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Treatment with hydrogen-rich water protects against thioacetamide-induced hepatic encephalopathy in rats through stabilizing liver–brain disturbance

Xujiao Wang[1,3], Xiao Liu[1,3], Peng Zhou[2,3], Jianguo Feng[2,3], Jing Jia[2,3], Bingqing Xie[4,5], Ye Chen[3,6] & Jun Zhou[2,3]✉

Hepatic encephalopathy (HE), a neuropsychiatric complication secondary to liver cirrhosis and hepatic failure, represents the leading cause of mortality in end-stage liver disease. While hyperammonemia remains the central pathogenic factor in HE progression, emerging evidence implicates oxidative stress, neuroinflammation, and neuronal apoptosis as critical synergistic contributors to HE pathogenesis. Hydrogen-rich water, known for its antioxidant, anti-inflammatory, and anti-apoptotic properties, has not been systematically investigated for therapeutic efficacy in HE management. In the current investigation, we successfully established a HE rat model by administering thioacetamide via intraperitoneal injection. By observing the general state and behavioral changes of the rats, detecting liver function and blood ammonia, and observing the pathological changes of liver and brain tissue, it was discussed whether hydrogen-rich water had a preventive and therapeutic effect on hepatic encephalopathy. Oxidative stress, inflammation and neuronal apoptosis were detected in plasma, prefrontal cortex and hippocampus to explore the possible mechanism of its protective effect. The results showed that hydrogen-rich water can improve the behavioral changes of the HE rats, reduce blood ammonia, reduce liver function damage, alleviate the pathological changes of liver and brain tissue, significantly inhibit the systemic and local oxidative stress and inflammation of the brain tissue of the HE rats, and reduce neuronal apoptosis. In summary, hydrogen-rich water might stabilize liver-brain disturbance in thioacetamide-induced HE rats by anti-inflammation, anti-oxidative stress and reducing neuronal apoptosis.

Keywords Hydrogen-rich water, Hepatic encephalopathy, Oxidative stress, Inflammatory response, Apoptosis

Hepatic encephalopathy (HE), a neuropsychiatric manifestation of hepatic insufficiency, presents with a spectrum of neurological impairments including affective disturbances, memory deficits, cognitive dysfunction, psychomotor retardation, attentional lapses, sleep-wake cycle abnormalities, and sensory processing impairments. In advanced stages, patients may progress to global neurological depression characterized by stupor or coma, severely compromising quality of life[1]. HE is a common complication of cirrhosis and liver failure, and about 50–70% of patients with portal hypertension and cirrhosis will eventually develop HE. HE is also the most common cause of death in patients with various liver diseases developing to the end stage, with a high mortality rate[2].

[1]Zigong Fourth People's Hospital, Zigong 643000, Sichuan, China. [2]Department of Anesthesiology, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, Sichuan, China. [3]Anesthesiology and Critical Care Medicine Key Laboratory of Luzhou, Southwest Medical University, No. 25 Taiping Road, Jiangyang District, Luzhou 646000, Sichuan, China. [4]Laboratory of Neurological Diseases and Brain Function, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, China. [5]Institute of Epigenetics and Brain Science, Southwest Medical University, Luzhou 646000, China. [6]Department of Traditional Chinese Medicine, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, Sichuan, China. ✉email: junzhou@swmu.edu.cn; scjunzhou@gmail.com

The mechanism of HE is complex, and there are various hypotheses including ammonia poisoning, blood-brain barrier (BBB) abnormalities, etc. The ammonia poisoning hypothesis is a core hypothesis recognized domestically and internationally, and ammonia is considered the main pathological factor of all types of HE[2]. HE patients are often accompanied by hyperammonemia due to the increased production and absorption of ammonia in the intestine of HE patients and the reduced ability to clear ammonia[3]. Because of its small molecular weight, ammonia is extremely easy to cross the BBB and enter the brain tissue. Therefore, the increase of blood ammonia will cause the increase of intracranial ammonia concentration, change the energy metabolism of brain tissue, cause neuron cells edema, inhibit nerve conduction function, and eventually lead to brain edema, cognitive dysfunction and neurological dysfunction[4]. Current research provides compelling evidence that oxidative stress and inflammatory responses are independent factors in the development of HE and have a synergistic effect with ammonia[5]. The experimental results demonstrated that antioxidant intervention can also reduce the symptoms of HE and enhanced spatial learning capabilities of HE rats. Treatment in the early comatose phase can also increase the survival rate of rats[6,7]. Clinical investigations have demonstrated that the onset of HE in cirrhotic patients is accompanied by an increase in inflammatory factors, and the level of inflammation in patients with HE is higher than that in counterparts[8]. Anti-inflammatory therapeutic interventions can attenuate cerebral edema, reduce the grade of HE, decelerate the progression of HE, improve survival rate, enhance motor and cognitive function, and facilitate the restoration of learning and memory capacities[9]. Hyperammonemia can initiate apoptotic pathways in experimental models of HE, and activation of oxidative stress and inflammation can also trigger pathophysiological events in the central nervous system that programmed cell death of neural cells[1,10]. These findings collectively demonstrate that oxidative stress, inflammation and neuronal apoptosis have synergistic effects with ammonia, which jointly promote the initiation and progression of HE[3].

Extensive preclinical and clinical studies have confirmed that hydrogen has anti-oxidation, anti-inflammation, anti-allergy and anti-apoptosis therapeutic properties, and can regulate the autophagic processes, while hydrogen-rich water and hydrogen have the same biological effects[11,12]. Hydrogen-rich water (HRW) has protective effects on organ ischemia-reperfusion injury, auditory neuropathy, type 2 diabetes, metabolic syndrome and other diseases[13–16]. HRW can improve the liver function of patients with hepatitis B by alleviating oxidative stress, alleviate non-alcoholic fatty liver disease in mice by antioxidant and anti-inflammatory effects, attenuate liver injury caused by obstructive jaundice, significantly reduce the incidence of hepatocarcinogenesis, reduce the maximum tumor volume, and also have protective effects on encephalopathy, liver ischemia reperfusion and liver resection injury[17–22]. Lactulose is a first-line therapeutic agent in the clinical treatment of HE, which can reduce the production and absorption of ammonia in the intestine. Recent investigations have revealed that lactulose can be decomposed by bacteria in the intestine, produce a large amount of hydrogen, and subsequently attenuate the level of oxidative stress in the body, which is an indirect antioxidant[23]. Lactulose treatment in rats undergoing partial hepatectomy can enhance the production of endogenous hydrogen, attenuate oxidative stress and excessive inflammation in the liver, and promote liver regeneration[19]. These findings suggest that the increase of endogenous hydrogen in lactulose may be one of the mechanisms of its protective effect on HE, and HRW is full of hydrogen.

Emerging evidence indicates that oxidative stress, neuroinflammation, and neuronal apoptosis exhibit synergistic interactions with ammonia toxicity, collectively driving the pathogenesis and progression of HE. Notably, HRW has demonstrated multifaceted therapeutic properties, including potent anti-inflammatory, antioxidant, and anti-apoptotic activities, with established protective effects against both hepatic and neurological disorders. Based on these mechanistic insights and therapeutic profiles, we hypothesize that HRW may exert significant protective effects against the development and progression of HE.

TAA exhibits selective hepatotoxicity with a dose-dependent correlation to hepatic dysfunction. The resulting hepatic pathology closely mimics the characteristic features observed in cirrhotic patients. Based on its pathophysiological relevance, TAA-induced liver injury has been formally recommended by the International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) as a validated experimental model for studying HE[24]. The dose of 900 mg/Kg can induce all stages of symptoms including coma in rats, and is the recommended dose for studying HE[25]. In this study, we established a HE rat model through intraperitoneal administration of TAA at 900 mg/kg. Comprehensive assessments were conducted, including: (1) monitoring of general physiological status and behavioral alterations, (2) evaluation of hepatic function and plasma ammonia levels, and (3) histopathological examination of liver and brain tissues. These investigations aimed to determine the potential preventive and therapeutic efficacy of HRW against HE. To elucidate the underlying mechanisms, we systematically analyzed oxidative stress markers, inflammatory mediators, and neuronal apoptosis in plasma, prefrontal cortex, and hippocampal tissues. Our findings provide substantial experimental evidence and theoretical foundation for the development of novel therapeutic strategies targeting HE pathogenesis.

Results

Effect of hydrogen-rich water on the results of Morris water maze in rats

Positioning navigation test was used to evaluate spatial learning ability of rats. During the positioning navigation training: with the increase of training times, the station seeking latency of rats in each group was gradually shortened (Fig. 1A). On the 6th day of the experiment, the search latency of T group was significantly longer than that of N group ($P<0.01$), and that of H5 group and H10 was significantly shorter than that of T group ($P<0.05$ or $P<0.01$) (Fig. 1B).

The swimming track, swimming speed and the number of crossing platform were used to evaluate the spatial memory ability of rats in space exploration experiment. On the 6th day of space exploration experiment (Fig. 1E), there was no statistical difference in swimming speed among all groups ($P>0.05$). Compared with N group, the percentage of time spent in the target quadrant and the frequency of crossing the platform of rats in T

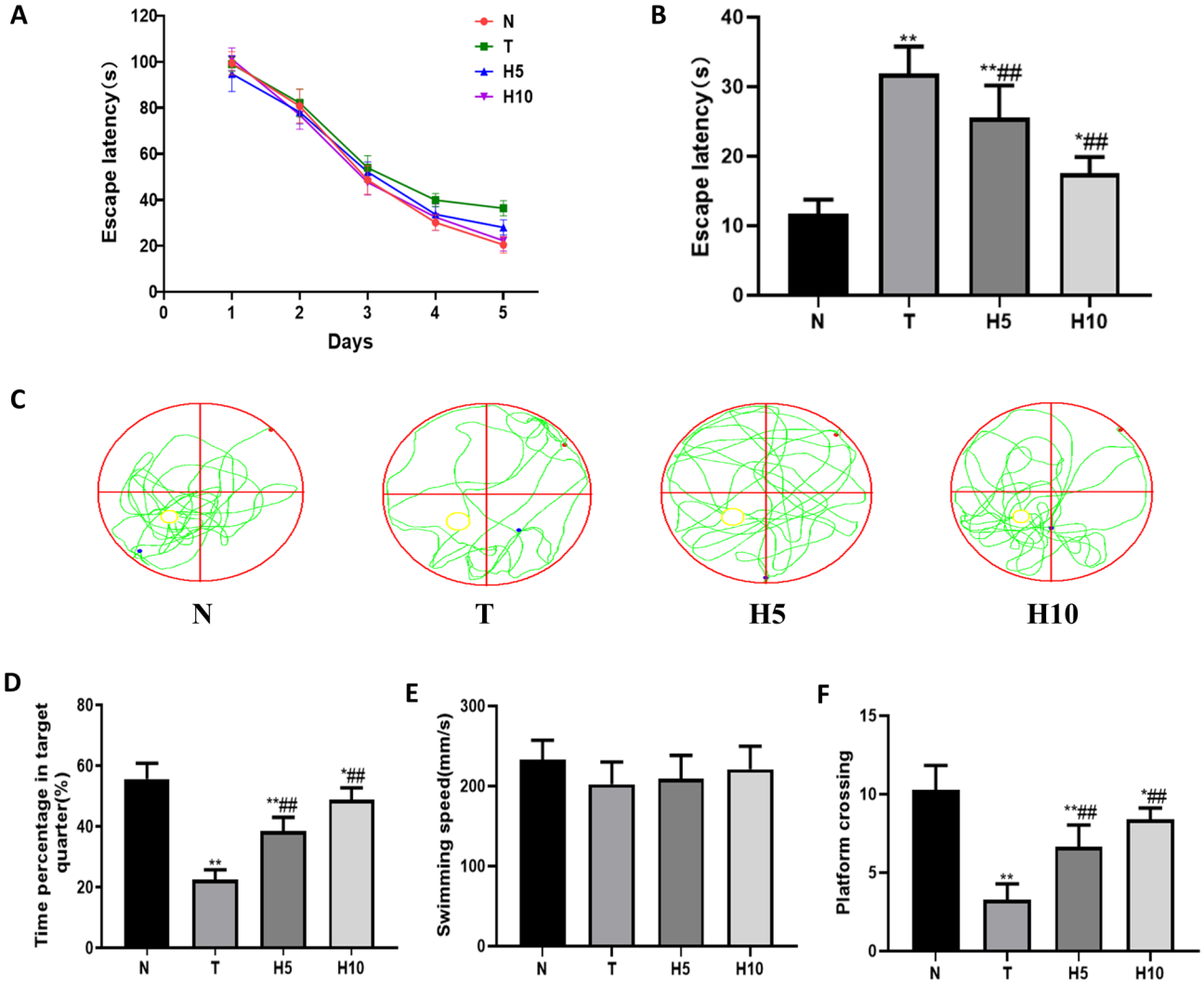

Fig. 1. Results of MWM experiment. **A** The change trend of station seeking latency of rats in each group 5 days before positioning navigation experiment; **B** Escape latency on the 6th day of the positioning navigation experiment; **C** Swim tracks for space exploration experiments; **D** Percentage of target quadrants; **E** Swimming speed; **F** Number of platform crossings. $^{*}P<0.05$, $^{**}P<0.01$ vs. N group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T group

group were significantly decreased ($P<0.01$), and were significantly increased after been given HRW ($P<0.01$) (Figure C–F).

Effects of hydrogen-rich water on general state observation, neurological score and opening experiment of rats

The rats in N group demonstrated consistent weight gain, maintained normal mental alertness, displayed active movement patterns, exhibited glossy fur, and produced well-formed stool. In contrast, The rats in T group showed significant weight loss, reduced food consumption, decreased spontaneous activity progressing to lethargy and eventual coma, along with dull or shedding fur and watery stool. The changes of rats in H5 group were alleviated compared with those in T group, but they were less energetic, lethargic, less exercise, less hair gloss and less stool. The rats in H10 group had no obvious weight loss, food intake was close to normal, mental performance was slightly poor, activity was less, hair was dull, stool was wet and soft.

Neurological scores in T group were significantly decreased compared with N group ($P<0.01$), and those in H5 group and H10 group were significantly increased compared with T group ($P<0.01$) (Fig. 2A). The opening experiment was used to evaluate the spontaneous motor ability of rats in each group, and the span times, total movement distance and upright times of rats in T group were significantly reduced compared with those in N group ($P<0.01$). H5 group and H10 group were significantly increased compared with T group ($P<0.05$ or $P<0.01$) (Fig. 2B–D).

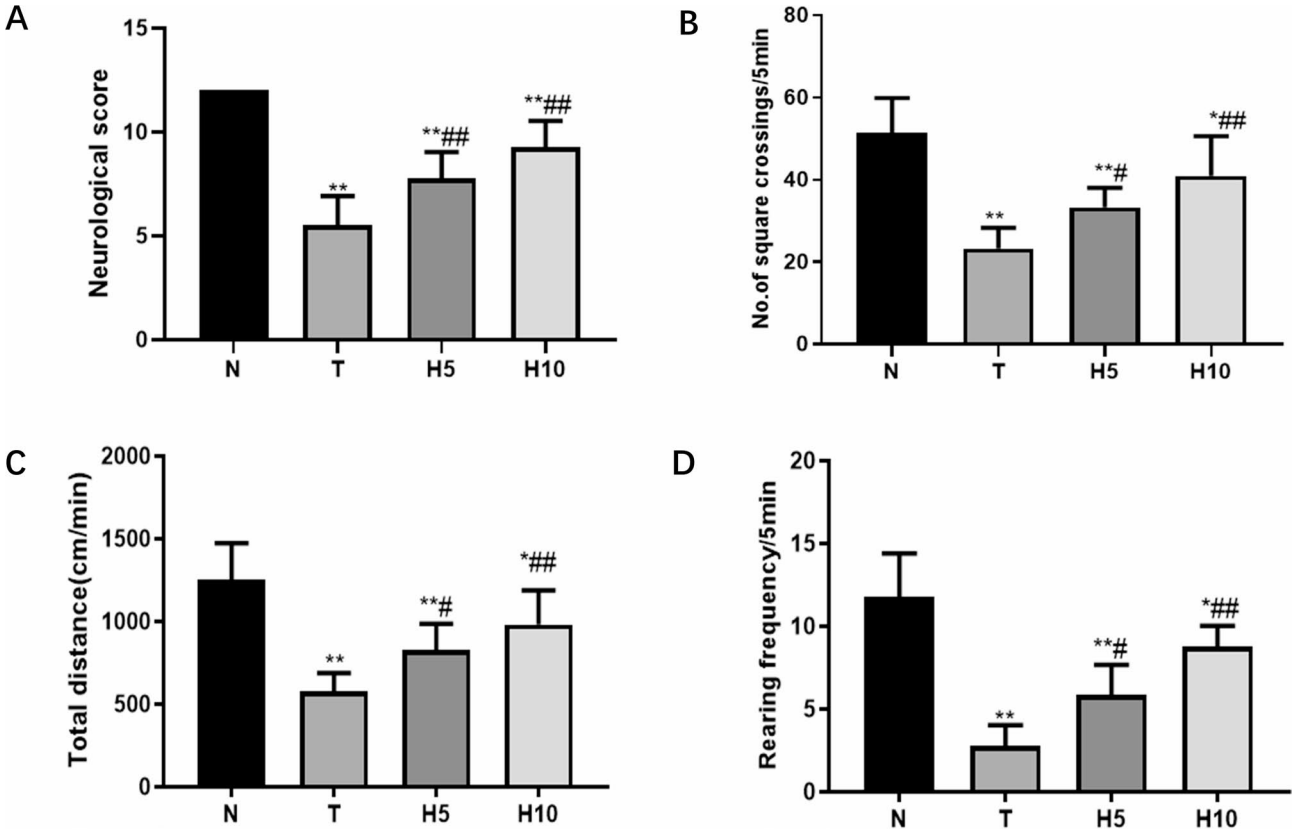

Fig. 2. Neurological scores and the opening experiment. **A** Neurological scores; **B** The number of spans; **C** The total distance traveled; **D** Number of upright positions. $^{*}P<0.05$, $^{**}P<0.01$ vs. N group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T group

Hydrogen-rich water reduced Amm levels and liver function damage in HE rats

The effects of HRW on plasma Amm, TBIL, ALT and AST levels: The plasma Amm, TBIL, ALT and AST levels in T group were significantly increased compared with those in N group ($P<0.01$); H5 and H10 groups were significantly lower than those in T group ($P<0.01$) (Fig. 3).

Hydrogen-rich water alleviated liver tissue damage in HE rats

The liver size of rats in N group was normal and the surface was smooth. In T group, the liver was enlarged, the edge was blunt, the surface had a coarse granular feeling, lack of luster, and necrosis was visible. The changes in H5 and H10 groups were less severe than those in T group (Fig. 4A).

Microscopic observation showed that the structure of hepatic lobules in group N was clear, the hepatic cords were arranged neatly, and there was no degeneration and necrosis of hepatic cells and no inflammatory cell infiltration. In T group, fibrous tissue hyperplasia occurred, normal liver tissue was destroyed and wrapped to form clumps, with structural changes similar to liver cirrhosis. The structure of liver lobules could not be distinguished, liver cords were disorganized, hepatocytes were edematous, plasma was pale and loose, necrosis appeared in some places, and a large number of inflammatory cells could be seen. H5 group could distinguish the structure of hepatic lobules, had mild fibrous tissue hyperplasia, inflammatory cell infiltration was less than T group, and the degree of cell degeneration and necrosis was less. The liver lobular structure of H10 group was basically normal, hepatocytes edema, a few showed balloon-like transformation, a small amount of inflammatory cell infiltration, no necrosis (Fig. 4B; Table 1).

Histomorphological changes of prefrontal cortex and CA1 region of hippocampus in HE rats induced by hydrogen-rich water

Prefrontal cortex: The prefrontal cortex of N group had clear structure and normal neuronal morphology; the structure of the prefrontal cortex in T group was obviously changed, the hierarchy was not clear, the shape of most neurons was irregular, the nuclei showed contraction, the staining was deepened, and the envelope was lightly stained; the prefrontal cortex structure and neuronal pathological changes of H5 and H10 groups were significantly improved compared with those of T group. The number of normal neurons in T group was significantly decreased compared with N group ($P<0.01$), and the number of normal neurons in H5 group and H10 group was significantly increased compared with T group ($P<0.01$) (Fig. 5A, B).

Hippocampal CA1 region: N group hippocampal neurons were arranged neatly with large and round nuclei; the arrangement of hippocampal neurons in T group was loose, the structure was disordered, some neurons

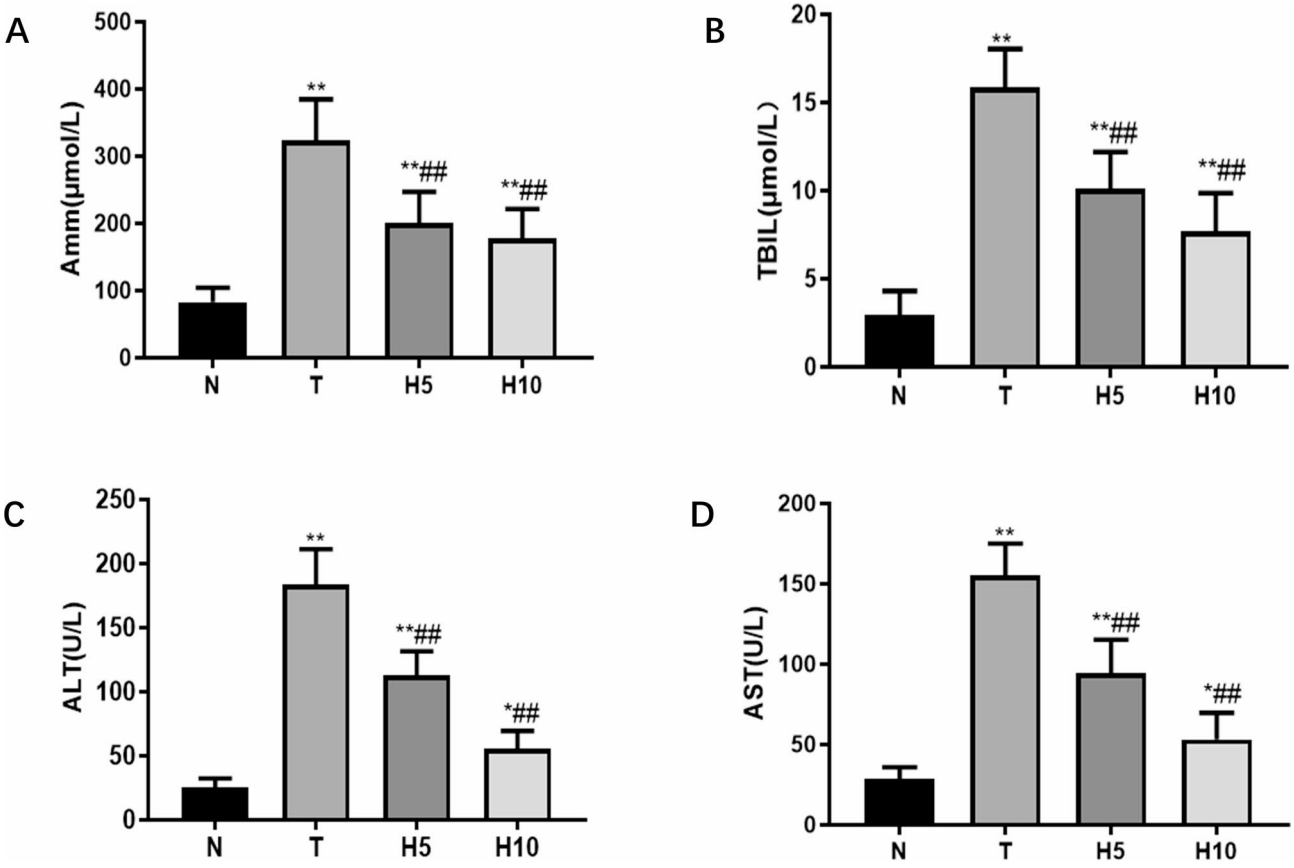

Fig. 3. Levels of blood ammonia and liver function in rats. **A** Ammonia content; **B** TBIL levels; **C** The vitality of ALT; **D** The vitality of AST. $^{*}P<0.05$, $^{**}P<0.01$ vs. N group; $^{\#\#}P<0.01$ vs. T group

were pyretic and deeply stained, and the number of normal neurons was significantly decreased compared with that in N group ($P<0.01$); the pathological changes of neurons in H5 group and H10 group were significantly improved compared with T group, and the number of normal neurons was significantly increased compared with T group ($P<0.01$) (Fig. 5A、C).

Hydrogen-rich water reduced the level of oxidative stress in HE rats

The activities of GPx and total superoxide dismutase (T-SOD) in plasma, prefrontal cortex and hippocampus of T group were significantly decreased compared with that of N group ($P<0.01$), and those of H5 and H10 groups were significantly increased compared with that of T group ($P<0.01$). The MDA content in plasma, prefrontal cortex and hippocampus of T group was significantly increased compared with that of N group ($P<0.01$), and the MDA content in H5 and H10 groups was significantly decreased compared with that of T group ($P<0.05$ or $P<0.01$) (Fig. 6).

Hydrogen-rich water alleviated the inflammatory response in HE rats

The contents of TNF-α, IL-1β and IL-6 in plasma, prefrontal cortex and hippocampus of T group were significantly increased compared with that of N group ($P<0.01$), and the contents of H5 and H10 groups were significantly decreased compared with that of T group ($P<0.01$)(Fig. 7).

Hydrogen-rich water alleviated the apoptosis of neurons in HE rats

Compared with N group, the number of neuronal apoptosis in the prefrontal cortex and CA1 region of hippocampus in T group was significantly increased, and the number in H5 group and H10 group was significantly decreased compared with T group. The apoptosis index of T group was significantly increased compared with N group ($P<0.01$), and H5 group and H10 group were significantly decreased compared with T group ($P<0.01$) (Fig. 8).

Discussion

Hepatic encephalopathy is a neuropsychiatric syndrome frequently associated with acute and chronic liver failure, indicating a state of deterioration of the disease. Ammonia is believed to be a central factor in the pathogenesis of HE, and ammonia entering the liver through the portal vein is predominantly metabolized to urea and glutamine through the urea cycle. As liver disease progresses, urea synthesis in the liver decreases,

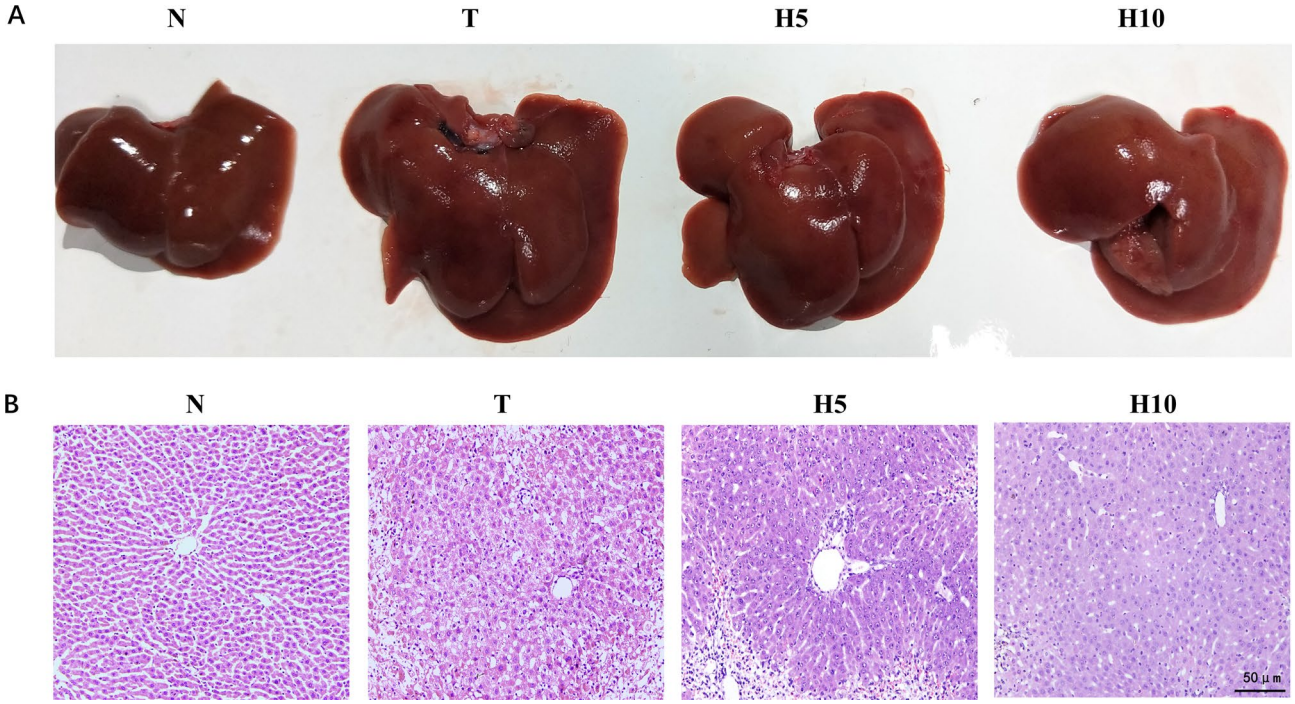

Fig. 4. Macroscopic and pathological changes of liver. **A** Pathological changes of the liver under visual observation; **B** Pathological changes of the liver were observed by light microscope (HE ×200), the scale was 50 μm

Group	*n*	(-)	(+)	(++)	(+++)	(++++)
N	8	8	0	0	0	0
T	8	0	1	1	3	3
H5	8	0	1	4	2	1
H10	8	0	4	3	1	0

Table 1. The degree of liver lesions under light microscope Normal (−), Hepacyte edema (+), ballooning degeneration (++), spotty necrosis accompanied inflammatory cell infiltration in portal area (+++), focal necrosis (++++)

resulting in compromised ammonia metabolism, leading to hyperammonemia[5]. Ammonia can easily cross the BBB and cause an increase in intracranial ammonia concentration, which is reduced by glutamine synthetase in astrocytes[4]. However, glutamine is a osmotically active substance, which can cause neuronal edema and disrupt neuronal metabolic homeostasis. When the concentration of ammonia increases, glutamic acid synthesis increases, and glutamic acid and ammonia synthesis glutamine increase, which will affect the energy metabolism process of cells and consume ATP, and the activity of Na+-K+ -ATPase is inhibited by ammonia, ATP production is reduced, energy source of the brain is reduced, and the body will eventually appear coma[26]. Elevated cerebral ammonia concentrations breaks the balance between excitatory neurotransmitter glutamate and inhibitory neurotransmitter GABA, causing neuropsychiatric disturbances[27].

TAA is a selective hepatotoxic chemical that can induce hepatocellular necrosis and hepatic dysfunction in a dose-dependent manner. TAA can cause elevated blood ammonia, neuronal edema, intracranial oxidative stress, inflammation and neurological impairment. The liver injury induced by TAA is similar to that in cirrhotic patients, and has been widely used in the study of the pathological mechanism of HE[25]. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (TBIL) levels are the most commonly used indicators to test liver function. In this experiment, the spatial learning ability of rats was evaluated by measuring the latency period of station seeking, the spatial memory ability of rats was evaluated by measuring the penetration index, the spontaneous motor ability of rats was evaluated by measuring the total distance of movement and the number of times of crossing the platform, and the exploration ability of rats was evaluated by measuring the number of upright times. Plasma levels of AST, ALT and TBIL were measured to assess liver function. In accordance with the methods described in the literature[28], the rat model of HE was established by intrabitoneal injection of TAA 300 mg/Kg/day for 3 consecutive days. It was observed that the rats in the model group showed deterioration of mental state, loss of appetite, weight loss, lethargy, ataxia, coma and other symptoms, increased blood ammonia, decreased liver function, and necrosis of liver tissue. The pathological sections showed the

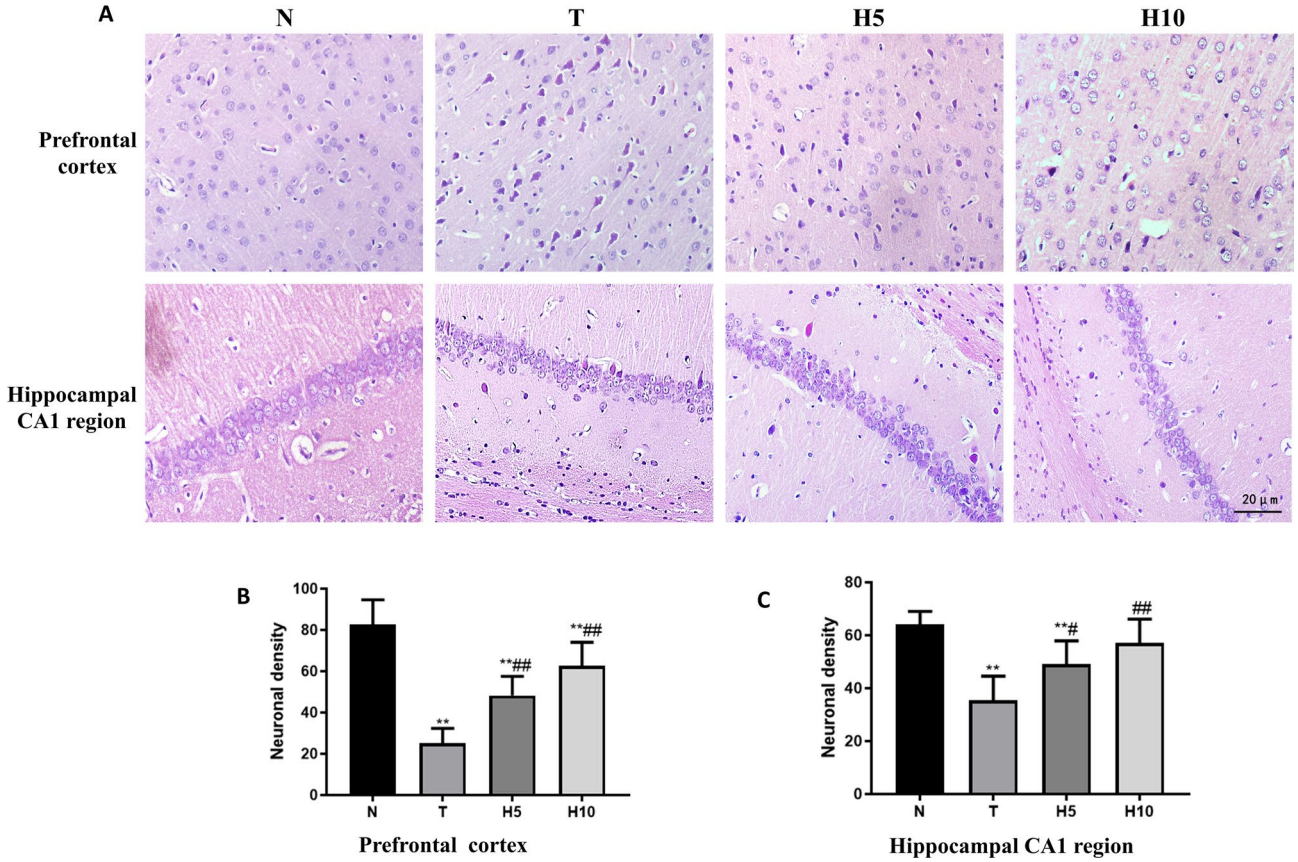

Fig. 5. Morphological changes of brain tissue. **A** Pathological changes of prefrontal cortex and hippocampal CA1 region (HE ×400), scale = 50 μm; **B** Normal neuron counts in the prefrontal cortex; **C** Normal neuron count in the CA1 region of the hippocampus. $^{**}P < 0.01$ vs. N group; $^{\#\#}P < 0.01$ vs. T group

disorder of liver tissue structure, degeneration and necrosis of liver cells, accompanied by neuronal injury, learning memory and spontaneous motor decline, which proved that we successfully established the HE model.

In this study, we observed hepatomegaly with visible necrotic foci on the hepatic surface in HE rat models. This finding contrasts with the characteristic hepatic atrophy typically observed in clinical cases of liver cirrhosis. The observed hepatomegaly may be attributed to the following pathophysiological mechanisms: (1) TAA metabolism generates substantial reactive oxygen species, inducing oxidative stress that damages hepatocyte membranes and organelles, leading to cellular edema; (2) subsequent inflammatory responses contribute to tissue edema; (3) portal venous flow obstruction results in hepatic congestion; and (4) sinusoidal endothelial cell injury accompanied by microthrombus formation causes sinusoidal congestion, collectively contributing to increased liver volume[29,30].The hepatic tissue may develop focal necrotic areas due to either ischemic injury or the direct cytotoxic effects of TAA. This pathological manifestation could result from compromised microcirculation leading to cellular hypoxia or through TAA-induced metabolic disturbances that ultimately trigger hepatocyte death[31].

HRW can release hydrogen in the body, and hydrogen has the characteristics of small molecular weight and strong permeability, can quickly diffuse to important organelles, and can quickly cross the BBB to brain tissue. Hydrogen does not have toxic side effects on the body, does not affect normal physiological parameters, nor does it affect arterial oxygen saturation or hemodynamics[32]. In clinical experiments, it was found that oral HRW could reduce the activity of ALT and AST, increase the activity of gamma-glutathione transferase, and increase the content of TBIL, but the changes remained within physiologically normal ranges[15]. Hydrogen is an ideal antioxidant that can enhance the activity of antioxidant enzymes and has selective antioxidant ability. It can eliminate strong oxidizing substances such as peroxynitrite (ONOO-) that are harmful to the body, while having no effect on weak oxidizing substances such as hydrogen peroxide (H2O2) that have normal physiological functions[33,34]. HRW has a good anti-inflammatory effect, can effectively inhibit inflammatory cells infiltration, reduce the production of pro-inflammatory factors, reduce inflammation. HRW also has a significant anti-apoptotic effect and can regulate the expression of pro-apoptotic factors and anti-apoptotic factors[35]. In this experiment, we observed that HRW treatment can ameliorate the symptoms of neuropsychiatric abnormalities in HE rats, improve neurological scores, reduce blood ammonia, alleviate liver and brain tissue damage, improve spatial learning and memory ability of HE rats, and enhance the ability of spontaneous movement and exploration of HE rats. To explore the possible mechanism of hydrogen-rich water alleviating injury in HE rats,

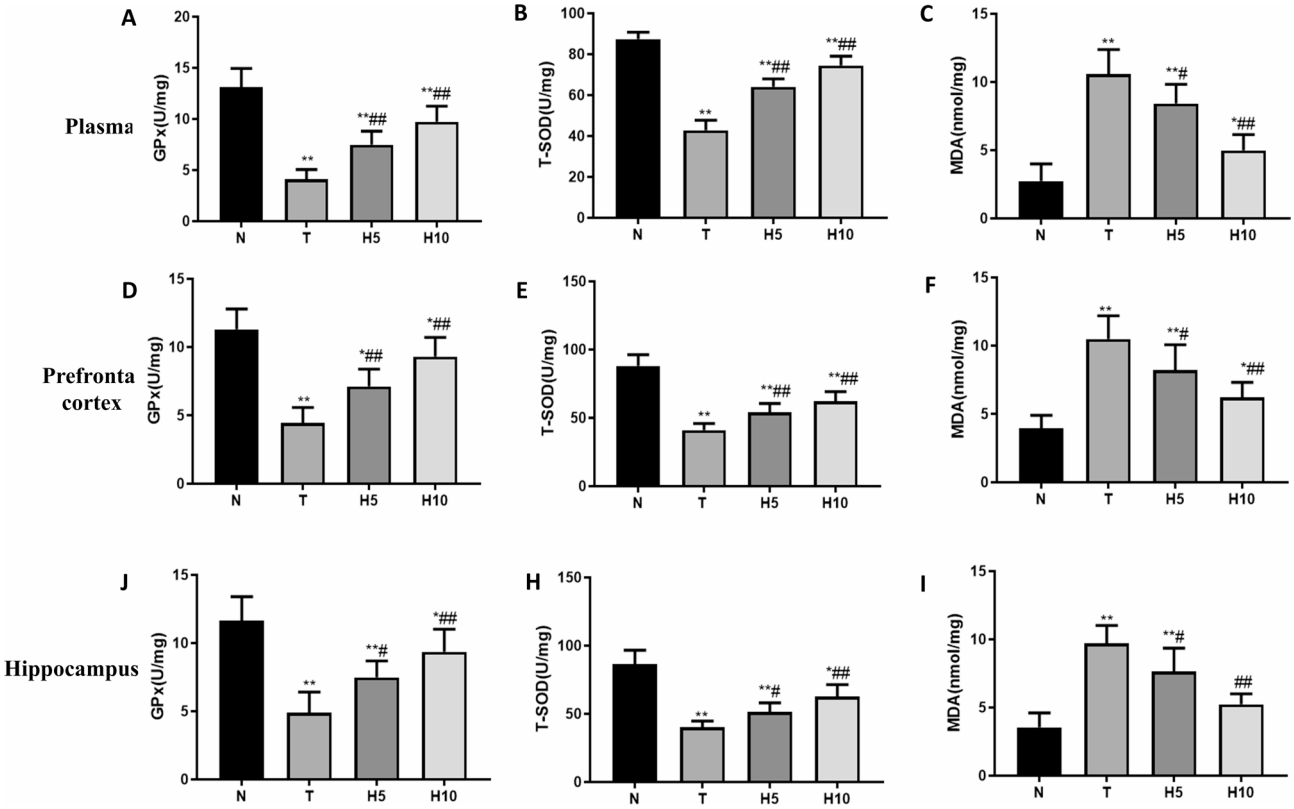

Fig. 6. Levels of oxidative stress in plasma, prefrontal cortex, and hippocampus. **A**–**C** Activity of GPx, T-SOD and content of MDA in plasma; **D**–**F** activity of GPx, T-SOD and content of MDA in prefrontal cortex; **J**–**I** Activity of GPx, T-SOD and content of MDA in hippocampus. $^{*}P<0.05$, $^{**}P<0.01$ vs. N group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T group

we examined oxidative stress, inflammatory response and neuronal apoptosis in plasma, prefrontal cortex and hippocampus.

In recent years, there is sufficient evidence that oxidative stress and inflammation are also important pathological mechanisms of HE, and ammonia jointly promote the occurrence and development of HE. Studies have shown that HE can cause central nervous system damage through oxidative stress, neuronal inflammation and apoptosis[1]. The prefrontal cortex has the functions of memory, analysis, judgment, thinking and executive control, and plays a very prominent role in behavior, consciousness and cognition. The hippocampus is mainly responsible for memory and learning, and is closely related to mood and sleep wakefulness. The patients with hepatic encephalopathy showed abnormal mood, memory impairment, cognitive function decline, learning and memory ability decrease and sleep disturbance, which may be related to the damage of prefrontal cortex and hippocampus[36]. The brain is one of the most vulnerable organs to oxidative damage due to its high metabolic rate, the need to consume a lot of oxygen, and the fact that brain tissue is rich in lipids. Malondialdehyde (MDA) is the most stable product of lipid peroxidation and represents the level of oxidative stress in the body. In order to prevent damage to reactive oxygen species (ROS), the brain must activate the antioxidant defense system, and antioxidant enzymes play a key role, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), whose activity reflects the body's ability to remove oxygen free radicals[37]. When the liver function is normal, the simple increase of blood ammonia can reduce the activity of antioxidant enzymes in the cerebral cortex and cerebellum of rats, and induce oxidative stress injury[38]. Furthermore, elevated ammonia concentrations induce cytotoxic edema in astrocytes, initiating a cascade of oxidative stress responses within the brain parenchyma that ultimately lead to neuronal dysfunction and neurological impairment[1]. Both astrocytic edema and inflammatory mediators can activate N-methyl-D-aspartate (NMDA) receptors, which can reduce the activity of antioxidant enzymes, weaken the body's antioxidant capacity, and cause neuronal damage and brain cell apoptosis[39–41]. A self-perpetuating pathological cycle is formed between astrocyte edema and oxidative stress response: astrocyte edema causes glutamate accumulation between neurons, and glutamate increases neuronal Ca2 + influx through activation of ionotropic glutamate receptors (NMDA), resulting in changes in mitochondrial osmotic pressure, and ultimately an increase in ROS. NMDA receptor activation and oxidative stress response can aggravate astrocyte swelling[3,42,43]. In this part of the experiment, we observed that compared with the normal group, the activities of GPx and T-SOD in plasma, prefrontal cortex and hippocampal tissue of rats in the model group were reduced, and the content of MDA was significantly increased, indicating that the oxidative stress level of HE rats increased, which was consistent with previous research results. However, the activities of GPx and T-SOD in plasma, prefrontal cortex and hippocampal tissue of rats were increased, while the content of MDA

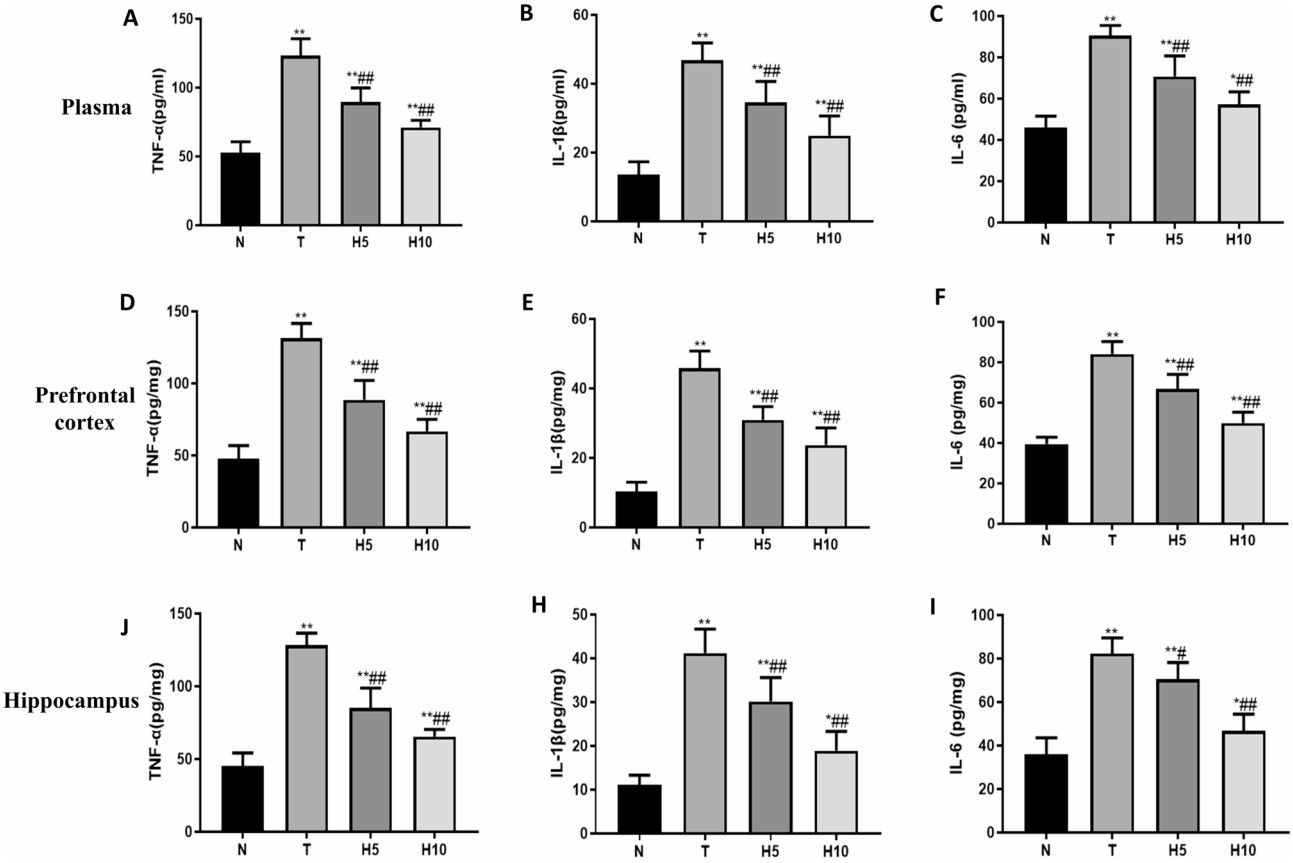

Fig. 7. Levels of inflammation in plasma, prefrontal cortex, and hippocampus. **A**–**C** The contents of TNF-α, IL-1β and IL-6 in plasma; **D**–**F** levels of TNF-α, IL-1β and IL-6 in the prefrontal cortex; **J**–**I** levels of TNF-α, IL-1β and IL-6. $^{*}P<0.05$, $^{**}P<0.01$ vs. N group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. T group

was significantly decreased after the administration of HRW, indicating that hydrogen-rich water can improve the activity of antioxidant enzymes in HE rats, inhibit lipid peroxidation, and alleviate the oxidative damage induced by TAA.

Systemic inflammatory response accelerates the progression of HE in patients with acute liver failure and can increase the stage of HE, indicating that inflammation is closely related to HE[44]. In this part of the experiment, we observed that compared with the normal group, the levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) in plasma, prefrontal cortex and hippocampal tissue of rats in the model group were significantly increased, indicating that the level of inflammation in HE rats was significantly increased, and hydrogen-rich water could significantly reduce the level of pro-inflammatory factors in HE rats and alleviate inflammatory damage. We observed that HE rats showed neuronal injury, learning and memory decline and spontaneous motor ability, and measured that the levels of TNF-α, IL-1β and IL-6 in plasma, prefrontal cortex and hippocampal tissue of HE rats were significantly increased, while treatment with HRW alleviated the symptoms of neuropsychiatric abnormalities and brain tissue injury of HE rats. The cognitive ability of HE rats was improved, and the levels of proinflammatory factors in plasma, prefrontal cortex and hippocampus were significantly reduced. We speculated that the reduction of TNF-α, IL-1β and IL-6 and other inflammatory factors may be related to the alleviation of cognitive dysfunction in HE rats. Inflammation is an important pathogenic feature of HE-induced brain injury. High levels of ammonia and free radicals can induce inflammatory genes and subsequently produce various inflammatory mediators. TNF-α, IL-1β, and IL-6, as the most important pro-inflammatory cytokines, are elevated in hepatic encephalopathy and trigger harmful inflammatory responses in the central nervous system. In HE patients and animals, elevated brain levels of these cytokines are associated with neuroinflammation and subsequent abnormal cognitive and motor activity[45,46]. Peripherally generated inflammatory mediators cannot cross the BBB, so they cannot have a direct impact on the brain, but the peripheral immune system can transmit signals to the brain, induce brain tissue to express pro-inflammatory factors, and promote oxidative stress and mitochondrial dysfunction, further aggravating neuroinflammation and neuronal damage tissue[47,48]. HRW may reduce the levels of TNF-α, IL-1β and IL-6 in HE rats through the following mechanisms, thereby alleviating the cognitive dysfunction of HE rats: (1) Antioxidant effect: Molecular hydrogen in HRW has a strong antioxidant capacity, which can clear excess ROS and inhibit the activation of pro-inflammatory signaling pathways such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) cascades, thus reducing the production of inflammatory factors, and the reduction of inflammatory factors can reduce the apoptosis and necrosis of neurons. Protect brain regions

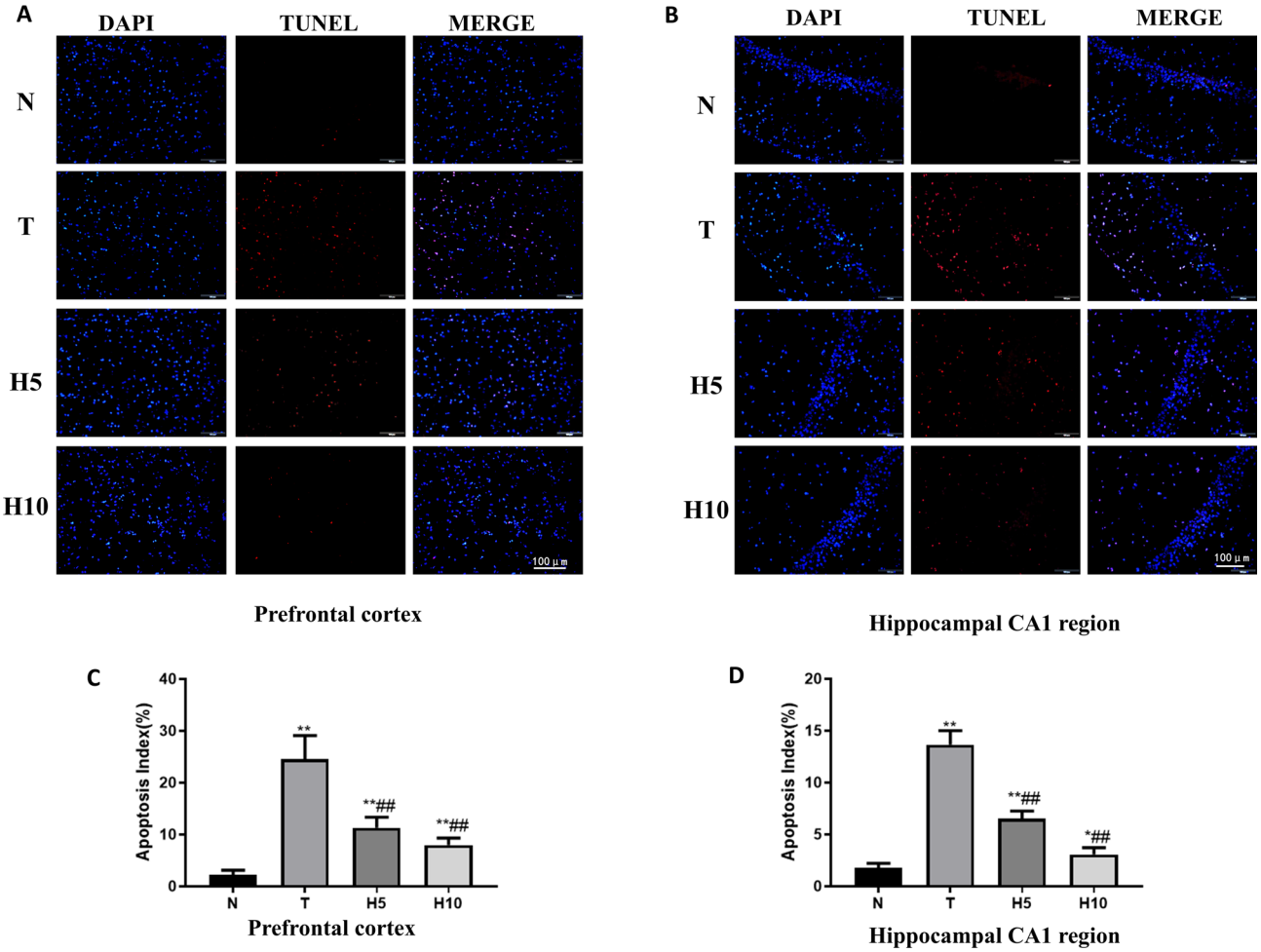

Fig. 8. TUNNEL dyeing results. **A**, **B** Results of TUNEL fluorescence staining in prefrontal cortex and hippocampal CA1 region (×200), nuclei were labeled with blue fluorescence (DAPI), TUNEL-positive cells were labeled with red fluorescence, the scale = 100 μm; **C**, **D** Apoptosis index in prefrontal cortex and hippocampal CA1 region. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. N group; $^{\#\#}P < 0.01$ vs. T group

related to learning and memory such as hippocampus and prefrontal cortex[49]. (2) Inhibition of microglia activation: Molecular hydrogen can inhibit the overactivation of microglia and reduce the release of TNF-α, IL-1β and IL-6, while the reduction of TNF-α and IL-1β can alleviate glutamate excitotoxicity, promote synaptic plasticity and long-term enhancement (LTP), and thus improve learning and memory ability[50]. (3) Protecting the BBB: By alleviating oxidative stress and inflammatory response, HRW may help maintain the integrity of the BBB and prevent peripheral inflammatory factors from entering the central nervous system (CNS). The reduction of inflammatory factors can improve the brain microenvironment and promote the repair and functional recovery of neurons[48,51]. Studies have shown that the increased levels of TNF-α, IL-1β and IL-6 in HE patients and animal models are closely related to cognitive dysfunction. Studies have shown that the plasma levels of TNF-α and IL-6 in HE patients are negatively correlated with cognitive function scores[52]. These findings are consistent with our findings that reducing inflammatory factors is associated with improved cognitive function.

Activation of oxidative stress and inflammation can initiate a series of pathophysiological events in the CNS and promote apoptosis of brain cells[10]. Apoptosis plays an important role in the pathophysiology of TAA-induced HE. Previous studies have shown that TAA induces up-regulation of caspase-3 and Fas expression in liver tissue, suggesting that TAA has pro-apoptotic activity[53]. It has also been reported that hyperammonemia can activate apoptotic pathways in experimental HE models[1]. We chose to establish a rat model of HE by intrabitoneal injection of 300 mg/Kg/day of TAA for 3 consecutive days to simulate the pathophysiological events associated with human HE. Our study showed that after TAA injection, brain tissue ammonia levels increased, GPx and T-SOD levels decreased, and MDA increased, indicating overproduction of ROS and lipid peroxidation in brain cells. This eventually raises the level of pro-inflammatory cytokines, which leads to apoptosis[54]. Previous studies have confirmed that oxidative cellular damage plays a direct role in the activation of apoptosis, and the overproduction of oxidized species leads to mitochondrial dysfunction, including the loss of mitochondrial membrane potential, the release of cytochrome c from mitochondria to cytoplasm, and the activation of caspase-3 and apoptosis[55]. Hematoxylin and eosin (H&E) staining showed that in the normal group, the prefrontal cortex and hippocampus were clearly structured, the neurons were in normal shape and arranged

neatly, and the nuclei were large and round. After TAA was given, the structure of the prefrontal cortex was significantly changed, and the hierarchy was not clear. Most neurons in the prefrontal cortex and hippocampus were irregular in shape, the nuclei showed shrinkage and deep staining, and the count of normal neurons was significantly reduced. The structural and neuronal pathological changes of frontal cortex and hippocampus were improved to varying degrees after the administration of HRW, and the number of normal neurons increased significantly. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay has strong sensitivity and specificity and is the preferred method for detecting apoptosis. Our TUNEL staining results showed that the apoptotic death of the brain cells in HE rats increased, and the number of apoptotic neurons in the frontal cortex and hippocampus CA1 region decreased significantly and the apoptotic index decreased after the administration of hydrogen-rich water. Therefore, our data showed that HRW has an anti-apoptotic effect in TAA-induced rat brain tissue.

As summarized in Fig. 9, our study demonstrates that treatment with HRW may protect against TAA-induced HE in rats through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms, offering new insights for therapeutic development. However, its specific mechanism and clinical application need further study. While our study provides valuable insights, several limitations should be acknowledged: (1) The current investigation did not elucidate the molecular mechanisms underlying the protective effects of HRW against HE. Future studies should focus on identifying specific signaling pathways and molecular targets involved in HRW-mediated neuroprotection and hepatoprotection. (2) Our findings in rodent models may have limited translational relevance due to inherent physiological and metabolic differences between rats and humans. Additionally, clinical HE patients often present with multiple comorbidities that may influence therapeutic outcomes. We recommend

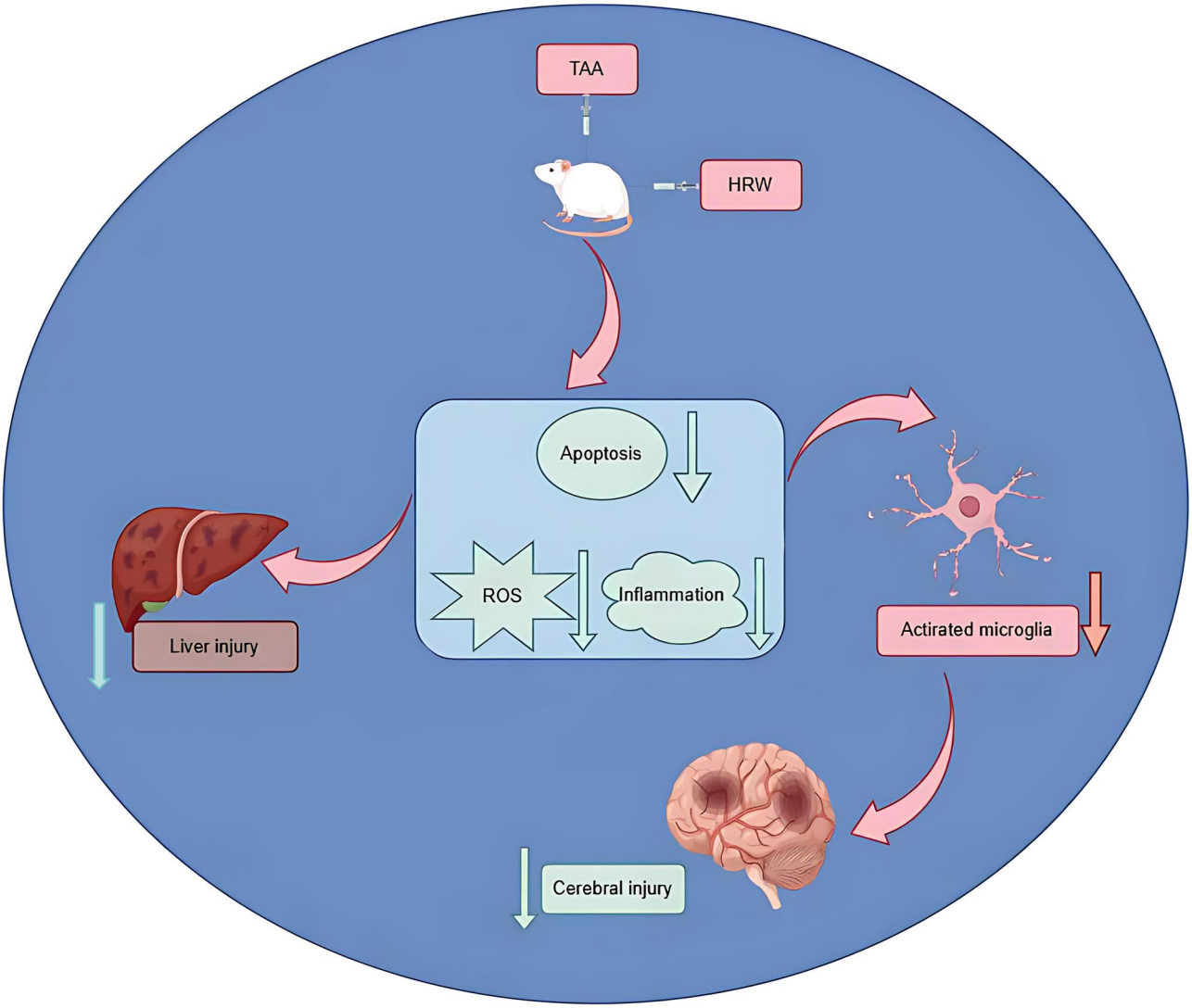

Fig. 9. Hypothetical target pathway. Oxidative stress, inflammation, and neuronal apoptosis collectively contribute to the onset and progression of hepatic encephalopathy. Hydrogen-rich water alleviates hepatic and cerebral damage in rats with hepatic encephalopathy through antioxidant effects, anti-inflammatory actions, and inhibition of cellular apoptosis

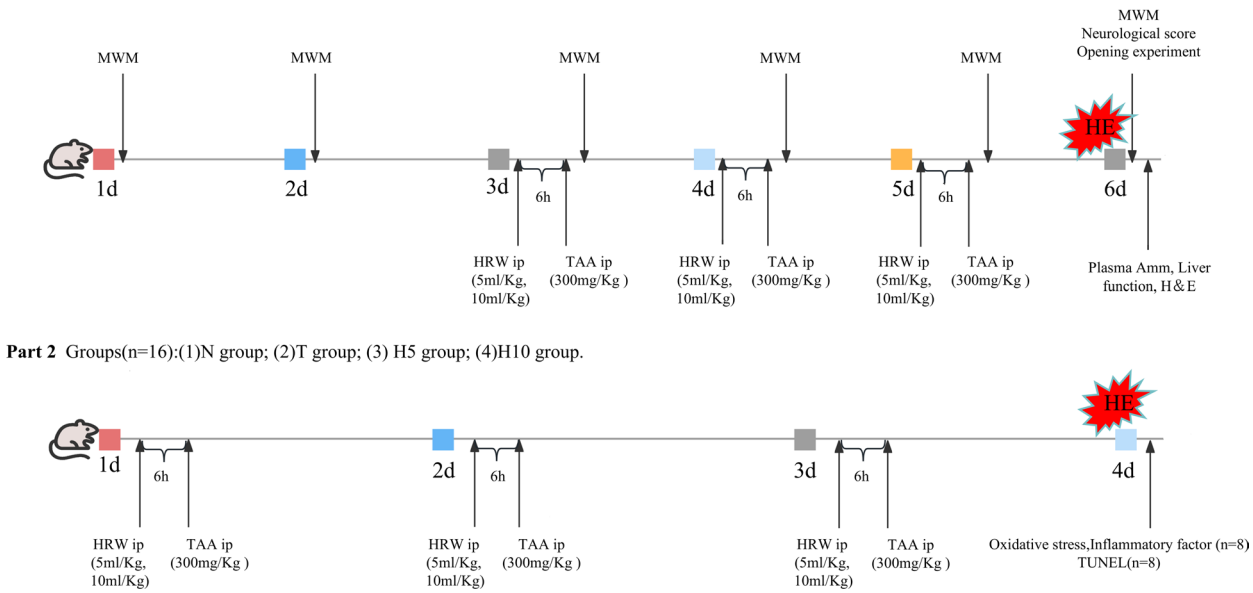

Fig. 10. Experimental design and animal groups. HRW: hydrogen-rich water; TAA: thioacetamide; ip: intraperitoneal injection; MWM: Morris water maze; HE: hepatic encephalopathy; H&E: hematoxylin and eosin; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; h: hour; d: day

subsequent investigations in higher mammalian models with closer hepatic physiology to humans, followed by early-phase clinical trials. (3) The study was limited to evaluating short-term effects of HRW on learning and motor functions in HE rats. Comprehensive assessment of long-term outcomes, including cognitive recovery and survival benefits, warrants extended treatment durations and observation periods in future research. (4) Although our data demonstrate the efficacy of HRW monotherapy in attenuating HE pathology in rats, clinical HE management typically involves lactulose and/or rifaximin as first-line therapies. The potential synergistic effects of HRW combined with standard clinical regimens remain to be investigated, which could potentially enhance therapeutic benefits for HE patients. These limitations highlight important directions for future research to further validate and optimize HRW-based therapeutic strategies for HE management.

Materials and methods

Feeding and grouping of animals

SPF-grade healthy adult male Sprague-Dawley (SD) rats weighing 220–250 g were purchased from the Experimental Animal Center of Southwest Medical University. They ate and drank water, exposed to light with normal circadian rhythm, indoor temperature was 22–24℃, and relative humidity was 40-60%. The animal manipulation involved in this experiment was approved by the Ethics Committee of Southwest Medical University and complied with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th edition, 2011). The study was conducted following the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010).

After feeding for one week, a total of 96 rats were randomly divided into 4 groups ($n = 24$): (1) normal group (N group); (2) Model group (T group); (3) hydrogen-rich water low-dose group (HRW 5 ml/Kg, H5 group); (4) hydrogen-rich water high-dose group (HRW 10 ml/kg, H10 group). When the rats died or were unable to complete behavioral testing, the rats were re-supplemented. The grouping and experimental procedures are detailed in the experimental flow diagram (Fig. 10).

Establishment and administration of the HE rat mode

Except N group, the other groups were intraperitoneally injected with 300 mg/Kg/day of TAA (Shanghai Yuanye Biotechnology Co., LTD., China), and HE rat models were established for 3 consecutive days[28]. N Group was intraperitoneally injected with equivalent volumes 0.9% NS, and H5 group and H10 group were intraperitoneally injected with HRW (Beijing Vitality Hydrogen Source Beverage Co., LTD., China) 5 ml/Kg and 10 ml/Kg, respectively, 6 h prior to TAA administration.

Animal behavior testing

Morris water maze (MWM)

The Morris water maze (MWM) test was conducted to assess spatial learning and memory capabilities, beginning two days prior to model establishment and continuing for five consecutive days. The maze (Shanghai XinRuan Information Technology Co. Ltd., Shanghai, China) consisted of a round tank (height 50 cm and diameter 150 cm), a platform (diameter 10 cm), and a camera analysis system. In the positioning navigation experiment

for five consecutive days (1d-5d), the rats were trained four times a day, each time in a different quadrant of 120 s to find a flooded platform, recording the escape latency, and the rats failed to find the platform within 120 s were manually guided to the platform. The space exploration experiment was carried out on the 6th day, namely, 3 days after modeling, the rats were put into the water facing the pool wall and sailed freely in the water for 120 s to record the escape latency and swimming speed. Then, the platform was withdrawn and the rats were allowed to sail freely in the water again for 120 s, recording the percentage of target quadrants and the number of times they crossed the platform[56].

General state observation and neurological score

After the first injection of TAA, the weight, food intake, mental state, hair, frequency of defecation, stool shape, autonomous activity and reflexes of the rats were observed daily. The diagnostic criteria for HE are: reduced autonomic activity, lethargy, delayed response, ataxia, coma and other symptoms in rats[57]. On the second day after the last injection of TAA, neurological functioning was assessed by measuring the pinna reflex, corneal reflex, tail flexion reflex, escape response reflex, righting reflex, and ataxia, which were assessed and scored on a scale of 0 (no reflex) to 2 (intact reflex). The neurological score at each time point was defined as the summation of these reflex scores[58].

Opening experiment

The opening test is often used to assess an animal's ability to move spontaneously and explore[59]. On the second day after the last TAA injection, the rats underwent the opening experiment ($n=8$). The opening test box is an uncovered wooden box with a black inner wall and a blue bottom plate, and the bottom plate is divided into 25 squares. The volume of the test box is 1 m×1 m× 0.5 m. Keep the laboratory quiet during testing, control the indoor lighting to be dim, and keep the central area of the opening bright. The rat was placed in the center of the experiment box, and the rat was released at the beginning of the experiment. The number of span times (both hind legs crossed the white line), the total distance of spontaneous movement and the number of upright times (both front legs raised) of the rat were recorded. The experimental duration was set at 5 min.

Specimen collection

On the second day after the last TAA injection, the rats fasted for 12 h and drank freely. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). After laparotomy, 5 ml of blood was collected by puncture of the inferior vena cava with a blood collection needle, and the plasma was left for 2 h and centrifuged at 3000 r/min for 10 min. The separated plasma was divided and stored at -80℃ for detection of plasma Amm and liver function.

Rats were humanely euthanized via intraperitoneal administration of an overdose of pentobarbital sodium (200 mg/kg). Eight rats were perfused through the left cardiac apex with 150 ml of pre-cooled normal saline (NS), followed by perfusion with 200 ml of 4% paraformaldehyde. After fixation for 30 min, the right lobe of the liver was cut off. The whole brain tissue was extracted from the severed head, and the prefrontal cortex and hippocampal tissues were separated on the ice. The prefrontal cortex and hippocampal tissues were placed in 4% paraformaldehyde, respectively, and fixed for 24 h for Hematoxylin-eosin staining. Sixteen rats were irrigated with 300 ml of pre-cooled NS, and the whole brain tissue was extracted from the rats on the ice. Eight complete brain tissues from each group were stored at -80℃ for TUNEL staining. Eight complete brain tissues from each group were separated from the prefrontal cortex and hippocampal tissues on the ice, and stored at -80℃ after packaging, which was used to make tissue homogenates to measure the expression levels of inflammatory factors and oxidative stress.

The content of amm and TBIL and the activity of ALT and AST were detected

The plasma after centrifugation was obtained, and the serum ammonia (Amm) and TBIL contents and the activity of alanine aminotransferase ALT and aspartate aminotransferase AST were detected with a specific detection kit (Nanjing Jiengcheng Bioengineering Institute, China) according to the manufacturer's instructions.

Hematoxylin-eosin staining

The liver was fixed with paraformaldehyde 24 h later, and the same part of the right lobe of the liver was cut, and the prefrontal cortex and hippocampus tissues of the brain were then dehydrated, paraffin embedded, and H&E staining was performed after section. Pathological changes of liver, prefrontal cortex and CA1 region of hippocampus were observed under light microscope. The grading was performed by pathologists blinded to the group allocation based on the severity of hepatic pathological changes[57]. Three high-power visual fields (×400) were randomly selected from each prefrontal cortex section. The number of normal neurons was counted and the average value was obtained. 3 slices of each hippocampal tissue were cut continuously. Under the ×400 microscope, the number of normal neurons in CA1 region was counted and the average value was obtained.

Determination of oxidative stress levels

GPx, T-SOD activity and MDA content of plasma and brain tissue homogenates were measured according to manufacturer's instructions using a specific detection kit (Nanjing Institute of Biological Engineering, China).

Determination of inflammatory factor levels

The levels of tumor necrosis TNF-α, IL-6 and IL-1β in plasma and brain tissue homogenate were determined by Enzyme linked immunosorbent assay (ELISA) kit (Shanghai Qiaodu Biotechnology Co., LTD., China).

TUNEL staining

TUNEL assay has strong sensitivity and specificity and is the preferred method for detecting apoptosis. The prefrontal cortex and hippocampus tissues of the brain after gradient sucrose dehydration were detected by in situ cell death detection kit (Roche, Switzerland), sliced, fixed, permeable, sealed, reacted, 4',6-diamidino-2-phenylindole (DAPI) stained, and sealed. The film was sealed and observed under fluorescence microscope. Three high-power visual fields (×200) were randomly selected from each prefrontal cortex section. The number of apoptotic cells and the total number of cells were counted and the average value was obtained. 3 slices of each hippocampal tissue were cut continuously. The number of apoptotic cells in CA1 region and the total number of cells were counted under the ×200 microscope at high power, and the average value was obtained. To calculate the Apoptosis Index (AI), AI = number of apoptotic cells/total number of cells ×100%.

Statistical analysis

All measurement data in this experiment were expressed as mean ± standard deviation (mean ± SD), and data were analyzed using GraphPad Prism 8.0.1 and SPSS 21.0 statistical software. Data met parametric assumptions, as confirmed by the Shapiro-Wilk test (for normality) and Levene's test (for homogeneity of variances). Subsequently, a one-way ANOVA was performed, followed by Tukey's HSD test for post hoc pairwise comparisons. $P < 0.05$ indicated that the difference was statistically significant.

Data availability

Data is provided within the manuscript.

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Author contributions

Xujiao Wang, Xiao Liu, and Peng Zhou conducted the experiments, data collection, and analysis, and wrote and edited the manuscript editing. Ye Chen and Bingqing Xie performed experiments and edited the manuscript. Jianguo Feng, and Jing Jia assisted with data interpretation and manuscript editing. Jun Zhou was responsible for study conception and design, data analysis and interpretation, study supervision, and manuscript revision. All authors have read and approved of this manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to J.Z.

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