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Title: Hydrogen-rich saline reduces delayed neurologic sequelae in experimental carbon monoxide toxicity

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Keywords: hydrogen; delayed neurologic sequelae; CO poisoning; oxidative stress; inflammation; myelin basic protein

Corresponding Author: Dr Xuejun Sun, Ph.D, MD

Corresponding Author's Institution: Second Military Medical University

First Author: Qiang Sun, Ph.D

Order of Authors: Qiang Sun, Ph.D; Jianmei Cai, Ph.D; Jiangrui Zhou, Ph.D; Hengyi Tao, MD; JH Zhang, PH.D, MD; Wei Zhang, MD; Xuejun Sun, Ph.D, MD

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Abstract: Objective: We investigated the feasibility and efficacy of hydrogen-rich saline therapy on delayed neurologic sequelae in rats after severe acute carbon monoxide (CO) poisoning.

Design: Controlled animal study.

Setting: Research laboratory of a university hospital.

Subjects: Sprague-Dawley rats weighing 250 ± 20 g.

Interventions: The rats were exposed to 1000 ppm CO in air for 40 minutes and then to 3000 ppm for another 20 minutes until they lost consciousness. Rats were intraperitoneal injected with hydrogen-rich saline or normal saline (10ml/Kg) for six times after resuscitation at 0, 12, 24, 36, 48, and 60 hr, respectively. The rats without CO poisoning were used as normal controls.

Measurements and Main Results: CO poisoning induced brain tissue inflammation, cell death, and cognitive dysfunction (Morris Water Maze) at one week after the CO insult. Hydrogen-rich saline treatment significantly attenuated degraded myelin basic protein (MBP), decreased the expression of Iba1, a microglial marker, reduced DNA oxidation, and suppressed pro-inflammatory cytokine interleukin Interleukin-1β (IL-1β) , Interleukin-6 (IL-6) and TNF-a in the cortex and hippocampal tissues when compared with those in normal saline-treated rats. These histological and biological improvements were accompanied with an improvement in the Morris water maze test.

Conclusions: This observation demonstrated that hydrogen-rich saline peritoneal injection improves histological and functional assessment of CO encephalopathy in a rat model. Hydrogen saline has potentials as a novel and alternative therapy for severely CO-poisoned patients with delayed neurologic sequelae. The therapeutic effects of hydrogen-rich saline may be related to antioxidant and anti-inflammatory actions.

Suggested Reviewers:

Dear Editors:
May 19, 2010

Please find enclosed our manuscript entitled ***Hydrogen-rich saline reduces delayed neurologic sequelae in experimental carbon monoxide toxicity***?

Hydrogen has been proved to be a novel antioxidant through its selectively reducing of the hydroxyl radical. Both in vivo and in vitro studies support the protective effect of hydrogen on injuries caused by oxidative stress. Our previous study has demonstrated that hydrogen-rich saline could reduce brain injury induced by ischemia and hypoxia insult in rats. Carbon monoxide (CO) poisoning is one of the most common gas intoxications confronting emergency physicians worldwide. Among survivors, more than half of those with serious poisoning develop a delayed neurologic sequelae which is lack of effective treatment. The pathophysiology of CO poisoning is related not only to hypoxia but also to excitatory amino acid and oxidative stress. In this study we investigated the effects of hydrogen-rich saline on the prevention of delayed neurologic sequelae in experimental carbon monoxide toxicity. We have shown that hydrogen-rich saline which is safe, easy to administer, cost-effective, has therapeutic effect on CO poisoning induced encephalopathy by reducing oxidative stress and inflammatory cascades in brain tissues. Hydrogen-rich saline has potentials as an alternative treatment for neurologic injury after severe acute CO poisoning.

This article is original, and not currently under consideration for publication elsewhere. The authors have no conflicts of interest to declare.

We shall look forward to hearing from you at your earliest convenience.

Thank you very much!

Sincerely yours,

Xue-jun Sun MD, Ph.D

Department of Diving Medicine, Faculty of Naval Medicine
Second Military Medical University
800 Xiang Yin Road
Shanghai, 200433, P.R. China
Tel: +86-21-81871141
Fax: +86-21-65492382
E-mail: sunxjk@hotmail.com

Hydrogen-rich saline reduces delayed neurologic sequelae in experimental carbon monoxide toxicity

Qiang Sun[a], Jianmei Cai[b], Jiangrui Zhou[c], Hengyi Tao [a], John H Zhang[d], Wei Zhang[b*], Xuejun Sun[a*]

[a] Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai, 200433, P. R. China; [b] Department of Neurology, Changhai Hospital, 174 Changhai Road, Shanghai 200433, PR China; [c] Laboratory of Stress Medicine, Department of Nautical Medicine, Second Military Medical University, 200433, P. R. China; [d] Department of Neurosurgery, Loma Linda University, Loma Linda, CA 92350, USA;

Qiang Sun and Jianmei Cai contributed equally to this work

The work was performed in the Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai, China.

The reprints will be not be ordered

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* Corresponding author. Wei Zhang and Xuejun Sun

Tel & Fax: +86-21-65492382.

E-mail address: van_zhang@sina.com (Wei Zhang);

sunxjk@hotmail.com (Xuejun Sun)

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improvement in the Morris water maze test.

Conclusions: This observation demonstrated that hydrogen-rich saline peritoneal injection improves histological and functional assessment of CO encephalopathy in a rat model. Hydrogen saline has potentials as a novel and alternative therapy for severely CO-poisoned patients with delayed neurologic sequelae. The therapeutic effects of hydrogen-rich saline may be related to antioxidant and anti-inflammatory actions.

Keywords: hydrogen; delayed neurologic sequelae; CO poisoning; oxidative stress; inflammation; myelin basic protein

Introduction

Carbon monoxide (CO) poisoning is one of the most common gas intoxications confronting clinical toxicologists, intensivists and emergency physicians worldwide (1). The clinical symptoms of CO poisoning are often nonspecific and diverse (2). Among survivors, more than half of those with serious poisoning develop a delayed neurologic sequelae (DNS) between 3 days and 4 weeks after CO poisoning (3). The pathophysiology of CO poisoning is related not only to hypoxia but also to excitatory amino acid and oxidative stress (4-6). Reactive oxygen species produced by activated neutrophils, mitochondria, and xanthine oxidase lead to lipid peroxidation which render delayed neurological injuries (7).

Hyperbaric oxygen therapy has been shown to significantly reduce the incidence of neuropsychiatric sequelae after CO poisoning when compared with treatment using normobaric oxygen therapy (8). However, the therapeutic value of hyperbaric oxygen is debatable (9-10) and there are side-effects (11). Hydrogen is a gaseous molecule without known toxicity, which chelates hydroxyl radical and affords neuroprotection in ischemia–reperfusion injury. Further studies demonstrated similar protective effects of hydrogen in ischemia-reperfusion injuries caused by oxidative stress in brain, liver, heart (12-14) and intestines (15). Hydrogen also provides anti-inflammatory effect in acute pancreatitis (16), colon inflammation (17), and liver inflammation. Recently, we hypothesize that hydrogen could act as a novel and effective

treatment of acute carbon monoxide poisoning (18). The aim of this study is to confirm our abovementioned hypothesis and to evaluate the feasibility and efficacy of hydrogen therapy for severe acute CO poisoning encephalopathy.

Materials and methods

Experimental Animals

Adult male Sprague–Dawley rats, weighing 250 ± 20 g, were provided by the Experimental Animal Center of the Second Military Medical University in Shanghai China. They were housed at a temperature of 22–24 °C and 12 hr light/dark cycle controlled environment with free access to food and water. All experimental procedures were approved by The Experimentation Ethics Committee of the Second Military Medical University in Shanghai, China. Rats were randomly assigned to the following groups: normal group (n=14); normal+hydrogen saline group (n=7); CO+saline (n=14) and CO+ hydrogen saline group (n=14).

Drugs

Hydrogen was dissolved in physiological saline for 6 hr under high pressure (0.4 MPa) to a supersaturated level using a hydrogen-rich saline producing apparatus constructed in our lab. The saturated hydrogen saline was stored under atmospheric pressure at 4 ºC in an aluminum bag with no dead volume and was sterilized by gamma radiation. The hydrogen level in the

saline was measured by gas chromatography using the method described by Ohsawa (19). The average saline hydrogen level was 0.86 mmol/L. Hydrogen saline was freshly prepared every week to ensure that a concentration of more than 0.6 mmol/L was maintained.

Animal Manipulations

CO poisoning was performed according to the published protocol (4) in a 7-liter Plexiglas chambers. Rats breathed 1000 ppm CO for 40 min then 3000ppm for up to 20 min, until they lost consciousness, then they were removed to breath room air and regain consciousness. Hydrogen-rich saline or normal saline (10ml/kg) was administered intraperitoneally for six times after resuscitation at 0, 12, 24, 36, 48, and 60 hr, respectively.

Nissl Staining

Perfusion-fixed brain tissues were further fixed overnight in the solution (4% paraformaldehyde in PBS), processed for embedding in paraffin and cut into 4-μm-thick serial sections. For Nissl staining, the 4-μm 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.

Immunohistochemical staining

For immunostaining, brain sections immersed in 0.3% H_2O_2 in 95% ethanol for 30 min and rinsed in PBS. Blocked in PBS containing 1.5% normal goat serum and 1% BSA for 2 hr at room temperature and stained with anti-Iba1 or anti-MBP (Abcam,UK) overnight at 4°C, followed by HRP or FITC-conjugated goat anti-rabbit IgG antibody for 2 hr at room temperature. They were then incubated with 50 mM Tris–HCl (pH7.2) containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2. Slides were observed under Fluorescence Microscope (LYMPUS BX-60).

Determination of TNF-α, IL-1β, IL-6 and 8OH-dG protein levels in brain tissues

Brain tissues were collected, weighed, and washed in normal saline and then homogenized immediately in 10 volumes of normal saline at 4°C. After centrifugation, supernatants were collected and stored at -80°C. Levels of TNF-α, IL-1β, IL-6 and 8OH-dG were measured with a commercial ELISA kit (Biosource, Camarillo, CA) according to the instructions of the manufacturer. The absorbance was read on a microplate reader (Denley Dragon, Wellscan MK 3, Thermo, Finland) and the concentrations were calculated based on a standard curve.

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 90 sec. All rats were allowed to rest on the platform for 20 sec and each rat was allowed 4 trials per day for 4 days. Two 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.

Statistical analysis

Values were presented as mean ± SD. Statistical analysis was done using the SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). For comparison of changes in Morris water maze test between groups, two-way ANOVA followed by Student-Newman-Keuls tests were utilized. For comparison of Nissl staining, TNF-α, IL-1β, IL-6 and 8-OHdG levels, differences between groups were determined with a one-way ANOVA followed by Student-Newman-Keuls tests. P value less than 0.05 was considered statistically significant.

Results

Morris water maze

The escape latency was measured at 9 day after CO poisoning. The result of ANOVA revealed significant improvement by hydrogen-rich saline on escape latency (P<0.05). As displayed in Figure 1, the escape latency remained unchanged with hydrogen-rich saline alone (Normal+hydrogen). However, the escape latency in CO+hydrogen group was shorter than that in CO+saline group, indicating that hydrogen-rich saline significantly improved spatial recognition and learning declined by CO poisoning ($^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group).

Nissl staining

Representative samples of Nissl staining from the cerebral cortex and hippocampus were done at 7 day after CO poisoning. As shown in figure 2, most neurons of cortex both in CO+saline and CO+hydrogen groups and of CA1 sector in CO+saline group were shrinked with intracellular space enlarged and had deep color staining, a part of neurons disappeared. The cells with eumorphism in the cortex and CA1 sector were significantly preserved in CO+hydrogen. The number of Nissl staining cells in cortex and hippocampus of CO+saline group was lower than that of CO+hydrogen group ($^{*}P$ <0.05 *vs.*normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group).

MBP staining

The brain slices were stained with MBP to identify neurofiber connectivity and linear diffusion. MBP poisitive neurofiber of cortex and hippocampus in CO+saline and CO+hydrogen group lined up in a diffusion manner, diminished branches, more cavities between fibers (Figure. 3). Hydrogen-rich saline significantly protected the connectivity of MBP positive neurofiber in cortex and hippocampus compared to that in CO+saline group.

Ib1 fluorescence staining

In order to determine whether microglia is activated in the brain at 7 day after CO poisoning, sections cerebral cortex were prepared from rat brain for immunohistochemical staining with anti-Iba1 antibody. Figure 4 demonstrated that the expression of microglia defined by Iba-1 positive staining in normal group is relatively low. Hydrogen-rich saline significantly reduced the expression of microglia when compared with that in CO+saline group.

TNF-α, IL-1β and IL-6 levels in brain issue

As shown in figure 5, TNF-α, IL-1βand IL-6 concentrations of the cerebral cortex and hippocampus were significantly decreased in CO+hydrogen group when compared with those in CO+saline group ($^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group).

8-OHdG levels in brian issue

8-OH-dG concentrations in the cerebral cortex and hippocampus of brain were significantly elevated in CO+saline group when compared with those in CO+hydrogen group. Hydrogen-rich saline treatment markedly lowered CO poisoning-induced elevation of 8-OHdG concentrations (Figure. 6; $^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group).

Discussion

To our knowledge this is the first study that demonstrated hydrogen-rich saline significantly prevented delayed neurologic sequelae in rats after severe acute CO poisoning. The protective effect was supported by significant cognitive improvement, marked preservation of structure of brain, reduced degraded MBP and DNA oxidation, inhibition of Iba1 expression, and attenuated pro-inflammatory IL-1β, IL-6 and TNF-a cytokine levels. This study demonstrated that hydrogen rich saline could be a novel and effective therapy for CO poisoning encephalopathy. This observation is especially important because except the controversial hyperbaric oxygen treatment (20), no other effective treatment is available for CO poisoning encephalopathy.

Carbon monoxide increases cytosolic heme levels, leads to oxidative stress, and binds to platelet heme protein and cytochrome *c* oxidase, interrupts cellular respiration and causes production of reactive oxygen species, which in turn lead to neuronal necrosis and apoptosis (1). Hydrogen selectively reacts with hydroxyl radical and peroxynitrite, exerts protective effects, without interactions with other reactive oxygen species, such as superoxide anion and H_2O_2 (19). In the present study, hydrogen-rich saline significantly reduced product of DNA lipid oxidation 8-hydroxydeoxyguanosine (8-OHdG), which is consistent with previously reports (13).

Carbon monoxide exposure also leads to inflammation which contributes to CO poisoning encephalopathy (5, 6, 21). In our study,

hydrogen-rich saline treatment significantly attenuated the enhanced expression of pro-inflammatory cytokine IL-1β , IL-6 and TNF-a in the cortex and hippocampus tissues when compared with those in saline-treated rats. CO poisoning activated microglia which is involved in inflammation and cell death (22-23). Hydrogen-rich saline treatment reduced the expression of microglia marker, Iba1, in brain tissues after CO poisoning, indicated hydrogen saline decreased microglia activation. In addition, it has been reported that an adaptive immunologic response to chemically such as carbon monoxide altered MBP, which is the major myelin protein of central nervous system, contributes to neurologic sequelae at least in animal models (4-5). Hydrogen-rich saline treatment preserved the degraded MBP by CO poisoning, indicating hydrogen-rich saline reduced inflammatory reaction caused by CO poisoning.

Conclusions

In this study, we have shown that hydrogen-rich saline which is safe, easy to administer, cost-effective, has therapeutic effect on CO poisoning induced encephalopathy by reducing oxidative stress and inflammatory cascades in brain tissues. Hydrogen-rich saline has potentials as an alternative treatment for neurologic injury after severe acute CO poisoning.

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Figure and Legends

Figure 1. Escape latencies which were observed at 9 days after CO poisoning. The escape latencies in CO+saline group were significantly longer than that of CO+hydrogen and control group. There was no differences between normal+hydrogen and normal group (n=7, $^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen).

Figure 2. Nissl staining of cortex and hippocampus and cell counting at 7 days after CO poisoning. (A) Nissl staining. Cortex and hippocampus in each group are shown at two different magnifications (×10, ×20). The cells with eumorphism in the cortex and CA1 sector were significantly preserved in CO+hydrogen. (B) Cell counting. The number of Nissl staining cells in cortex and hippocampus of CO+saline group was lower than that of CO+hydrogen group (n=7, $^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group).

Figure 3. Myelin basic protein (MBP) staining of cortex and hippocampus. Cortex and hippocampus in each group are shown at two different magnifications (×10, ×20). MBP-positive myelinated fibres of cortex and hippocampus were released after CO poisoning. However, it was relatively better-preserved in CO+hydrogen group compared to that in CO+saline group. Immunopositive fibers are stained in brown.

Figure 4. Iba1 fluorescence staining of rat brain. Sections of cerebral cortex were stained with the anti-Iba1 antibody (a microglial marker) 7 days after CO poisoning. Hydrogen-rich saline significantly reduced the expression of microglia when compared with that in CO+saline group.

Figure 5. Levels of Tumor necrosis factor-alpha (TNF-α), Interleukin-1β (IL-1β) , Interleukin-6 (IL-6) in cortex and hippocampus. (A) TNF-α levels; (B) IL-1β levels; (C) IL-6 levels. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA. Data are expressed as mean ± SD. N=7, $^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group.

Figure 6. Levels of 8-hydroxydeoxyguanosine (8-OHdG) in cortex and hippocampus. The levels of 8-OH-dG were detected by ELISA. Data are expressed as mean ± SD. N=7, $^{*}P$ <0.05 *vs.* normal; $^{\#}P$ <0.05 *vs.* CO+hydrogen group.

Figure 1

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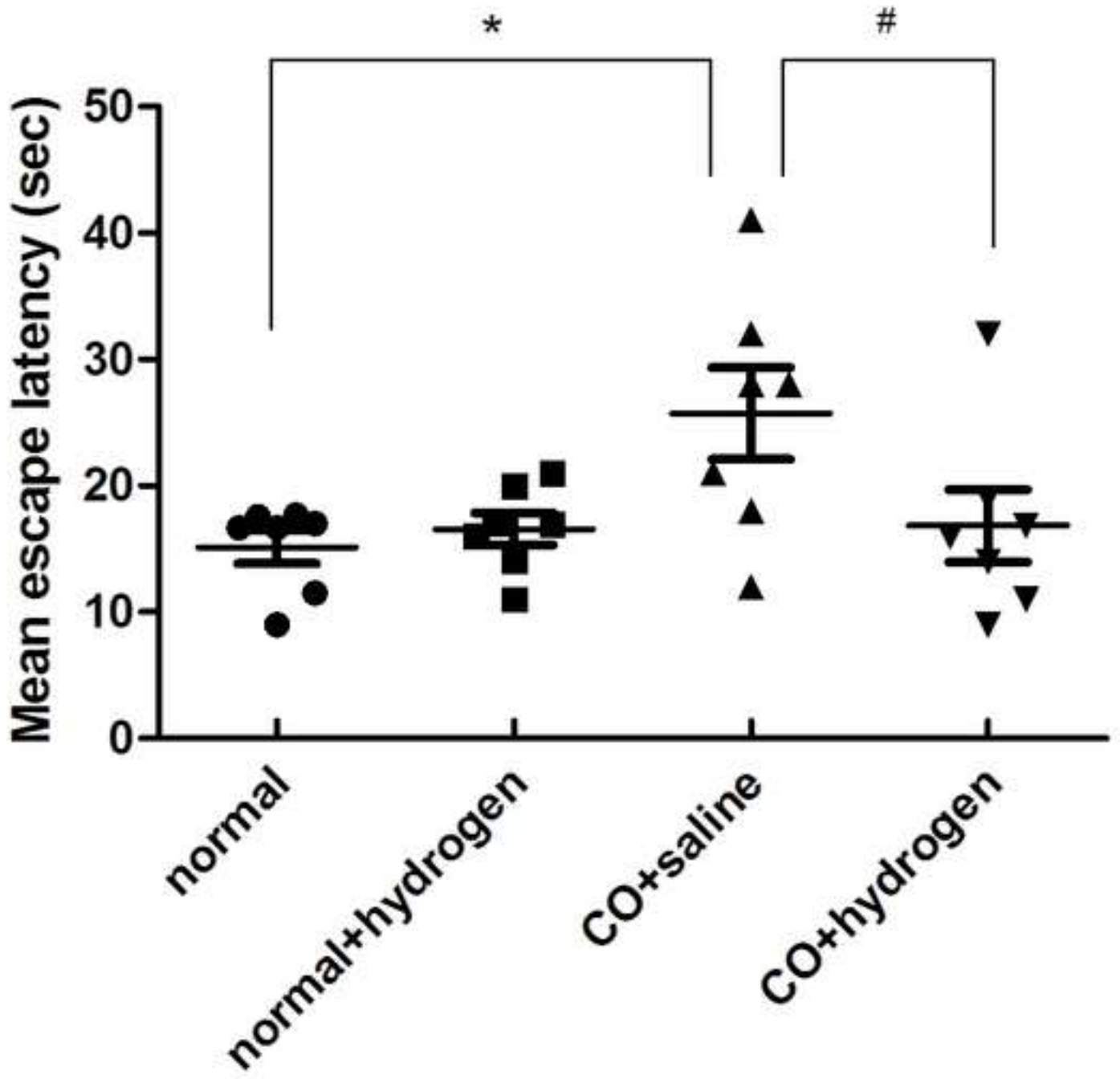

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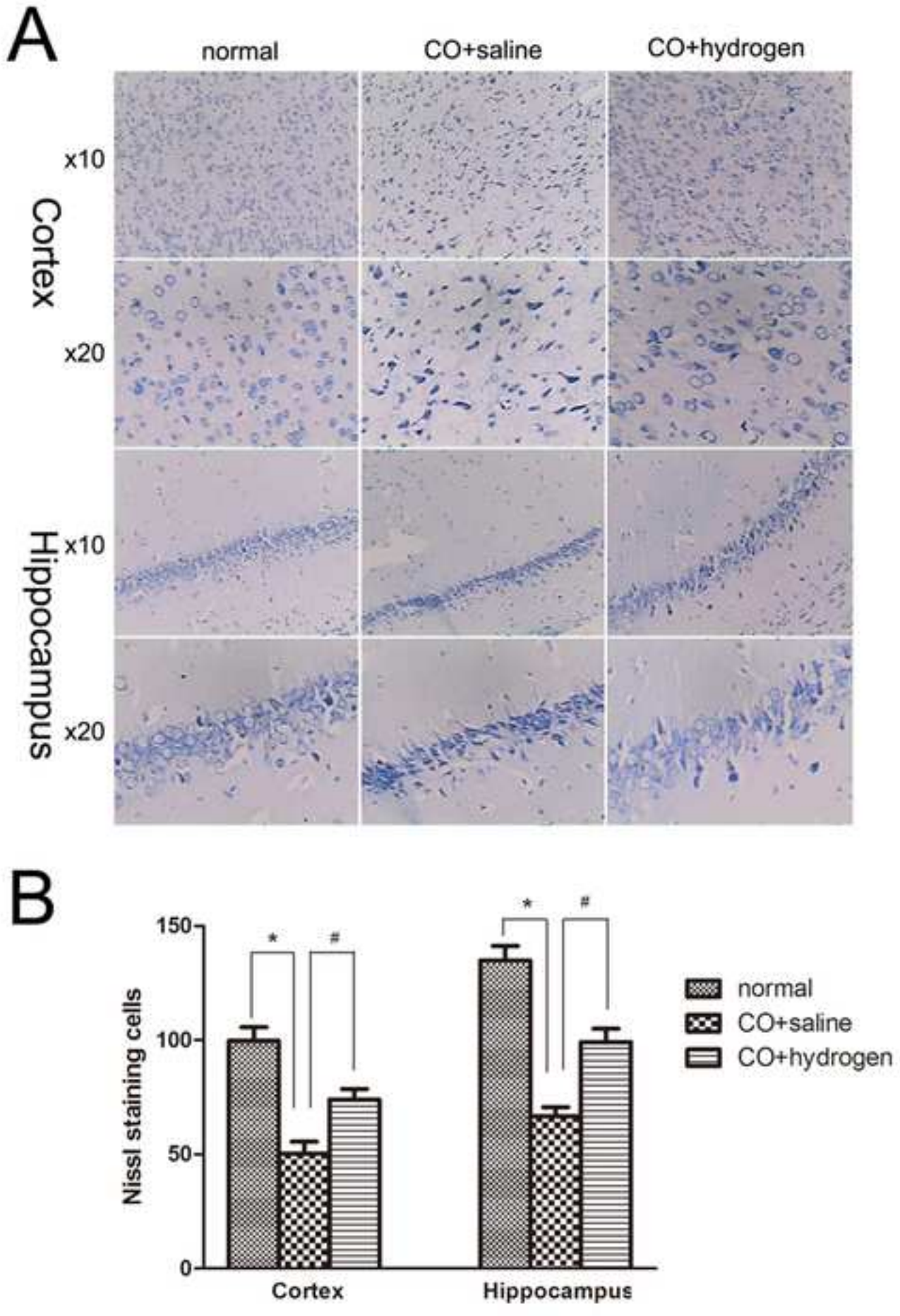

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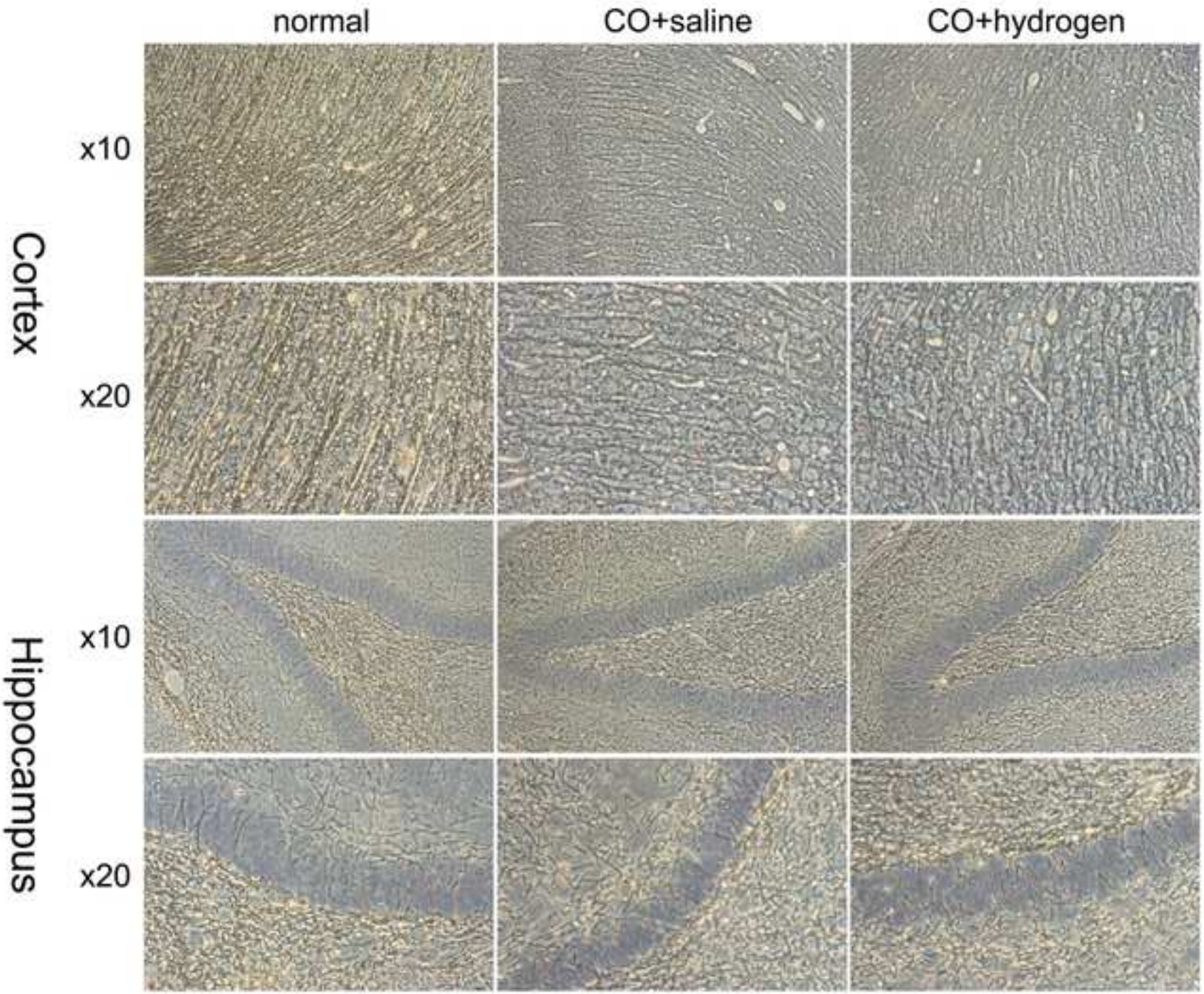

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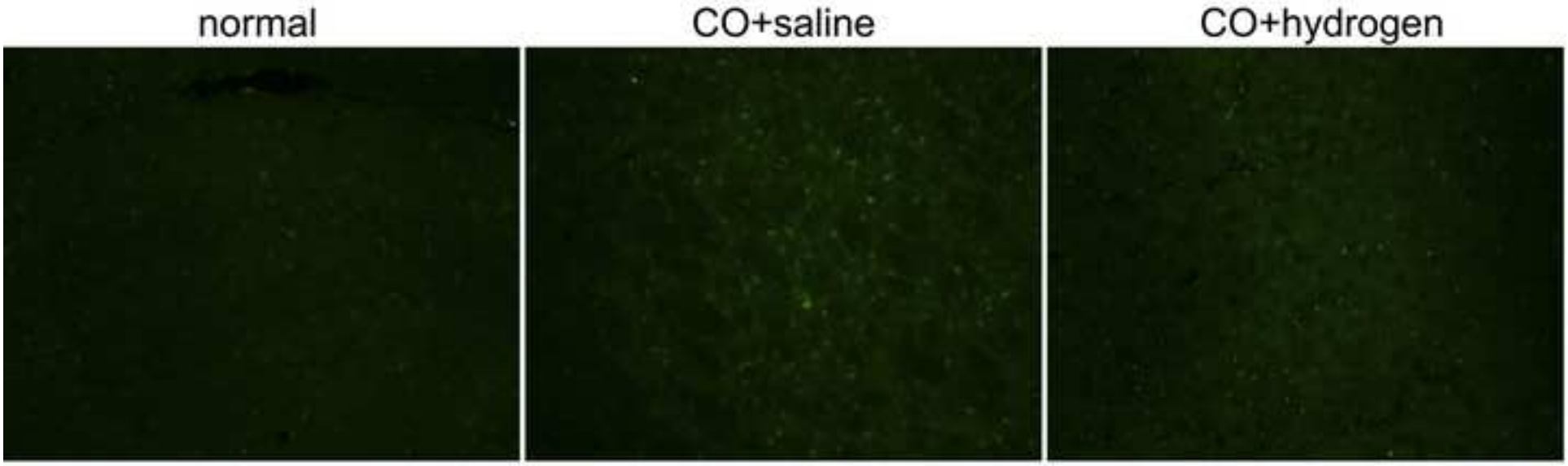

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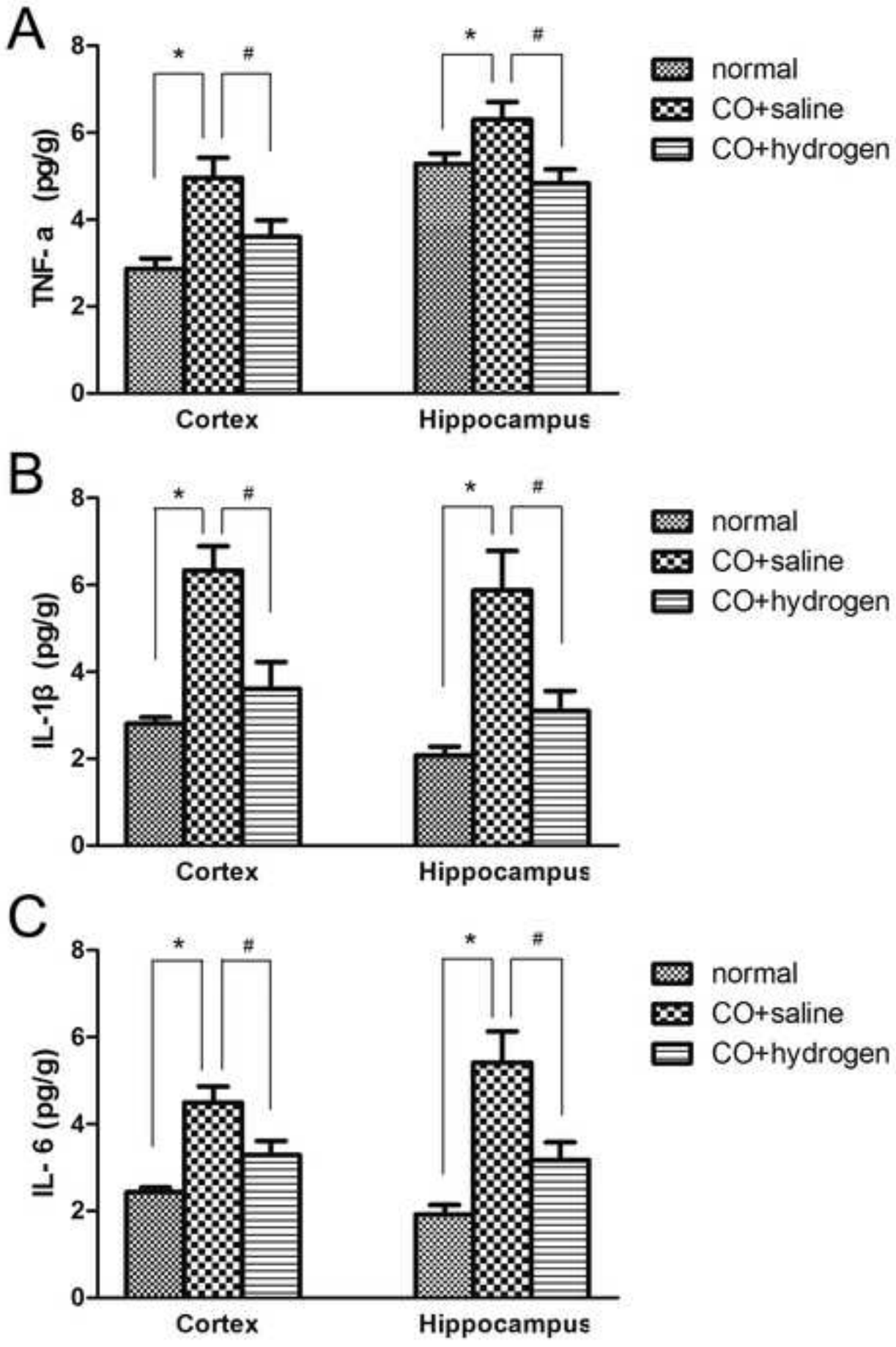

Figure 6

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