

Neuroprotective Effects of Hydrogen Saline in Neonatal Hypoxia–ischemia Rat Model

Jianmei Cai^{ab1}, Zhimin Kang^{a1}, Kan Liu^a, WenWu Liu^a, RunPing Li^a, John H. Zhang^c, Xu Luo^{b*}, Xuejun Sun^{a*}

^a Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai, 200433, PR China;

^b Department of pathophysiology, Qingdao Medical University, Shandong, 266021, PR China;

^c Department of Neurosurgery, Loma Linda University, Loma Linda, California, USA

*Corresponding author. Fax: +86-21-65492382.

E-mail address: sunxjk@hotmail.com (X. J. Sun)

1 Jianmei Cai and Zhimin Kang contributed equally to this work

Abstract

Cerebral hypoxia–ischemia (HI) represents a major cause of brain damage in the term newborn. This study aimed to examine the short and long-term neuroprotective effect of hydrogen saline (H₂ saline) using an established neonatal HI rat pup model. Seven-day-old rat pups were subjected to left common carotid artery ligation and then 90 min hypoxia (8% oxygen at 37 °C). H₂ saturated saline was administered by peritoneal injection (5 ml/kg) immediately and again at 8 h after HI insult. At 24 h after HI, the pups were decapitated and brain morphological injury was assessed by 2,3,5-triphenyltetrazolium chloride (TTC), Nissl, and TUNEL staining. Acute cell death, inflammation and oxidative stress were evaluated at 24 h by studying caspase-3 activity, MDA measurement as well as Iba-1 immunohistochemistry in the brain. At 5 weeks after HI, spontaneous activity test and Morris water maze test were conducted. We observed that H₂ saline treatment reduced the caspase activity, MDA, Iba-1 levels, the infarct ratio, and improved the long-term neurological and neurobehavioral functions. H₂ saline has potentials in the clinical treatment of HI and other ischemia-related cerebral diseases.

Keywords: hypoxia–ischemia; hydrogen; caspase; Iba-1; neurobehavioral function

1. Introduction

Cerebral hypoxia–ischemia (HI) represents a major cause of brain damage in the term newborn. Although the mechanisms involved in HI were not completely understood, neuronal cell death either necrosis or apoptosis may play a critical role (Martin et al., 2000; Northington et al., 2001). Specially, apoptosis represents a treatable target which may occur in penumbra areas for days after the initial insult (Pulera et al., 1998). However, there is no specific treatment which is available to HI patients (Perlman, 2006).

Inflammation and oxidative stress are the two major causes of apoptosis identified after ischemic brain injury including neonatal HI (Kriz, 2006). Microglia was involved in the inflammatory process induced by the HI (Stoll et al., 1998). Allograft inflammatory factor-1 (AIF-1) in microglia is an index for the activation of microglia (Postler et al., 2000). Oxidative stress after HI damages to DNA, membrane and proteins and contribute to apoptotic changes. The malondialdehyde (MDA) is the product of lipid membrane oxidation and a marker of the oxidative damage.

Hydrogen gas was found to be protective in the brain, heart and liver after ischemia-reperfusion damage (Ohsawa et al., 2007; Fukuda et al., 2007; Hayashida et al., 2008). Hydrogen gas neutralizes free radicals and reduces oxidative stress. However, application of hydrogen gas presents a clinical issue for safety and convenience. In this study, we produced and tested long term neuroprotective effect of intraperitoneal application of saturated hydrogen saline (H₂ saline) in an established neonatal HI model.

2. Results

2.1 Nissl staining

We tested three different doses of hydrogen saline in the treatments to identify

the proper dose in the Nissl staining. Figure 1 shows representative samples of Nissl staining from the cerebral cortex and hippocampus of pups 24 h after HI insult. Extensive neuronal changes in the cortex and CA1 sector of the hippocampus were noticed with features of considerable dark, pyknotic neurons in HI group (B1-4). More Nissl stained cells (D1-4) were observed in H₂W group than in HI group. We used 5 ml/Kg injection for the following studies because it was more effective than the other two dosages.

2.2 TTC staining

Figure 2 shows representative photographs of TTC-stained sections from 8-day-old rats in each group, 24 h after the initial HI insult. The infarct ratio in HI group (10.8%) is markedly higher than that in H₂W group (0.99%) which is not significant from normal controls.

2.3 Content of MDA

The content of MDA in each group was detected at 24 h after HI. The content of MDA in HI group (9.00 ± 0.92) significantly increased compared to the control rats (3.52 ± 1.50). However, H₂ saline administration dramatically suppressed the production of MDA (4.5 ± 1.96) in rats after HI when compared that in the HI group.

2.4 TUNEL staining

Photos in Figure 3 were the representative graphs with different magnifications in TUNEL staining in samples collected at 24 h after HI. At higher magnification, the nuclei of cells were clearly stained in both hippocampus and cortex. The results indicated that TUNEL-positive cells were markedly increased in cortex and hippocampus after HI insult (B1-4). While the administration of H₂ saline

dramatically reduced the number of TUNEL-positive cells (C1-4). A few TUNEL-positive cells were identified in samples from normal control pups (A1-4).

2.5 Caspase-3 activity

The activity of caspase-3 was measured at 24 h after HI insult as shown in Figure 5. The activity was 1.18 ± 0.23 in cortex and 0.93 ± 0.22 in hippocampus in HI group. H₂ saline significantly suppressed the activity of caspase-3 in the cortex (0.12 ± 0.09) and hippocampus (0.09 ± 0.10) which were consistent with the results of TUNEL staining.

2.6 Immunohistochemical analysis

Iba1-positive cells had small nuclei, scant cytoplasm, and thin, branched processes in Figure 4. This kind of cell was found throughout the parenchyma of the white and grey matter. The distinctive morphology and widespread distribution of these cells were highly consistent with the classical descriptions of ramified microglia. H₂ saline treatment dramatically decreased the number of Iba1-positive cells (B1,B2). Samples were collected at 24 h after HI.

2.7 Body weight

There was no disparity in body weight between the three groups in the first 15 days after HI insult (Figure 5). In the later 20 days, a significant difference was found between the HI and H₂W group, while there was no statistic difference between control and H₂ saline treatment group.

2.8 Function test

The Postural Reflex Test shows an HI insult affects the sensorimotor function of the rats. Score 0 represent normal sensorimotor function, whereas Score 1 and Score 2 represent a deficiency in sensorimotor function. All of the control animals scored a Score 0. 22.22% of the HI animals scored a Score 0, 77.78% a Score 1 and 66.67% a Score 2. 63.64% of the HI+H₂W scored a Score 0, 36.36% a Score 1 and 9.09% a Score 2. A Chi-squared test showed that there was a significant difference in the distribution between the groups.

2.9 The locomotor activity

In order to display the time dependency of a behavior, spontaneous activity was tested. Fig 6 shows the trace of spontaneous behavior and the statistic. The observation indicated all rats experienced HI insult moved less as time in the chamber increased. However, the rats in HI group were more active and the move time for H₂W group, not different from normal controls, was more than that in HI group. The average total distance in H₂W group was less than that in HI group.

2.10 Morris Water Maze

The escape latency was measured at 24 h after HI insult. The escape latency was 12±1.23 sec in control group and 27±3.21 sec in HI group. Administration of H₂ saline shortened the time (15±1.89 sec) required for rats to reach the platform compared with HI group during the water maze testing. But the time in rats of H₂ saline group was not different from that in normal rats. H₂ saline improved spatial recognition and learning that had declined by HI.

3. Discussion

We investigated the neuroprotective effect of peritoneal administration of

saturated H₂ saline in neonatal HI rats. The short term results indicated H₂ saline treatment significantly reduced the infarct ratio, increased the number of survival neurons, reduced the number of apoptotic cells, suppressed caspase-3 activity, prevented activation of microglia, and decreased the level of oxidative stress (MDA). These short term effects were translated into long-term neurological functional improvements at 5 weeks after HI insult. These observations indicated that H₂ saline may be a potential therapeutic option for neonatal brain disorders.

The key neuroprotective effect of hydrogen is neutralizing free radicals, especially the hydroxyl radical ($\cdot\text{OH}$) and peroxynitrite anion (ONOO^-) (Ohsawa et al., 2007). The $\cdot\text{OH}$ is the neutral form of the hydroxide ion and the normal product in the cell metabolism. $\cdot\text{OH}$ has a high reactivity, making it a very dangerous radical with a very short in vivo half-life of approximately 10^{-9} s (Valko et al., 2007). In addition, cells produce both the superoxide anion ($\text{O}_2^{\cdot-}$) and nitric oxide (NO) during the oxidative burst triggered during inflammatory processes (Dedon and Tannenbaum, 2004). Under these conditions, NO and $\text{O}_2^{\cdot-}$ may react together to produce significant amounts of oxidative active molecule, ONOO^- , which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation (Valko et al., 2007). ONOO^- also reacts with carbon dioxide (CO_2) present in biological fluids to form reactive intermediates that can oxidize thiols and nitrate phenolic compounds, such as tyrosine (Valko et al., 2007). After neonatal hypoxic and ischemic brain injury, reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as the $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, hydrogen dioxide (H_2O_2), NO, ONOO^- , appear to play a critical role in cell death. Among the ROS, $\cdot\text{OH}$ and ONOO^- are much more reactive and react indiscriminately with nucleic acids, lipids and proteins. The brain has potent defenses including dietary free-radical scavengers (ascorbate, α -tocopherol), the endogenous tripeptide glutathione, and enzymatic antioxidants against

ROS. However, there isn't known detoxification system for $\cdot\text{OH}$ and ONOO^- . Recently, Ohsawa et al (2007) found that molecular hydrogen can selectively reduce $\cdot\text{OH}$ and ONOO^- in cell-free systems and exert a therapeutic antioxidant activity, in a rat middle cerebral artery occlusion model. Therefore, the ability of hydrogen to reduce or eliminate $\cdot\text{OH}$ and ONOO^- may be responsible for the neuroprotective effect observed in this study.

A Japanese team reported that hydrogen gas serves as an antioxidant to provide protective effects to ischemic insult in the brain and liver by selectively scavenging $\cdot\text{OH}$ and ONOO^- (Ohsawa et al., 2007; Fukuda et al., 2007). They also found that inhalation of hydrogen gas reduced infarct size in the rat model of myocardial ischemia–reperfusion injury (Hayashida et al., 2008) and intragastric administration of H_2 saline prevented the stress-induced decline in learning and memory caused by chronic physical restraint (Nagata et al., 2008). In our previous study, we found inhalation of 2% hydrogen also provided the protective effects on the same HI neonatal model (Cai et al., 2008). Although gas containing 2% hydrogen is safe for clinical practice, this strategy was not convenient because of the requirement of sealed chamber or face mask. Therefore, we produced H_2 saline which can be injected quickly without safety risks.

We selectively studied two major mechanisms of HI injury, inflammation and oxidative stress. Inflammatory response can be mediated by activated microglia, the resident immune cells of the CNS, which normally respond to neuronal damage including neonatal HI and remove the damaged cells by phagocytosis (Stoll et al., 1998). Iba1 is a 147-amino-acid calcium-binding protein widely used as a marker for microglia (Ito et al., 2001). The Iba1 gene and protein are identical to AIF-1, a protein involved in various aspects of inflammation (Deininger et al., 2002). AIF-1 has been reported to play a role in microglia activation in neuro-inflammatory disorders and ischemic brain injury

(Postler et al., 2000). Expression of AIF-1 was upregulated in response to apoptotic neuronal cell death and degeneration of injured central motoneurons (Tanaka et al., 1998), and sensory neurons (Ito et al., 1998). In the present study, H₂ saline reduced brain injury possibly by suppression of inflammatory response induced by HI. In addition, H₂ saline may reduce neuronal apoptosis to decrease the expression of AIF-1. We have observed similar results using hydrogen gas in another study (Cai et al., 2008). Furthermore, oxidative stress may lead to inflammation or may be enhanced by inflammatory actions. H₂ saline reduced oxidative stress in the neonatal brain after HI may contribute to the reduction of inflammation and apoptosis.

Even though the different pharmacokinetics between hydrogen gas and hydrogen saline are not clear and deserve further investigation, the advantages of hydrogen saline over hydrogen gas application are the safety issues, the easiness of application, and the possible higher concentrations of hydrogen in saline than could be used in gas. It is established that hydrogen at a concentration above 4% is inflammable and dangerous, and application of hydrogen gas requires either a sealed chamber or mask. In a previous study that hydrogen water was administered into the stomach of rats (Nagata et al., 2008). Even though this gastric administration is safe, but hydrogen in water tends to evaporate and loss hydrogen in the stomach or intestine, and it is difficult to control concentration and absorption. Therefore, we used peritoneal injection that we can quickly withdraw and immediately inject hydrogen saline into animals to avoid losing hydrogen into environment. This hydrogen saline preparation is superior to hydrogen gas or hydrogen water, and higher and more accurate concentrations of hydrogen can be applied.

A feature of this study is the evaluation of long term neurological and neurobehavioral functions. We have found that H₂ saline does not only prevent or reduce early pathological changes such as infarction or biochemical

changes such as inflammation and oxidative stress, but also produce long lasting functional improvement. This observation provides strong support for future clinical trials of H₂ saline in neonatal HI or other brain injuries. Taken together, peritoneal administration of H₂ saline reduces brain injury after neonatal HI possibly by attenuating the inflammation and oxidative stress, leading to reduction of apoptosis and improvement of long term neurological and neurobehavioral functions.

4. Experimental procedures

4.1 Experimental groups

7-day-old Sprague-Dawley rat pups were randomly assigned to the following three groups: 1) control group (no carotid ligation or hypoxia) (n=40), 2) HI group (carotid ligation and hypoxia) (n=80), 3) HI+H₂W group (carotid ligation, hypoxia and H₂ saturated saline treatment) (n=80). Pups in each group were obtained from different litters to obtain parity within the groups. The Animal and Ethics Review Committee at the Second Military Medical University evaluated and approved the protocol used in this study.

4.2 H₂ saline therapy paradigms

Purified H₂ was dissolved into normal saline for 2 h under 0.6 MPa. H₂ saturated saline was administered by peritoneal injection (5 ml/kg) immediately and again at 8 h after HI insult.

4.3 Hypoxia–ischemia model

The model used in this study was based on the Rice–Vannucci model (Vannucci and Vannucci, 1997). Pups were housed with the dam under a 12:12 h light–dark cycle, with food and water available *ad libitum* throughout the studies. These neonatal rats were anesthetized by inhalation with Diethyl Ether. The rats were kept at a temperature of 37 °C as the left common carotid artery was exposed and ligated with 5-0 surgical sutures. After operation, the

pups were returned to the holding container. Anesthesia and surgery time averaged 5 min per pup. Surgery was completed for an entire litter, and the pups were allowed to recover within their dams for 1 h (for rehydration via nursing). Then they were placed in a jar perfused with a humidified gas mixture (8% oxygen balanced nitrogen) for 90 min. Both the jar and mixture were kept at 37 °C to maintain a constant thermal environment. All surviving pups were returned to their dams after hypoxia exposure.

4.4 Measurement of infarct ratio

At 24 h after administration of H₂ saline, the pups were decapitated and the left brain hemispheric volumes were measured. Briefly, the brains were quickly removed after decapitation and placed in cold saline for 5 min, cut at 2-mm intervals from the frontal pole into 5 coronal sections. After incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 37 °C, the brain slices were fixed in 4% formaldehyde for 24 h. The volumes of each of the sections were summed by an image analysis system (ImageJ, a public domain image analysis program, developed at the National Institutes of Health). The percentage of infarction (infarct ratio) was calculated by dividing the infarct volume by the total volume of the slices.

4.5 Nissl staining

For Nissl staining, the 4- μ m sections were hydrated in 1% toluidine blue at 50 °C for 20 min. After rinsing with double distilled water, they were dehydrated and mounted with permount. The cortex and the CA1 area of hippocampus from each animal were captured and Imaging-Pro-Plus (LEIKA DMLB) was used to perform quantitative analysis of cell numbers.

4.6 TUNEL staining

TUNEL staining was performed on paraffin-embedded sections by using the in situ cell death detection kit (Roche). According to standard protocols, the

sections were dewaxed and rehydrated by heating the slides at 60 °C. Then these sections were incubated in a 20 µg/ml proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37 °C. Dried area around sample and added Converter-AP on samples for 1 h at 37 °C. After rinsing with PBS (5 min, 3 times), sections were coloured in dark with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyphosphate (BCIP).

4.7 Cell counting

Six visual fields (0.6 mm²) of the cerebral cortex and CA1 were photographed in each section. The number of staining cells in each field was counted at higher magnification (×40). The data were represented as the number of cells per high-power field.

4.8 Caspase-3 activity assay

Brain samples from the cortex and hippocampus were taken from the impaired hemispheres of neonatal rats 24 h after H₂ saline administration. The activity of caspase-3 was measured with caspase-3/CPP32 Fluorometric Assay Kit (BIOVISION Research Products 980 Linda Vista Avenue, Mountain View, CA 94043 USA). Briefly, brain samples were homogenized in ice-cold cell lysis buffer and kept at 4 °C for 1 h. Brain homogenate was centrifuged (Eppendorf, 5810R) at 12,000 g for 15 min at 4 °C. The supernatant was collected and stored at -80 °C until use. Protein content was measured by using the Enhanced BCA Protein Assay Kit. 20-200 µg cell lysates were incubated in a 96-well plate with 2×Reaction Buffer (50 µl). The reaction was started by adding 1 mM DEVD-APC substrate (5 µl). After incubation in the dark at 37 °C, the plate was read in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter.

4.9 Detection of MDA

Lipid peroxidation levels were measured with the thiobarbituric acid (TBA) reaction. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to thiobarbituric acid (TBA) with MDA at 535 nm. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution was added to 0.5 ml homogenate in centrifuge tube and placed in a boiling water bath for 15 min. The mixture was cooled and centrifuged at 1000× g for 10 min. Next, 2 ml of the supernatant was added to 1 ml of 6.7 g/l TBA solution in a test tube, and placed in a boiling water bath for 15 min. The solution was then cooled and its absorbance was measured with a spectrophotometer (UV-WFZ75, Shanghai, China). TBARS levels were expressed as nmol/mg protein in the brain.

4.10 Immunohistochemical procedures

Rats were perfused through the left ventricle of the heart with PBS and then with 4% paraformaldehyde in PBS. The fixed brains were immersed in 20% sucrose in PBS overnight, quickly frozen with dry ice powder, sliced into 12–14 µm sections with a cryostat, and stored at –80°C. For the Iba1 staining, sections were washed in PBS, incubated in 0.3% H₂O₂ in methanol for 30 min to inhibit endogenous peroxidase activity, washed in PBS, and blocked in PBS containing 1.5% normal goat serum and 1% BSA for 2 h at room temperature. The sections were then incubated with a rabbit anti-Iba1 polyclonal antibody (Abcam, UK) overnight at 4°C, washed in PBS, and incubated with a HRP-conjugated goat anti-rabbit IgG antibody for 2 h at room temperature. They were then incubated with 50 mM Tris-HCl (pH 7.2) containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂. For control staining, normal rabbit IgG was used as the primary antibody.

4.11 Function test

The postural reflex test (Bona et al., 1997) was used to evaluate functional recovery in the pups 5 weeks after injury. The examiner was blinded to the

experimental protocols. The pups were held by the tail 50 cm above the table. Normal rats extend both forelimbs toward the table (Score 0). Pups with brain damage flex the forelimb contralateral to the damaged hemisphere (Score 1). Thereafter, the pups were put onto the table, and a lateral pressure was applied behind the shoulder of the pup until the forelimbs slid. This was repeated several times, and a reduced resistance to lateral force toward the contralateral side was considered abnormal (Score 2).

4.12 Spontaneous activity test

Locomotor activity testing was performed once per animal in 42×42 cm chambers equipped with a 16×16 grid of infrared LED-photodetector pairs in the x and y planes and an additional elevated set in the x plane to record rearing movements as described previously by Reed et al (Reed et al., 2002). Total distance, rest time and move time were recorded for a total of 3 min for each animal.

4.13 Spatial Learning

Morris water maze testing was performed in a tank of 122 cm diameter with the water temperature maintained at 21 °C. The water was tinted with white tempera paint to obscure the platform. A 10 cm×10 cm platform was hidden 1 cm below the surface of water. Entry points were varied. Each trial lasted until either the rats had found the fixed platform or for a maximum of 3 min. All rats were allowed to rest on the platform for 20 s and each rat was allowed 4 trials per day for 4 days. 2 days after training, the test was performed again and the examiner determined the time of swimming until the rats reached the platform. The time spent in each quadrant was recorded and retention of the spatial training was assessed.

4.14 Data analysis

All quantitative data are expressed as mean±SD. The significance of

differences between means was verified by ANOVA followed by Tukey test. For analyzing the results of cell counting, a non-parametric Kruskal–Wallis ANOVA was used followed by Dunn's test. Chi-square analysis was used for the test based on the scoring system, spontaneous activity and water maze tests. Significance is defined as a probability of 0.05 or less.

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Fig. 1. Nissl staining of cerebral cortex (A1-E2), hippocampus (A3-E4) and cell counting, at 24 h after HI. (A) Representative slides of Nissl staining. Cortex and hippocampus in control, HI groups and H₂ saline groups at two different magnifications (A1-E1, A3-E3: ×10; A2-E2, A4-E4: ×40). More neuronal loss and dead cells appeared in the HI group after injury. In CA1 sector of control and H₂W group, the cell outline was clear and the structure was compact. Cells were big and have abundant cytoplasm and Nissl body (white arrow). In HI group, cells arranged sparsely and the cell outline was fuzzy. The cells with eumorphism were significantly reduced. (B) Cell counting. The number of Nissl staining cells in cortex and hippocampus of HI group was lower than that of H₂W groups, especially in 5 ml/Kg group (*P<0.05; **P<0.01). (n=10 in control; n=20 in HI; n=20 in HI +H₂W)

Fig. 2. TTC staining and infarct ratio. (A) Representative slides of TTC-stained coronal sections were derived from 8-day-old neonatal rats after H₂ saline treatment. Marked cerebral infarction was observed in the HI group. (B) The infarct ratio was 10.8% in HI group and 0.99% in H₂W group (**P< 0.01). (n=8 in control; n=16 in HI; n=16 in HI +H₂W)

Fig. 3. TUNEL staining of cortex (A1-C2) and hippocampus (A3-C4) and TUNEL-positive cell counting at 24 h after HI. (A) TUNEL staining. Cortex and hippocampus in each group after H₂ saline administration are shown at two different magnifications (A1-C1, A3-C3: ×10; A2-C2, A4-C4: ×40). The TUNEL-positive material was localized in the nuclei of the neurons. In samples collected from the HI group, the damaged cells were characterized by a round and shrunken morphology. The processes disappeared and the neuronal body became rounded with strong TUNEL staining in the nucleus (white arrow). An occasional TUNEL-positive cell was found in control and H₂W group. (B) Cell counting. The cortex and hippocampus of HI group had a higher proportion of TUNEL-positive cells than that of H₂W group. H₂ saline significantly reduced the number of TUNEL-positive cells (**P<0.01). (n=10 in control; n=20 in HI; n=20 in HI +H₂W)

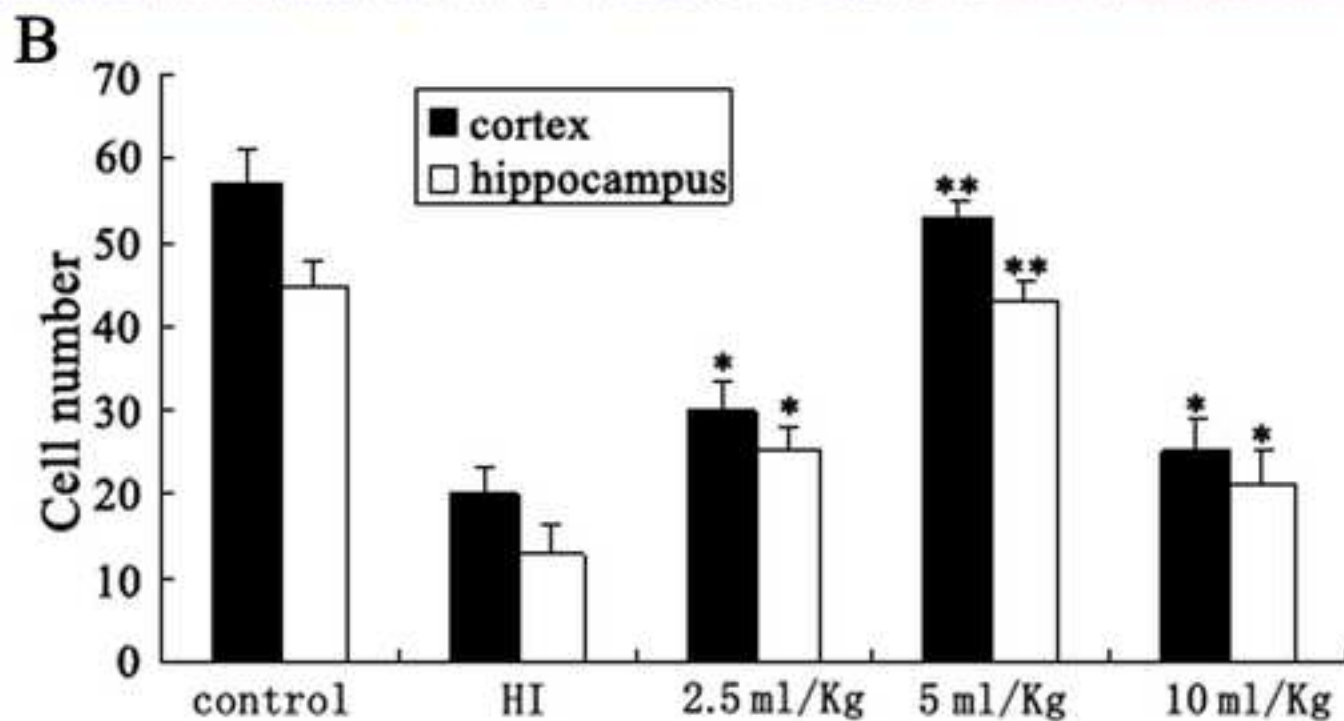
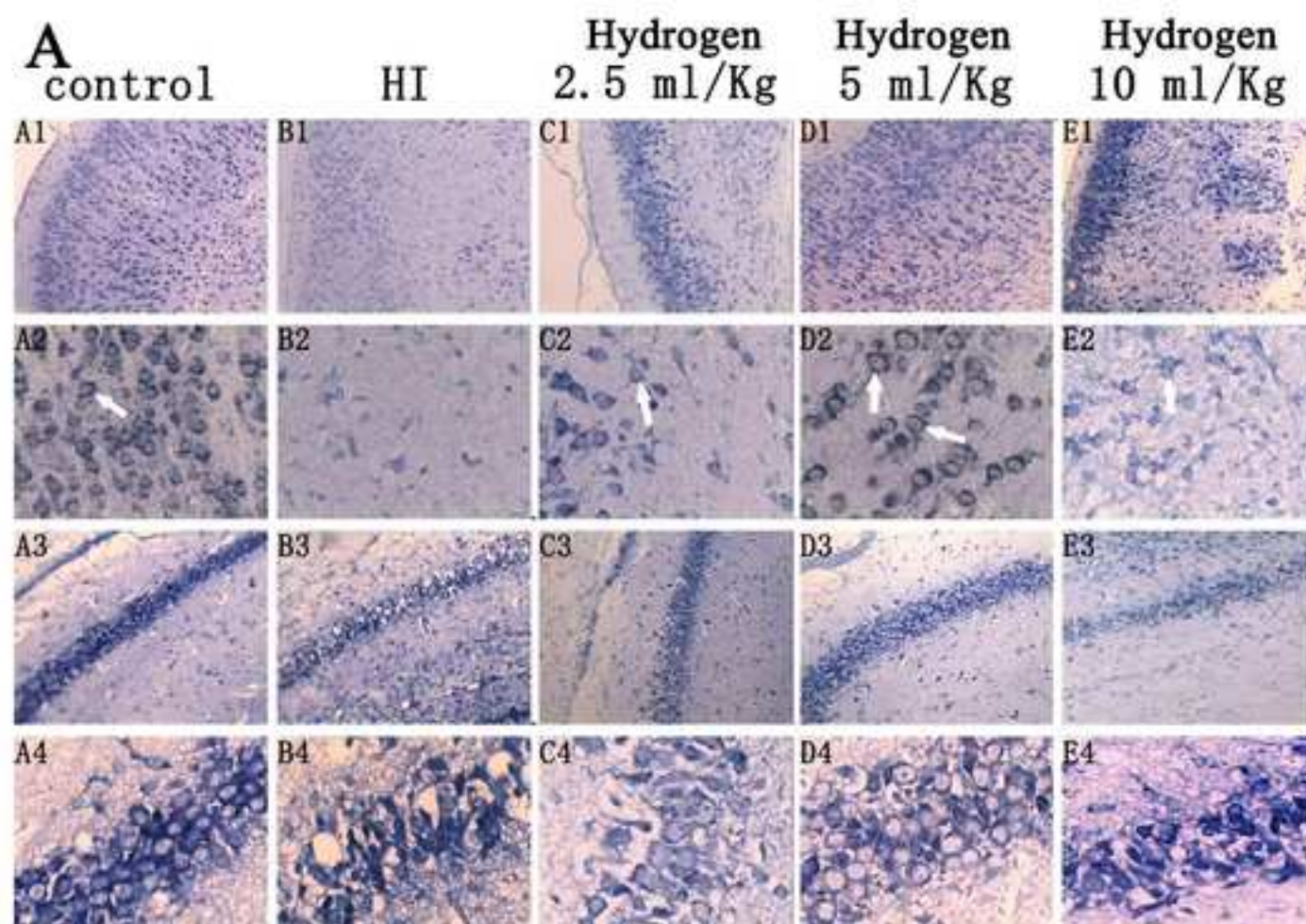
Fig. 4. Representative microscopic pictures stained with anti-Iba1 antibody at 24 h after HI. Iba1-positive cells had small nuclei, scant cytoplasm, and thin, branched processes. H₂ saline treatment reduced the number of Iba-1 positive cells.

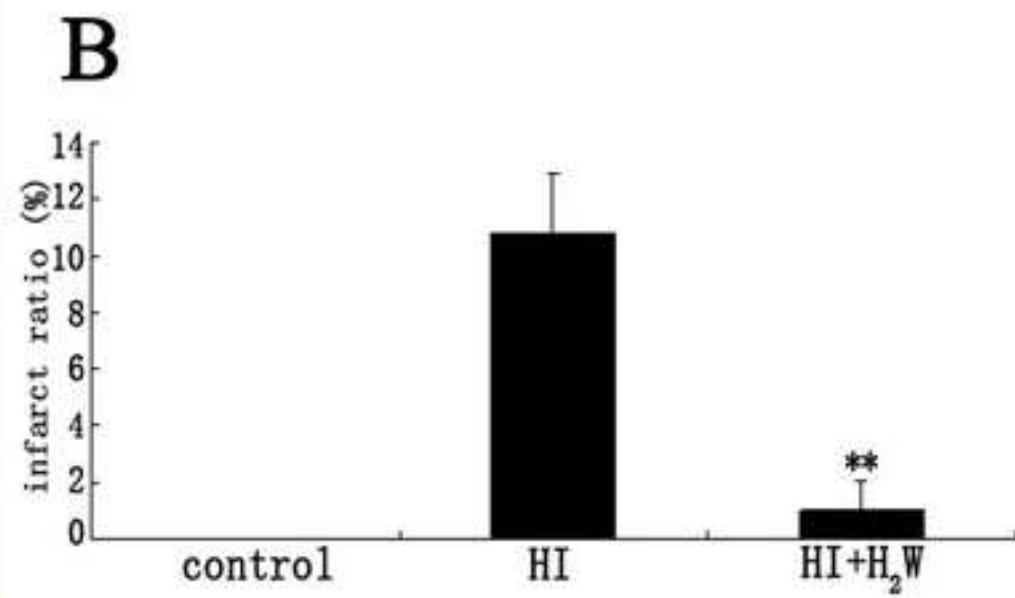
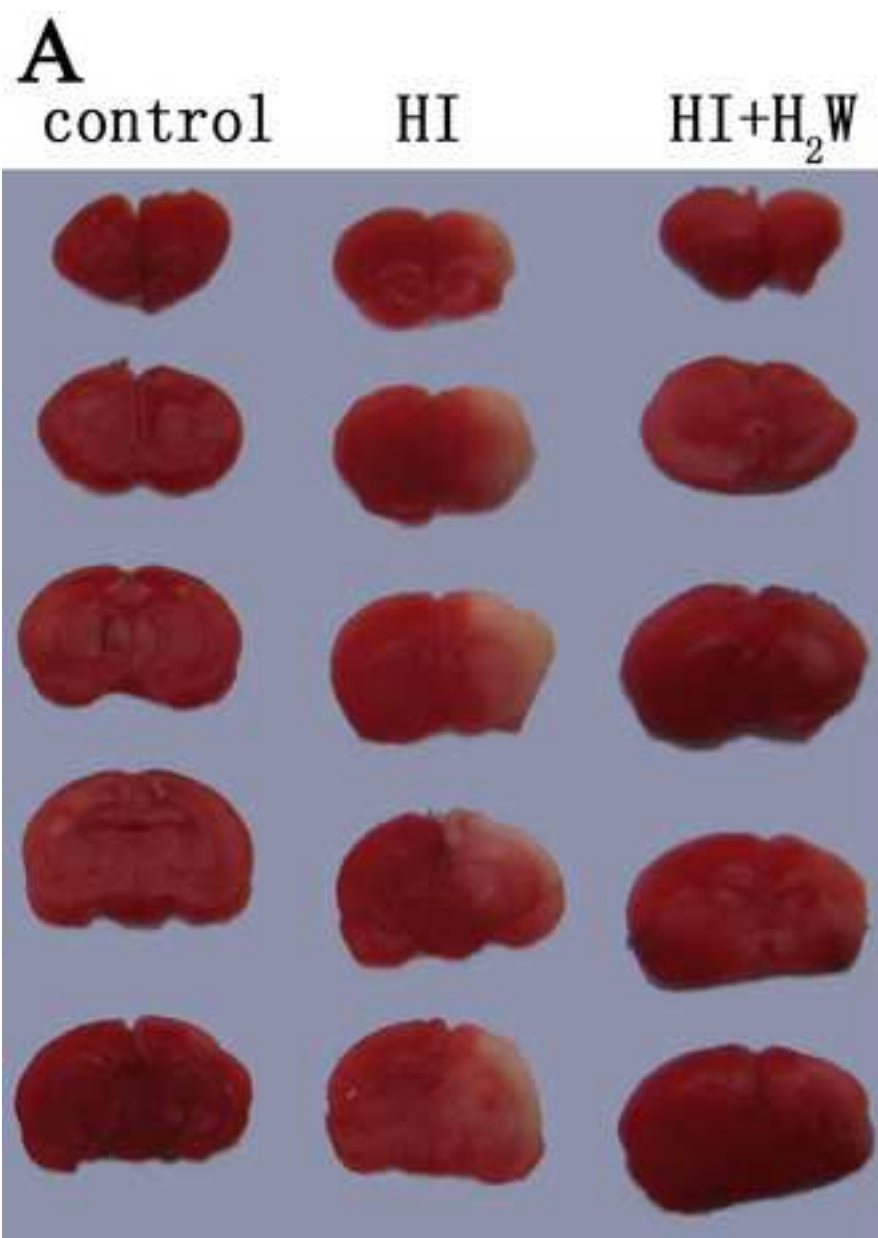
Fig. 5. Body weight. The difference in body weight between the three groups was not observed in the first 2 weeks after HI insult. However, the disparity was obvious between HI and HI+H₂W group at 2 weeks after HI insult (*P<0.05; **P<0.01).

Fig. 6. Spontaneous activity study. Rats in the HI group were more active than

those in other two groups. In HI group, the move time was more and the total distance was longer than those in HI+H₂W group at 5 weeks after HI (**P<0.01). (n=12 in control; n=24 in HI; n=24 in HI +H₂W)

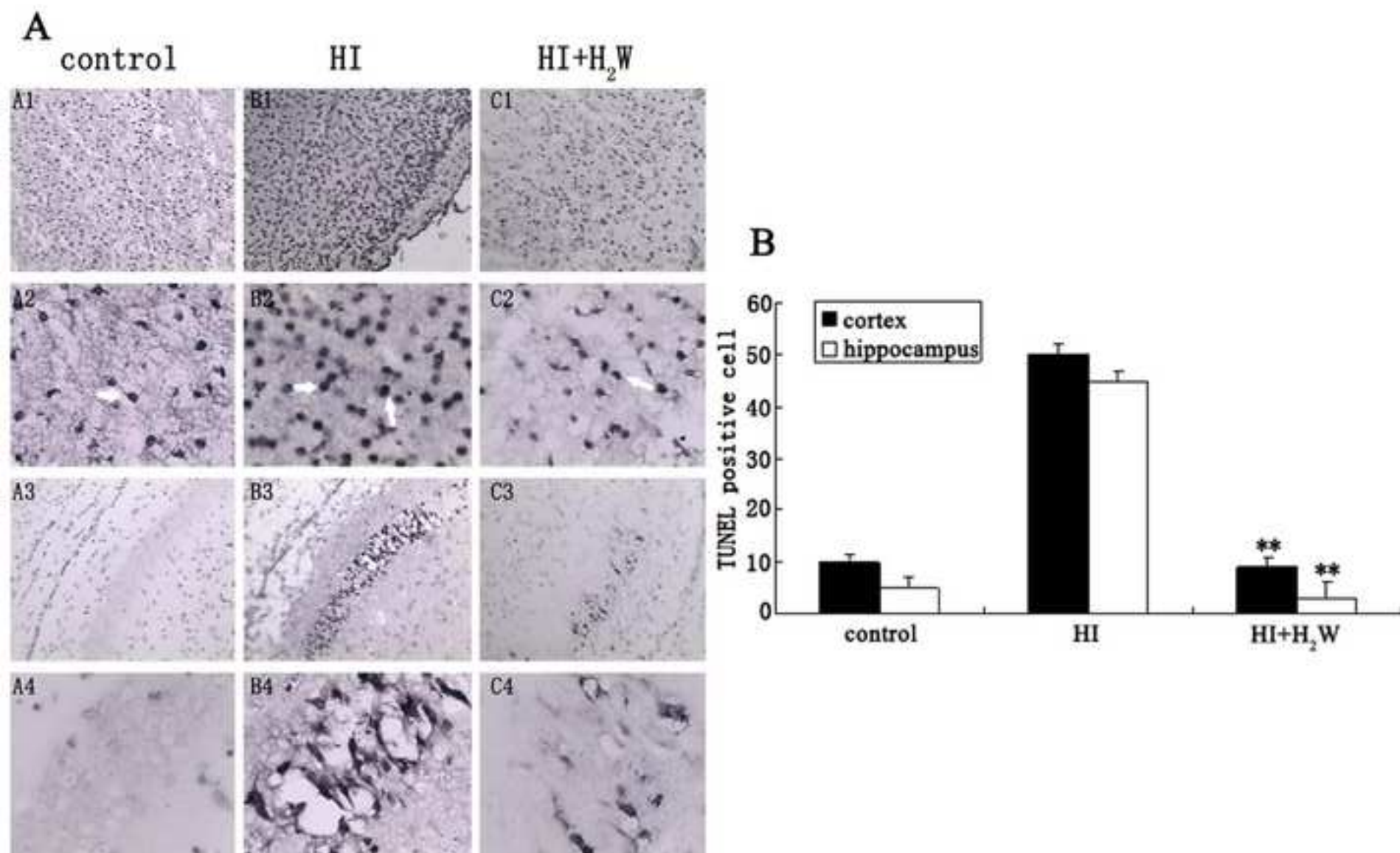
Figure
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Figure

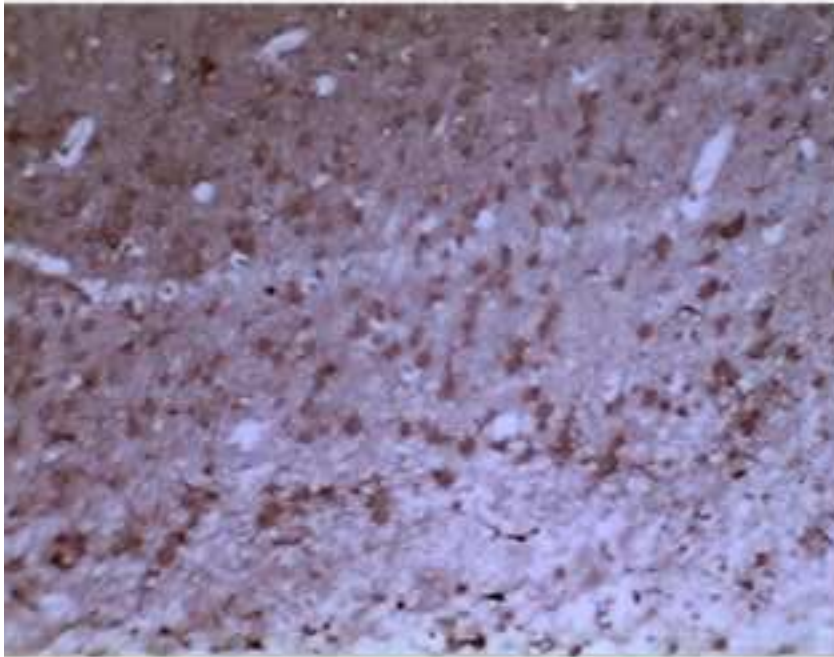
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HI



HI+H₂O

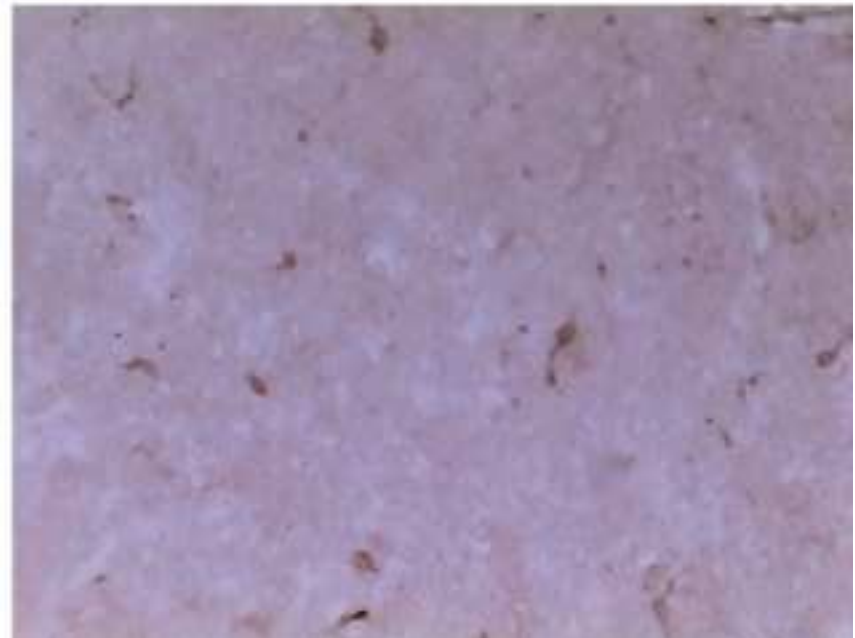
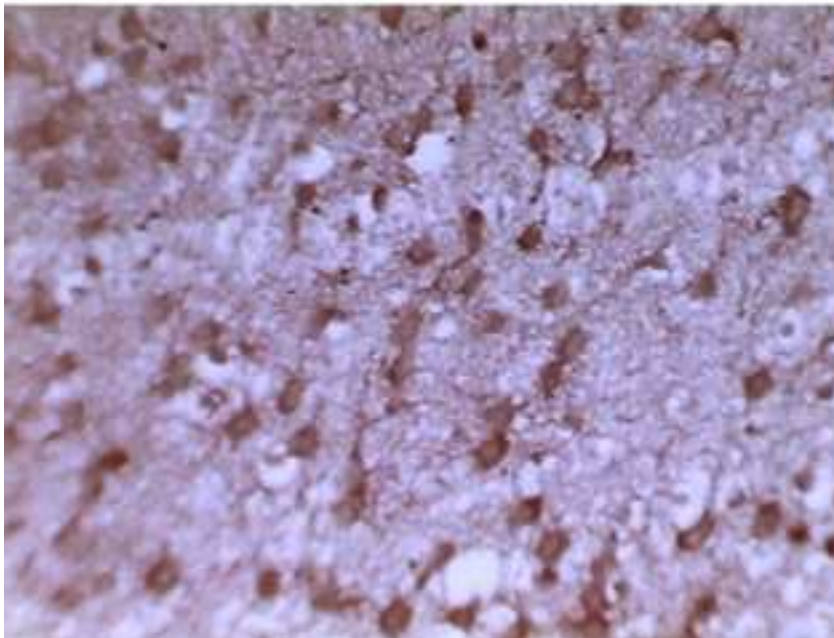


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