24 h after injury, the expression of HIF-1α and VEGF in TBI and TBI+HW gradually increased, and stay at a high level at 7th d. Compared to sham group, the expression of HIF-1α and VEGF in TBI and TBI+HW significantly increased (P<0.01); compared to TBI group, the expression of HIF-1α and VEGF in TBI+HW also significantly increased (P<0.01/P<0.05) (**Figure 3**).

Reverse transcription polymerase chain reaction (RT-PCR)

The results showed that after 24 h after injury, the expression of HIF-1α mRNA and VEGF mRNA in TBI and TBI+HW gradually increased, and stay at a high level at 7th d. Compared to sham group, the expression of HIF-1α mRNA and VEGF mRNA in TBI and TBI+HW significantly increased (P<0.01). Compared with the TBI group, the expression of HIF-1α mRNA and VEGF mRNA in TBI+HW also increased (P<0.01/P<0.05) (**Figure 4**).

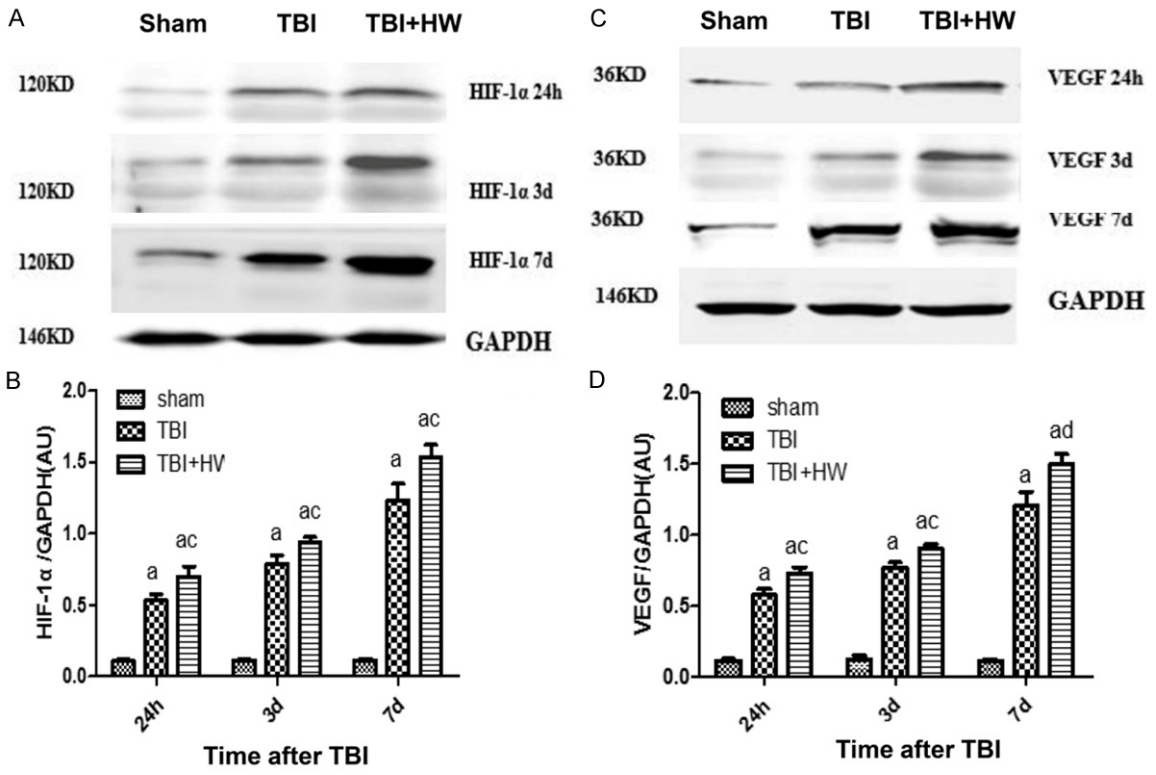

Figure 3. Expression of HIF-1α and VEGF protein in lesion boundary brain tissue of Sham, TBI and TBI+HW rats at different time-points. A, B. Expression of HIF-1α protein. C, D. Expression of VEGF protein (compared to Sham, aP<0.01, bP<0.05; compared to TBI, cP<0.05, dP<0.01).

Discussion

TBI-challenged rats showed distinct brain injuries characterized by the enhancement of blood-brain barrier (BBB) permeability, the increase of lesion volume and brain edema as well as behavioral symptoms (neurological dysfunction) [5]. Furthermore, a variety of cellular and molecular responses took place in brain after ischemic stroke and TBI, including excitotoxicity, reactive free radical generation, inflammation, ischemia/reperfusion injury, and result in neuronal death [9]. Secondary ischemia and hypoxia of brain tissue after traumatic brain injury are the major factors leading brain damage, the damage of nerve cells in the center of brain trauma is irreversible, while the damage of lesion boundary brain tissue is reversible, given these, reducing the secondary brain injury is the key of craniocerebral trauma treatment. The generation of new-born blood vessels in the lesion boundary brain tissue plays an important role in the repair of damaged nerves [9]. The central nervous system (CNS) vascular system in adults is extremely stable under physiological conditions, however, it can be activated both structurally and functionally in response to acute pathological conditions including ischemic stroke and traumatic brain injury (TBI) [17]. Numerous studies [3, 18, 19] have demonstrated that angiogenesis actively occurs after ischemic stroke and TBI and contributes to oxygen and nutrients supply for damaged brain tissue, as well as the formation of new neurons and synapse, which is an important protection mechanism of craniocerebral injury.

Hypoxia-inducible factor 1 (HIF-1) plays an essential role in angiogenesis and growth by regulating the transcription of several genes which promotes angiogenesis and growth in response to hypoxia stress and changes of growth factors, and VEGF is the strongest promoting vascular growth factor and the downstream goal gene of HIF-1α [20]. Now more than 20 kinds of proangiogenic factors have been separated and purified, such as hypoxia-inducible factor, vascular endothelial growth factor (VEGF), insulin-like growth factor, trans-

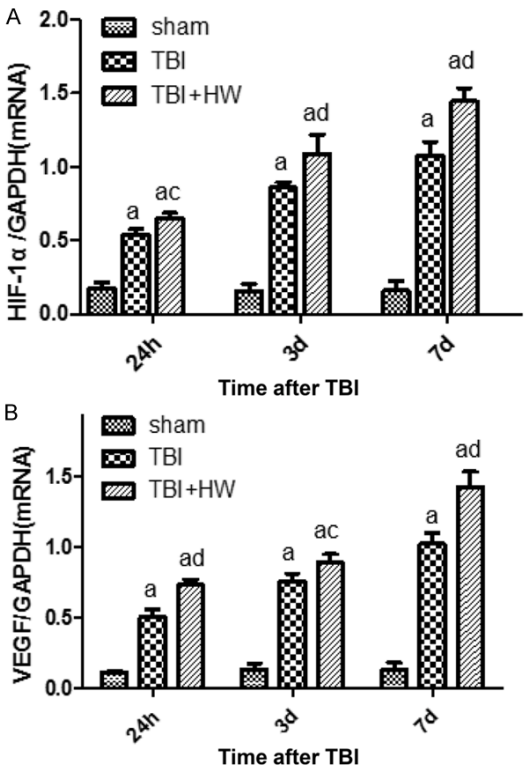

Figure 4. Expression of HIF-1α and VEGF mRNA in lesion boundary brain tissue of Sham, TBI and TBI+HW rats at different time-points. A. Expression of HIF-1α mRNA. B. Expression of VEGF mRNA (compared to Sham, aP<0.01, bP<0.05; compared to TBI, cP<0.05, dP<0.01).

forming growth factor and angiogenesis factor, etc. HIF-1α is not only the key regulation factor of angiogenesis, but also the key upstream transcription factor of blood vessel growth signals. Lu et al [21] found that neurogenesis induced by TBI is related to HIF-1α and VEGF: HIF-1α was proved to directly act on the VEGF promoter and the up-regulation of HIF-1α finally activates the expression of VEGF, then induces angiogenesis in ischemia area, leading the promotion of neurogenesis and recovery of behavioral function after TBI, while after using HIF-1 alpha inhibitors, the expression of VEGF and its mediated neural protection were suppressed. Thau-Zuchman et al [22] proved that after infusing exogenous VEGF to lateral ventricle, cell proliferation under the ventricle and around the damaged cerebral cortex was obviously increased, nerve formation and vascular regeneration were significantly increased, and brain edema volume reduced in TBI rats.

Endothelial progenitor cells (EPCs), the precursor cells of mature endothelial cells, normally reside in bone marrow hematopoietic system, and few in peripheral circulation system, however, bone marrow derived EPCs can release to peripheral circulating blood in response to ischemia and hypoxia, and gather in ischemic area, then proliferate and differentiate into endothelial cells to participate in vascular intima restoration and new capillary network establishment as well as improvement of blood supply for ischemic tissue [11]. CD34, CDl33 and precursor cells of vascular endothelial growth factor receptor 2 (VEGFR-2) phenotype appeared in EPCs surface, and CD34 is usually as one of the main cell surface markers for identifying EPCs cell [23]. Part CD34+ cells isolated from peripheral blood can be cultured in vitro and proliferate and differentiate into vascular endothelial cells; while CD34+ cells in the body can gather to ischemic tissues and damaged vascular intima, then proliferate and differentiate into vascular endothelial cells to reduce blood vessel disorders [24]. Now, tissue-resident ECs and EPCs have been proved to be the key source of endothelial cells for vascular repairmen [23].

Hydrogen is a colorless, odorless, diatomic gas with a certain reducing quality. A study by Ohsawa et al [25] reported that inhaled H2 (1% to 4% concentration) markedly decreased oxidative stress, effectively removed free radicals and protected brain against ischemia/reperfusion injury in rats, and the effect of 2% H2 was better. It was also demonstrated that 2% H2 or hydrogen-rich water decreased oxidative stress and inflammation in the intestine and kidney after organ transplants [26-28]. Furthermore, some studies showed H2 and materials related hydrogen including hydrogen sulfide and hydrogen-rich saline treatment attenuated cerebral ischemia/reperfusion injury and oxidative stress and brain edema of TBI rats, as well as have anti-inflammatory and anti-apoptosis effect, can significantly improve neural function in rats [14, 15, 29]. Hydrogen dissolved in physiological saline to be hydrogen-rich saline, which has the function of antioxidant stress and decreasing cerebral edema, etc. [5, 13]. The results of Hou et al [14] proved that hydrogen-rich saline improved cognitive performance, reduced malondialdehyde (MDA) level and enhanced molecules levels which are rela-

tive to brain-derived neurotropic factor (BDNF)-mediated synaptic plasticity after mild TBI.

This study aimed to detect the effect of hydrogen-rich water on angiogenesis in the boundary tissue of injuried brain, the results showed that for sham group rats, brain organizations appeared normal under optical microscope after HE staining. For TBI and TBI+HW groups, at 24 h after brain injury, brain tissue appeared severe edema, bleeding, many degeneration and necrosis neurons, few CD34+ cells and little blood capillary hyperplasia; at 3rd d, brain edema was more severe, CD34+ cells and blood capillary hyperplasia increased; at 7th d, brain edema attenuated, CD34+ cells and blood capillary hyperplasia increased significantly which was in accordance with Beck's et al [30] results that endothelial cells proliferate as early as 12-24 h following stroke, leading to peri-infarcted angiogenesis 3 days following the ischemic injury. All these results showed CD34+ cells and blood capillary hyperplasia were positively correlated, which is consistent with Guo's et al [24] opinion that hydrogen-rich water improved angiogenesis. NSS scores of TBI+HW rats significantly lower than TBI rats showing neurologic deficits of TBI rats were more severe and hydrogen-rich water contributed to against neurologic deficits. In comparison with TBI group, brain edema was slighter in TBI+HW group at 24 h and 3rd d after brain injury, besides, degeneration and necrosis of neurons reduced, at 7th d, CD34+ cells and blood capillary hyperplasia increased significantly, all these demonstrated that hydrogen-rich water treatment improved angiogenesis and neurogenesis. Moreover, after 24 h after rat brain injury, protein and mRNA of HIF-1α and VEGF in brain cortex around the wound gradually increased, and stay at a high level till at 7th d, which proved there is positive correlation between the expression of HIF-1α as well as VEGF and angiogenesis and neurogenesis. All the results showed that local nerve cells were of degeneration and necrosis, blood vessels were damaged, blood-brain barrier was broken and regional brain tissue hypoxia after TBI. In addition, HIF-1α directly acted on the VEGF promoter and elevated VEGF expression, meantime VEGF promoted CD34+ cells migrate to damaged brain tissue from bone marrow, then proliferate and differentiate into mature vascular endothelial cells; at last participate in the formation of newborn blood capillary network, the repair of damaged vascular intima and blood-brain barrier, the improvement of tissue ischemia and the recovery of neural function. After comparing TBI with TBI+HW, hydrogen-rich water promoted the expression of HIF-1α and VEGF as well as the formation of new-born blood vessels in the lesion boundary brain tissue.

In conclusion, TBI can promote angiogenesis via up-regulating the expression of HIF-1α and VEGF; besides, hydrogen-rich water played a positive effect on the promotion progress and improved nerve function of trauma rats.

Acknowledgements

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Disclosure of conflict of interest

None.

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