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Abstract

***Background*—**We have previously shown that hydrogen (H_2) inhalation, commenced at the start of hyperoxic cardiopulmonary resuscitation (CPR), significantly improves brain and cardiac function in a rat model of cardiac arrest (CA). Here, we examine the effectiveness of this therapeutic approach when H_2 inhalation is commenced upon the return of spontaneous circulation (ROSC) under normoxic conditions, either alone, or in combination with targeted temperature management (TTM).

***Methods and Results*—**Rats were subjected to 6 min VF CA followed by CPR. Five min after achieving ROSC, post-CA rats were randomized into four groups: mechanically ventilated (MV) with 26% O_2 and normothermia (control); MV with 26% O_2, 1.3% H_2 and normothermia (H_2); MV with 26% O_2 and TTM (TTM); MV with 26% O_2, 1.3% H_2 and TTM (TTM + H_2). Animal survival rate at 7 d after ROSC was 38.4% in the control group, 71.4% in the H_2 and TTM groups, and 85.7% in the TTM+H_2 group. Combined therapy of TTM and H_2 inhalation was superior to TTM alone in terms of neurological deficit scores at 24, 48, and 72 h post-ROSC, and motor activity at 7 d post-ROSC. Neuronal degeneration and microglial activation in a vulnerable brain region was suppressed by both TTM alone and H_2 inhalation alone, with the combined therapy of TTM and H_2 inhalation being most effective.

***Conclusions*—**H_2 inhalation was beneficial when commenced after ROSC, even when delivered in the absence of hyperoxia. Combined TTM and H_2 inhalation was more effective than TTM alone.

Key words: cardiopulmonary resuscitation, heart arrest, ischemia/reperfusion injury/neuroprotection, antioxidant, hydrogen gas

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Introduction

Ischemia–reperfusion (I/R) is a critical cause of rapid and acute oxidative stress in post-cardiac arrest syndrome (PCAS). The reactive oxygen species (ROS) generated by reperfusion of the ischemic brain are therefore a potential target for preventing ischemic brain injury. In 2007, Ohsawa *et al.*[1] discovered that hydrogen gas (H_2) has antioxidant and anti-apoptotic properties that protect the brain against I/R injury by selectively neutralizing hydroxyl radicals. Since then, the efficacy of H_2 on I/R injury has been studied extensively.[2, 3] Inhalation of 1-4% H_2 reduces infarct size in rat models of acute cerebral, and coronary artery, occlusion, with 2% H_2 being most effective.[1, 4] These pioneering studies were followed by publications from a number of groups worldwide demonstrating 1.3%-3% inhaled H_2 protects against acute oxidative stress.[5-12]

We demonstrated previously that inhalation of 2% H_2 commenced at the beginning of hyperoxic (98% O_2) cardiopulmonary resuscitation (CPR), and until 2 hours after the return of spontaneous circulation (ROSC), improved survival and neurological deficit score (NDS) in a rat model of cardiac arrest.[12] This effect was comparable to that of targeted temperature management (TTM) alone (33°C).[12] Several issues require further investigation before clinical application of these findings becomes feasible. First, there was no direct evidence for preservation of brain function at a histological level. A brief episode of global brain ischemia produces selective and often extensive neuronal loss in vulnerable brain structures in humans and rodents, such as the hippocampal CA1 pyramidal neurons. In addition, cell death does not occur immediately, but is delayed for days (delayed neuronal death). Accordingly, previous reports assessed CA1 pyramidal neuron necrosis at 10 and 30 days[13] or at 7 days[14]

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post-ischemia. In our previous study, although H_2 inhalation improved neurological outcome (based on the NDS) at 24 hours after ROSC, this phenomenon was not associated with a reduction in either brain edema or hippocampal CA1 pyramidal neurons at this early time point. Second, in our previous study inhalation of H_2 commenced at the beginning of CPR, but in a clinical setting hypothermia is applied after ROSC. Therefore, this present study investigated whether the benefit of H_2 inhalation is similar when commenced after ROSC. Third, because all animal groups in our previous study were ventilated using 98% O_2, H_2 may protect only from the harmful effects of hyperoxia. We wish to investigate therapy benefit under normoxic conditions.

We therefore investigated whether H_2 inhalation without hyperoxia improves neurological outcome in a rat model following resuscitation from CA, independent of TTM.

Materials and methods

Animal preparation

Seventeen-week-old male Wistar ST rats weighing an average of 408 g were used in this study after obtaining institutional approval from the Animal Ethics Committee, and were housed in a rodent facility under 12 h light–dark cycle conditions.

The rats were fasted overnight except for free access to water, and then were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg). The trachea was intubated via a tracheostomy with a 14-gauge cannula and mechanically ventilated (MV) with a tidal volume (TV) of 0.65 ml per 100 g body weight, a respiratory rate of 100/min, and FiO_2 of 0.21 (Ventilator: SN-480-7, Shinano, Japan). Polyethylene catheters (PE50,

Natsume, Japan) were inserted into the left femoral arteries and veins, and flushed intermittently with saline solution containing 2.5 IU/ml bovine heparin. Arterial blood pressure was measured and an electrocardiogram (ECG) was recorded by subcutaneous needle electrodes. Core temperature was monitored by a rectal temperature probe (BAT-10, Physitemp Instruments Inc., NJ, USA) and maintained using a heating plate (SCP-85, AsOne, Japan) throughout the experiment as appropriate temperature management.

Ventricular fibrillation and CPR model

Ventricular fibrillation (VF) was induced by electrical stimulation via a transthoracic epicardium electrode, as previously described.[15] This stimulator (Isostim, World Precision Instruments Inc., FL, USA) was used to perform direct and constant electrical stimulation of the epicardium with crude current, continuous single stimulation, a delay of 100 ms, a wave width of 1 ms, a frequency of 50 Hz, an intensity of 1 mA, and stimulation duration of 3 min. The MV was stopped and disconnected from the tracheal tube after onset of VF. CA was defined by an abrupt blood pressure drop, disappearance of the pulse wave signal, and VF on the ECG recording. After 6 min of VF induction, advanced cardiac life support started, in which the rats were ventilated (0.65 ml/100 g, 100 breaths/min) and chest compressions (200/min) were started by the investigator's finger, paced by a metronome. Chest compressions were adjusted to a uniform rate, and a target aortic diastolic pressure of > 20 mm Hg.[16] Adrenalin (2 μg/100 g) and 0.1 ml sodium bicarbonate (8.4%) were immediately administered to the rats at the beginning of CPR and repeated at 3-min intervals as needed.[12, 17-20] The administration of intravenous fluid during CPR was limited to under 2 ml. Three min after commencing chest compressions, defibrillation was performed with direct-current

single-phase waves of 2 J when the ECG displayed VF. If the defibrillation failed, CPR was repeated and defibrillations were again performed 1 min after CPR. ROSC was defined as the return of supraventricular rhythm with a mean aortic pressure over 50 mm Hg for a minimum of 5 min. If spontaneous circulation was not restored in the rats after 6 min, CPR was considered to be a failure. The concentration of H_2 in the gas mixture was monitored using a Breath Gas Analyzer Model TGA-2000 (TERAMECS, Kyoto, Japan). Sham-operated controls were subjected to the same operative procedure, without electrical stimulation.

Premixed gas comprising H_2 gas and oxygen

The maximal concentration of flammable H_2 is 1.3% in mixed gas formulations comprising flammable gas and oxygen at greater than atmospheric concentration. The *High Pressure Gas Safety Act* in Japan states that flammable gas contained in the mixed gas cannot exceed one third of the lower explosion limit (4%). Our preliminary experiments revealed that although 2% inhaled H_2 is superior to 1% inhaled H_2 for reducing reactive oxygen metabolites in blood, both are essentially equivalent in suppressing systemic inflammatory activation, and equally improved the survival and functional outcomes in our rat model of CA with VF (data not shown). Therefore, we decided to use premixed gas comprising 1.3% H_2 and 26% O_2 in the following experiments.

Experimental protocol

Five min after achieving ROSC, animals were randomized into four groups (2:1:1:1 ratio): MV with 26% O_2 and normothermia (control: Ctl group, n = 13); MV with 26% O_2, 1.3% H_2 and normothermia (H_2 group, n = 7); MV with 26% O_2 and TTM (TTM group, n = 7); and MV with 26% O_2, 1.3% H_2 and TTM (TTM + H_2 group, n = 7). Gas inhalation was

continued for 2 h (**Figure 1**). In animals of the TTM groups, rapid cooling was started after randomization and induced with ice packs and an electric fan. Body temperature reached 33°C within 15 min. Once the target temperature was reached, it was maintained for 2 h, followed by a slow rewarming period at a rate of 1.5°C/h, after which the animals were maintained at 37°C until the end of the experiment. Normothermic animals were maintained at 37°C. Arterial blood pressure, ECG recordings, intrathoracic pressure, and rectal temperature were monitored for 4 h. No inotropic agent was administered. After a recovery period of 4 h, rats were weaned from the ventilator, all vascular catheters and tracheal tubes were removed, and surgical wounds were sutured. After each experimental period, rats were returned to their cages with easily accessible food and water, and were observed in a rodent facility with a controlled room temperature of 22°C. Buprenorphine (0.01 mg/kg) was injected intramuscularly daily to ensure the surgical pain relief during the recovery period for all groups.

We used 43 consecutive rats for the survival study. Among them, 9 rats were excluded from further analyses (5 rats: ROSC was not achieved, 4 rats: CA was not induced due to technical failure), and randomization did not begin until ROSC. The survival time after CPR was recorded up to 7 d.

Neurological deficit evaluation

A single investigator who was unaware of each animal's group assignment performed all neurological deficit score (NDS) evaluations. Consciousness and breathing, cranial nerve reflexes, motor function, sensory function, and coordination were scored according to an NDS system (0-500 scale; 0 normal, 500 death or brain death), as described previously.[21]

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Y maze test

The Y-maze test is a gross test for spatial memory, and uses a Y-maze apparatus composed of three equally spaced arms (120°; 80 cm long × 30 cm high × 15 cm wide). This ethologically relevant test is based on the rodents' innate curiosity to explore novel areas. Briefly, rats were placed into one of the arms of the maze (start arm) and allowed to explore the maze with one of the arms closed for 15 min (training trial). After a 1-h intertrial interval, rats were returned to the Y maze by placing them in the start arm. Rats were allowed to explore freely all three arms of the maze for 8 min (test trial). A rat is considered to enter an arm if all four limbs enter into an arm compartment. The sequence of arm entries was recorded by video recorder. The dependent variables were motor activity, defined as the number of arms entered, and percent alternation, calculated as the number of alternations (entries into three different arms consecutively) divided by the total possible alternations (i.e., the number of arms entered minus 2) and multiplied by 100.[22] After each trial, the maze was cleaned with dilute alcohol and dried with paper towel.

Histopathological analysis

At 7 d after ROSC, rats were decapitated. The left sides of the brains were quickly removed and fixed with Zamboni's solution. The brains were histologically compared with those of sham-operated rats. Coronal tissue slices (6-μm thickness) of paraffin-embedded brain tissue (at the level of the hippocampus) were stained for histological evaluation. To examine changes in the neurons, astrocytes, and microglia after global cerebral ischemia–reperfusion, we performed immunohistochemical staining with an anti-neuronal nuclei antibody (NeuN, Catalog No. MAB377, Millipore, Temecula, CA, USA) for neurons, anti-ionized calcium-

binding adapter molecule 1 antibody (Iba-1, Catalog No. 019-19741, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for microglia/macrophages, and biotin-conjugated microtubule-associated protein 2 antibody (MAP2, Catalog No. M9942, Sigma-Aldrich, St. Louis, MO, USA) for axonal damage, Fluoro-Jade C (FJC) labeling[23] (Fluoro-Jade C Ready-to-Dilute Staining Kit, Catalog No. TR-100-FJ, Biosensis, Thebarton, SA, Australia) for degenerative neuronal cells, and glial fibrillary acidic protein (GFAP) staining (anti-GFAP antibody, Catalog No. AB5804, Millipore, Temecula, CA, USA) for activated astrocytes.

In each NeuN-, Iba1-, and MAP2-stained section, 2 slide fields were randomly examined using a defined rectangular field area (0.14 mm^2). MAP2 staining is reported as the area's relative intensity of staining (%), calculated using automated counting software (Image J 1.46r, National Institutes of Health, USA). Fluorescence microscopic examination of FJC and GFAP-staining was performed on 2 slide fields of defined field area (0.05 mm^2). Images were analyzed using Image J. Automated counting data for each stained section was reported as degenerating cells in blue and activated astrocytes in red.

Statistical analysis

Measurements are reported as mean ± SEM. For single comparisons, we performed an unpaired two-tailed Student's t-test; for multiple comparisons, we used an analysis of variance (ANOVA) followed by Tukey's correction for post hoc comparisons. Physiologic data (rectal temperature, mean arterial pressure, heart rate) were examined by a mixed-effects model for repeated measures analyses comprising treatment group, time, and treatment-by-time interaction as factors and random intercept for each subject. NDS were on an ordinal scale and were analyzed by Kruskal-Wallis with Mann-Whitney U analyses between multiple

groups. The Kaplan–Meier analysis and log-rank test were used to calculate the survival rates. Statistical significance was considered at a two-sided p value of < 0.05. Statistical analyses were performed using SPSS® software (SPSS Inc., Chicago, IL, USA).

Results

H_2 therapy commenced after ROSC under normoxic conditions improved animal survival and neurological recovery in post-CA rats

There were no differences among the four experimental groups in hemodynamics, blood gases, or chemistries at baseline, during CPR, or during post-cardiac arrest care following ROSC (**Supplementary Table 1, Supplementary Table 2**, **Figure 2b and 2c**). There were also no significant differences in the number of gasps during CA, diastolic pressure during CPR, or the number of defibrillations required to establish ROSC among the four experimental groups (**Supplementary Table 1**). Rectal temperature was rapidly reduced from 37.0 ± 0.0°C to 32.8 ± 0.2°C at 15 min after ROSC in hypothermic animals, and the normothermic animals showed no significant changes in rectal temperature during the experiments (**Figure 2a**).

The survival rate at 7 d after ROSC was 5 out of 13 (38.4%) rats in the Ctl group; 5 out of 7 (71.4%) rats survived in the H_2 group; 5 out of 7 (71.4%) rats survived in the TTM group; and, 6 out of 7 (85.7%) rats survived in the TTM+H_2 group ($P < 0.05$ *vs.* Ctl group, **Figure 3**).

The NDS was evaluated at 24, 48, 72 h and 7 d after ROSC (**Table 1**). NDS values at 72 h after ROSC were better in the H_2 group (185 ± 82, $P < 0.05$), the TTM group (217 ± 74,

$P < 0.05$), and the TTM+H_2 group (90 ± 68, $P < 0.01$) than in the Ctl group (395 ± 40), while scores in the TTM+H_2 group were better than in the TTM group ($P < 0.05$). As a practical example, rats with a NDS of 395 were unresponsive, immobile, reacted minimally to stimuli and were associated with high mortality. Rats with NDS of 217 generally had an increased respiratory rate, sluggish responses to pain, and disordered coordination, but retained some mobility, whereas rats with a NDS of 90 appeared alert, reacted briskly to stimuli and had mild respiratory impairment. NDS values at 7 d were also better in the H_2 group (166 ± 87, $P < 0.05$), the TTM group (185 ± 84, $P < 0.05$), and the TTM+H_2 group (88 ± 69, $P < 0.01$) than in the Ctl group (389 ± 43) (**Table 1**). Improved neurological outcome following H_2 and hypothermia treatment was consistent when NDS values were evaluated separately for survivors only (**Supplementary Table 3**). To confirm the prognostic value of NDS, we examined the relationship between NDS category at 24 h after ROSC, and mortality. A higher NDS at 24 hours after ROSC was associated with a poorer survival (**Figure 4**).

Rats were also subjected to a Y-maze test to assess motor activity and spatial memory. Motor activity at 7 d after ROSC was significantly lower in the Ctl ($P < 0.01$ *vs.* sham) and TTM groups ($P < 0.05$ *vs.* sham) than in the sham group (**Figure 5a**), while motor activity at 7 d after ROSC was non-significant in the H_2 and TTM+H_2 group compared to the sham group. The Ctl group appeared deficient in spatial working memory as tested by spontaneous alternation in the Y-maze compared to the sham group ($P < 0.05$), whereas spatial working memory at 7 d after ROSC became non-significant in the other groups compared to the sham group (**Figure 5b**).

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H_2 inhalation rescued neuronal death, and suppressed microglia activation in the hippocampus and cerebral cortex

A transient period of global cerebral ischemia and reperfusion after CA is associated with delayed neuronal death and activation of astrocytes and microglia in selectively vulnerable areas such as the hippocampus and cerebral cortex, both in experimental animals and in humans.[24-27] The number of surviving NeuN-positive cells in the hippocampus CA1 from the Ctl group was significantly lower than that in sham-operated post-CA rats. The number of NeuN-positive cells in the hippocampus CA1 was significantly preserved in the H_2, TTM, and TTM+H_2 groups compared to the Ctl group (**Figure 6a**). MAP2, abundant in neuronal dendrites, functions in maintaining the cytoskeletal integrity of dendrites. Extensive loss of MAP2-immunoreactive dendrites was apparent in the hippocampus CA1 in the Ctl group. The intensity of MAP2-immunoreactive dendrites were significantly higher in the H_2, TTM, and TTM+H_2 groups compared to the Ctl group, suggesting maintenance of normal structural integrity of CA1 dendrites (**Figure 6b**). There were significantly more Iba1-positive activated microglia/macrophages in the CA1 in the Ctl and TTM groups compared to the other groups (**Figure 6c**).

We also evaluated neuronal degeneration and astrocyte activation in the cerebral cortex by FJC and GFAP immunostaining. FJC^+ degenerating neurons and $GFAP^+$ reactive astrocytes were conspicuous in the Ctl group, while the TTM+H_2 group showed significantly fewer degenerating neurons than controls (**Figure 7**).

Discussion

Inhalation of H_2 commenced before the ROSC has been shown to improve animal survival

and NDSs in a rat model of CA, to an extent comparable to TTM (33°C).[12] In this study, we demonstrated a beneficial effect of H_2 inhalation commenced after the ROSC, even in the absence of hyperoxia. H_2-treated animals exhibited improved NDS scores, with correlative reductions neuronal degeneration and microglial activation in regions typically vulnerable to ischemic injury.

To date, therapeutic hypothermia is the only approach proven to improve outcome in patient with PCAS, [28-30] when applied after the ROSC. However, Nielsen *et al.*[31] recently reported that therapeutic hypothermia at a target temperature of 33°C did not confer a benefit compared with a target temperature of 36°C in patients with PCAS. One explanation for the lack of benefit at the lower temperature is that improvement in patient intensive care over the past decade reduces any potential incremental benefit of a single intervention.[32] From the viewpoint of translating the present results into clinical benefit, it is particularly important that the combined therapy of TTM and H_2 inhalation was more effective than TTM alone. The NDS at 24, 48, and 72 h after ROSC demonstrated improved neurological outcome in the TTM+H_2 group compared to the TTM group. Motor activity was also better in the TTM+H_2 group than in the TTM group. Consistent with this, degenerative neuronal changes in the cerebral cortex and survival rate at 7 d after ROSC were both significantly improved by the TTM+H_2 therapy compared to the Ctl group, but not by TTM alone.

The patients in the HACA trial[30] were allowed to passively cool to a temperature of 32-34°C and were maintained as such for 24 h, with passive rewarming occurring over a median period of 8 hours. In the Bernard study,[29] the patients were treated with TTM of 33°C for 12 hours, and active rewarming over a period of 6 hours. In an animal study, Ye *et al.*[33]

have demonstrated that a shorter duration of mild hypothermia, induced rapidly and early after ROSC, improved postresuscitation microcirculation, myocardial and cerebral functions, and survival as well as, or better than, prolonged duration of hypothermia in a rat model of cardiopulmonary resuscitation. Taking into account the high metabolic rate of rats compared to humans, we chose a 2 h duration for therapeutic hypothermia, followed by 2 h of rewarming.

Previously, we demonstrated that post-CA rats die of systemic I/R injury.[12] In that study, the left ventricular end-diastolic pressure gradually increased to ≥ 20 mmHg at 2 hours after ROSC in the control group. Histological analyses at 24 hours after ROSC exhibited contraction band necrosis, coagulation necrosis with cytoplasmic eosinophilia, loss of nuclei, and vacuolar degeneration surrounded by inflammatory cell infiltration in the myocardium of the post-CA control rats; all effects that could be associated with increased water content in the lung. The early death in these post-CA rats was also associated with a systemic inflammatory response as shown by the increased serum IL-6 levels at 2 hours after ROSC and impaired neurological function based on the NDS at 24 hours after ROSC.

The early mortality of Ctl group post-CA rats was comparable to other recently published findings. Using a rat model of 6-min-VF CA, Ma *et al.*[34] showed that 4 out of 10 animals survived more than 72 hours, while Janata *et al.*[18] showed better outcomes than ours. However, Janata's experimental protocol was different from ours in that the VF CA was induced by a 1-minute impulse, and if spontaneous defibrillation occurred, an additional 15-second impulse was delivered. In addition, after ROSC, sodium bicarbonate was given to treat metabolic acidosis and crystalloids, while boluses of vasopressin 0.005 IU/ kg and

epinephrine 5 μg/kg were given IV to keep MAP greater than 60 mm Hg. By contrast, we performed constant electrical stimulation of the epicardium for 3 minutes and there was no spontaneous reversion of VF.

H_2 therapy's mode of action is primarily suppression of oxidative stress. Cellular redox homeostasis is maintained by a delicate balance between ROS production and antioxidant defences.[35] When ROS are produced excessively or endogenous antioxidant capacity is diminished, indiscriminate oxidation leads to potentially damaging "oxidative stress". The hydroxyl radical (•OH) is a major trigger of the chain reaction forming free radicals,[36] and once occuring on biomembranes, it continues and expands causing serious cellular damage. In contrast, H_2O_2 and •NO function as signaling molecules that induce antioxidant enzymes. Notably, H_2 reacts with strong oxidants such as •OH in cells, but remains mild enough to neither disturb metabolic redox reactions nor affect signaling ROS. In addition, H_2 rapidly diffuses into tissues and cells. Thus, H_2 can be used as an effective antioxidant therapy.[37]

Inhaled H_2 acts rapidly; it may also therefore be suitable as a defense against acute oxidative stress elicited by ischemia/reperfusion.[1, 4, 11] Inhaled H_2 at therapeutic doses has no adverse effects on the saturation level of arterial oxygen (SpO_2) nor hemodynamic parameters, including blood pressure, heart rate, and left ventricular pressure.[4] H_2 may accumulate in the lipid phase more than in the aqueous phase, especially in unsaturated lipid regions that are the major target of the radical chain reaction. Thus, H_2 may confer an advantage in suppressing the radical chain reaction, avoiding lipid peroxide production and the consequent generation of oxidative stress markers, such as 4-hydroxyl-2-nonenal and

malondialdehyde. Additionally, •OH can modify deoxy-guanine (dG) to 8-OHdG. Indeed, we showed that inhalation of H_2 gas decreased these oxidative markers in rat models of cardiac ischemia-reperfusion injury and PCAS.[4, 12]

H_2 inhalation was protective and, in some aspects, superior to the use of hypothermia in other studies.[4, 12] For example, H_2 gas inhalation, but not therapeutic hypothermia, prevented PCAS-associated increases in left ventricular end-diastolic pressure and serum IL-6 at 2 hours after ROSC.[12, 38] These results are surprising because therapeutic hypothermia is believed to confer protection against reperfusion injury by multiple mechanisms,[39] including suppression of free radicals, enzymes, and excitatory and inflammatory reactions, in addition to the direct physical protection of membranes. In contrast, the cornerstone of H_2 therapy is selective ROS attenuation.

The reduction of oxidative stress by H_2 may lead to various effects including anti-inflammatory and anti-apoptotic responses via changes in gene expression,[40] signal transduction,[41-43] and mitochondrial membrane potential,[11, 37] although whether H_2 has a mode of action independent of its anti-oxidative properties is unknown. In a rat model of hyperoxic lung injury, H_2 reduced mRNA levels encoding several pro-inflammatory cytokines, including IL-1β, IL-6, tumor necrosis factor (TNF)-α, and intercellular adhesion molecule (ICAM)-1, in the lung.[40] H_2 inhalation significantly improved the survival rate and organ damage in a mouse model of cecal-ligation and puncture-induced sepsis[44] as well as zymosan-induced generalized inflammation.[45] H_2 also has anti-apoptotic properties, such as mitigating hyperoxia-induced lung epithelial cell apoptosis via the induction of Bcl-2 protein and suppressing hyperoxia-mediated induction of Bax protein.[40] Inhalation of H_2 also

reduced infarct size in a canine model of cardiac ischemia-reperfusion injury via opening of the mitochondrial K_{ATP} channels followed by inhibition of mitochondrial permeability transition pores.[11]

We recognize several limitations in our study. First, the small rodent brain has different metabolic and rheological properties from the complex human brain. Findings analogous to those from the rat CPR model are yet to be demonstrated in large-animal and clinical studies.[46] Second, pentobarbital used for anesthesia may adversely affect basic cardiac function.[47] Although there was no difference among experimental groups in the pentobarbital dose, or the time from premedication dosing to onset of CA, hypothermia might reduce the systemic clearance of pentobarbital and thus affect myocardial contractility. Third, the rats in the TTM group were subjected to hypothermia for only 2 h. Such a short duration of hypothermia has not been replicated in larger animals or preclinical work. Further studies are required prior to clinical application, especially if such a short therapeutic window is being proposed. Fourth, the experimental design does not allow conclusions to be drawn as to the most appropriate concentrations of H_2, or whether increased duration of H_2 ventilation would provide a greater degree of protection. Finally, it is possible that greater differences between TTM and H_2 therapy may have been revealed with a larger number of animals, or with a longer duration of therapy.

In conclusion, we have demonstrated for the first time that H_2 inhalation commenced after normoxic resuscitation improves neurological outcome, independent of TTM, in a rat model of CA. Our findings suggest a potentially novel and easily applicable solution to PCAS. Although further investigation is required, H_2 is expected to be an innovative

therapeutic tool for unmet medical needs that currently constitute a considerable health burden, particularly for critically ill patients. This study may pave the way for successful translation of H_2 inhalation therapy into clinical practice.

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Table 1. Neurological Deficit Score at 24, 48, 72 h, and 7 d.

Group	24 hours	48 hours	72 hours	7 days
Ctl (n = 13)	381 ± 34	384 ± 37	395 ± 40	389 ± 43
H_2 (n = 7)	155 ± 45**	218 ± 77*	185 ± 82*	166 ± 87*
TTM (n = 7)	254 ± 67	239 ± 69*	217 ± 74*	185 ± 84*
TTM + H_2 (n = 7)	80 ± 25**,#	115 ± 66**,#	90 ± 68**,#	88 ± 69**

Ctl, control; TTM, targeted temperature management. Values expressed as mean ± SEM. Significant differences: *P < 0.05, ** P < 0.01 compared to the Ctl group; #P < 0.05 compared to the TTM group.

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Figure Legends:

Figure 1. Experimental protocol for CPR and post resuscitation care in a VF-induced cardiac arrest model. CA, cardiac arrest; CPR, cardiopulmonary resuscitation; DC, direct current cardioversion; ROSC, return of spontaneous circulation; TTM, targeted temperature management.

Figure 2. Changes in rectal temperature, mean blood pressure, and heart rate during post-cardiac arrest care after ROSC. (a) Rectal temperature, (b) mean blood pressure, and (c) heart rate. The bars represent the mean and SEM. Ctl, control; TTM, targeted temperature management.

Figure 3. Kaplan-Meier analyses of cumulative survival at 7 days after ROSC. Significant differences: * $P < 0.05$ compared to the Ctl group. Ctl, control; TTM, targeted temperature management.

Figure 4. The relationship between NDS category and mortality. To examine if NDS score was associated with mortality, we combined the data sets from 4 groups and drew Kaplan-Meier survival curves according to NDS at 24 hours after ROSC. Animals were divided into 4 groups by NDS quartiles at 24 hours after ROSC. $P < 0.001$ for analysis of variance between groups. NDS, neurological deficit score; ROSC, return of spontaneous circulation.

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Figure 5. Effects of H_2 and TTM on motor activity and spatial memory at 7 d after cardiac arrest. (a) Motor activity and (b) spatial memory at 7 days after cardiac arrest in the Y-maze test. (n = 3-4) * $P < 0.05$, ** $P < 0.01$ compare to the sham-operated group. The bars represent the mean and SEM. Ctl, control; TTM, targeted temperature management.

Figure 6. Effect of H_2 and TTM on neuronal death and microglia activation in the hippocampus CA1 sector at 7 d after cardiac arrest. Representative photomicrographs of immunohistochemistry for (a) NeuN, (b) MAP2, and (c) Iba-1 in the hippocampus CA 1 sector of the sham-operated group (n = 3) and the experimental groups (n = 4-5) at 7 days after cardiac arrest. Bar = 50 μm, Significant differences: * $P < 0.05$, ** $P < 0.001$ compared to the sham-operated group, † $P < 0.05$, †† $P < 0.01$, § $P < 0.001$ compared to the Ctl group. The bars represent the mean and SEM. Ctl, control; TTM, targeted temperature management.

Figure 7. Effect of H_2 and TTM on neuronal death and astrocyte activation in the cerebral cortex at 7 d after cardiac arrest. Representative photomicrographs of the immunohistochemical localization of DAPI, Fluoro-Jade C (FJC), and GFAP staining in cerebral cortex. (a, e, i, m, q) Nuclear DNA was labeled with DAPI (Blue); (b, f, j, n, r) degenerating neurons were labeled with FJC (green); (c, g, k, o, s) reactive astrocytes were labeled with GFAP (red); (d, h, l, p, t) merged images. n = 3-6. Bar = 50 μm. * $P < 0.01$ compared to the sham-operated group, † $P < 0.01$ compared to the Ctl group. The bars represent the mean and SEM. Ctl, control; TTM, targeted temperature management.

Figure 1

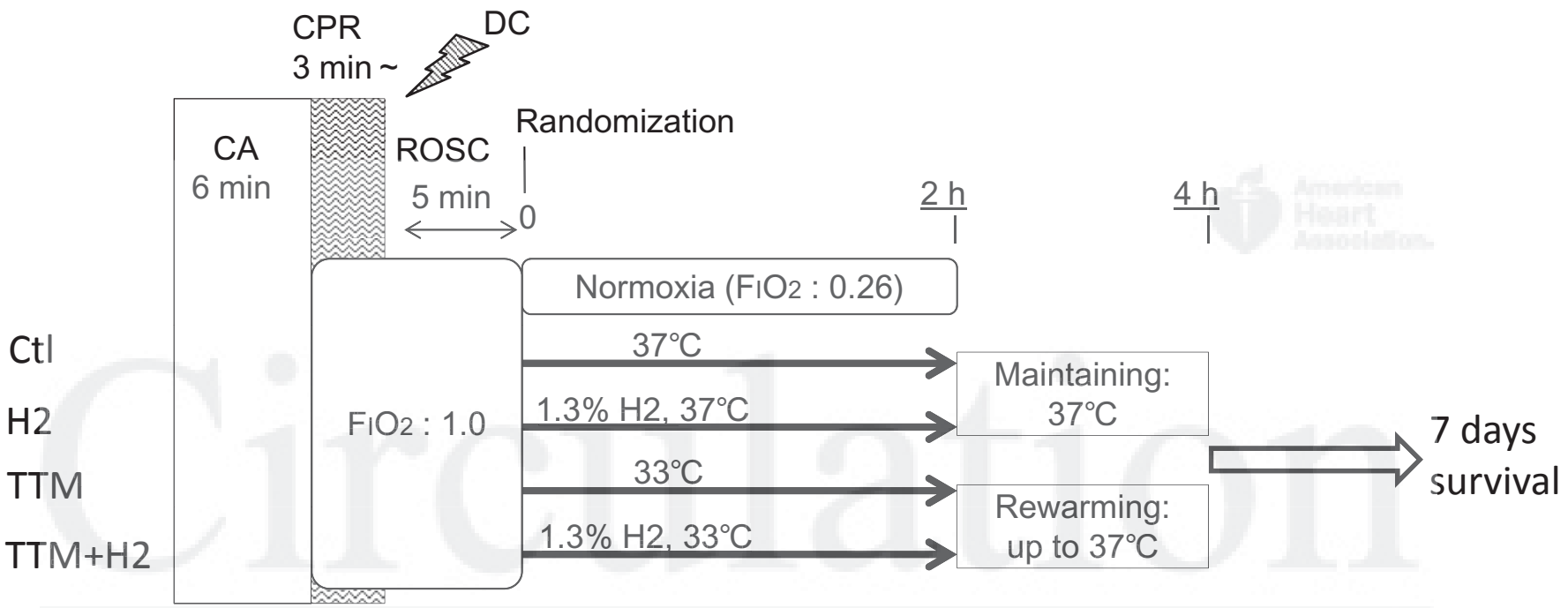


Figure 2

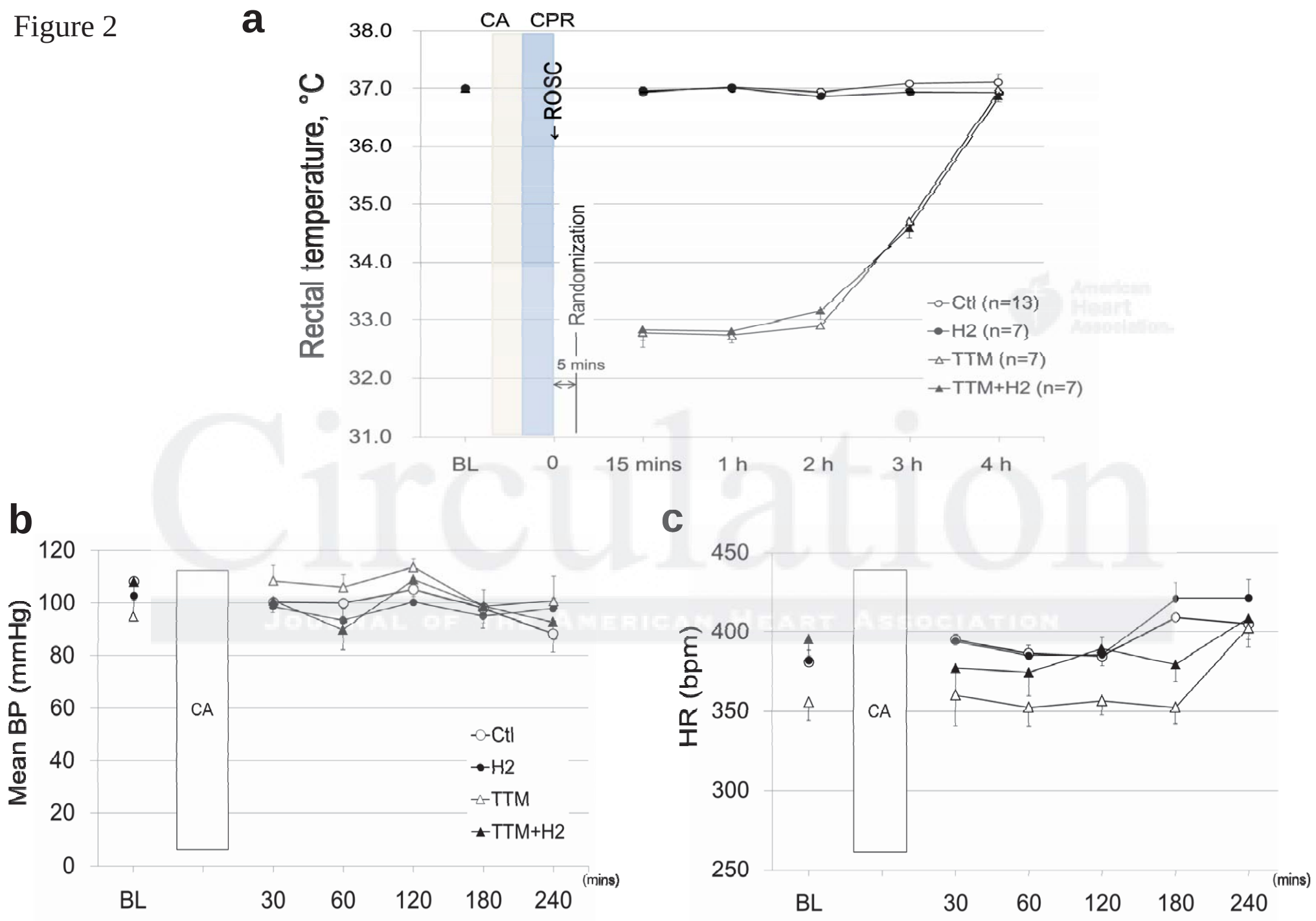


Figure 3

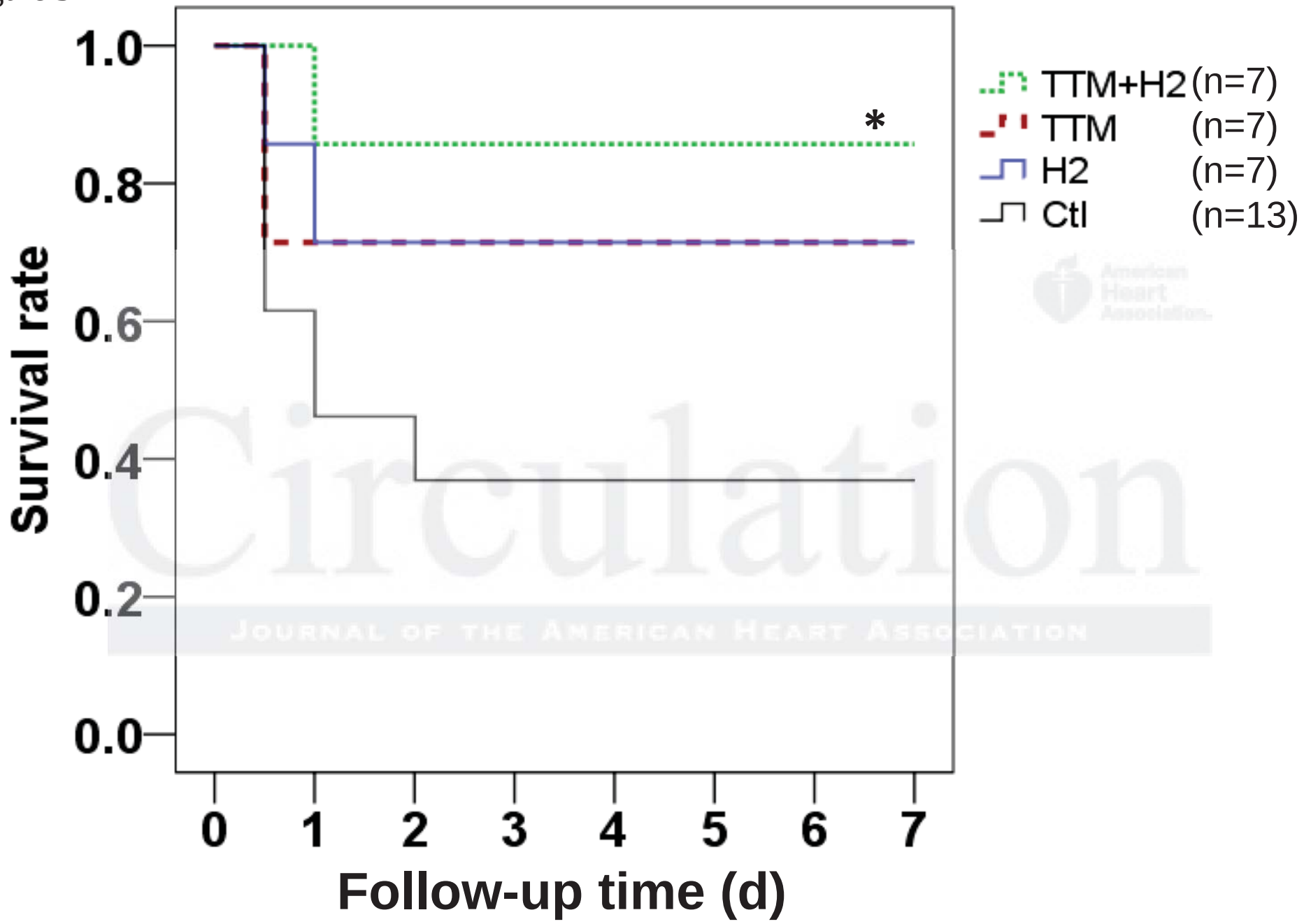
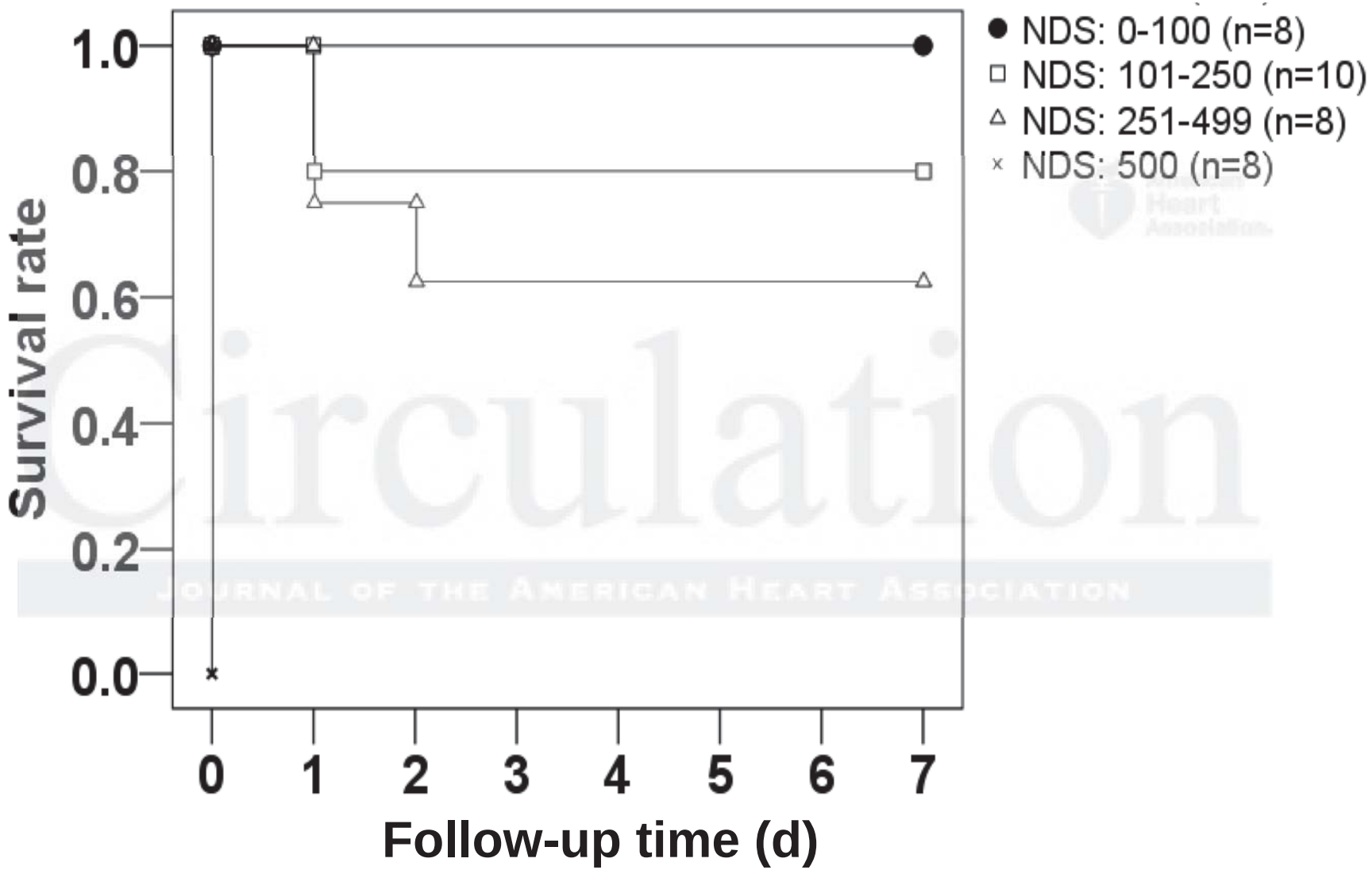


Figure 4



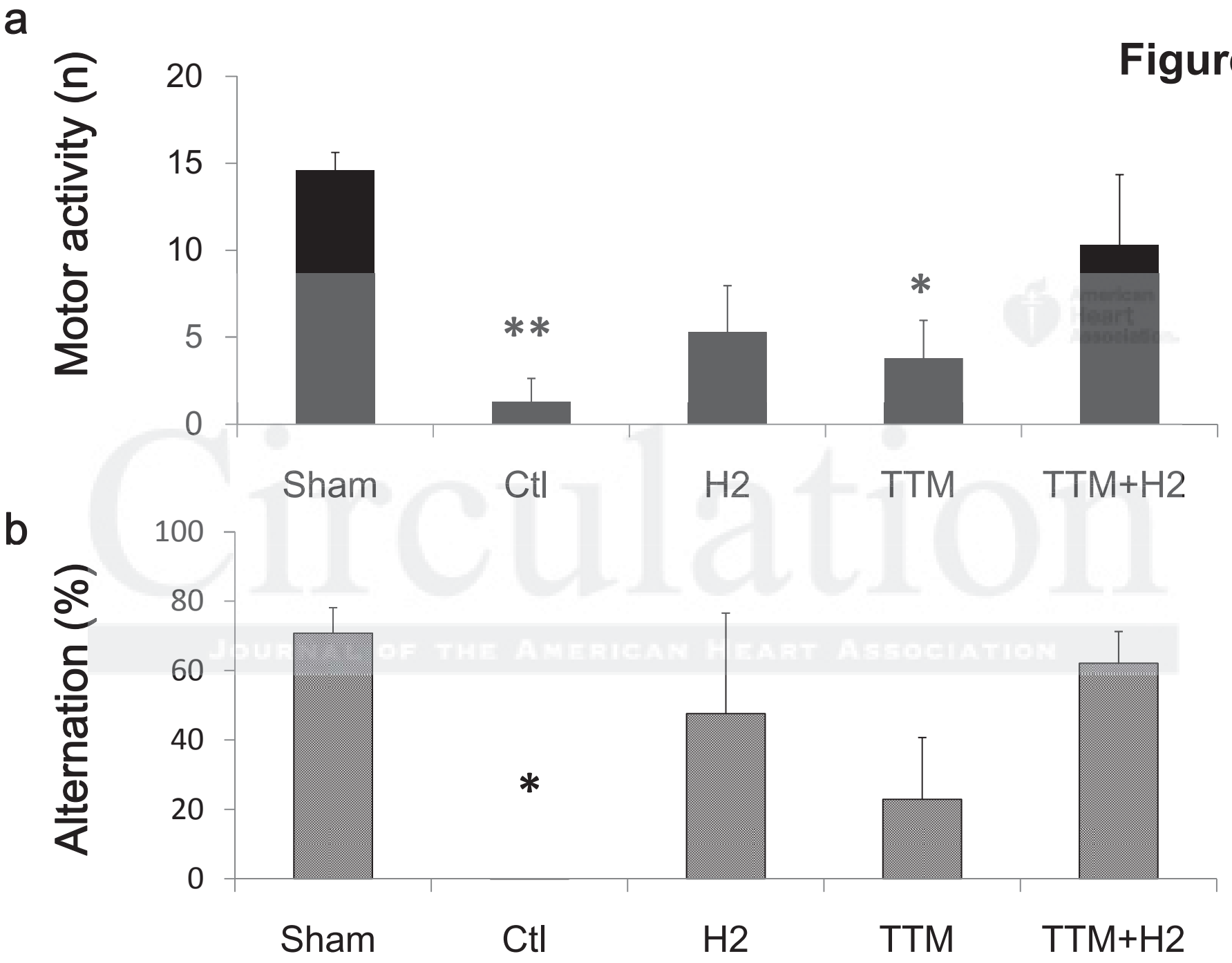


Figure 5

Figure 6

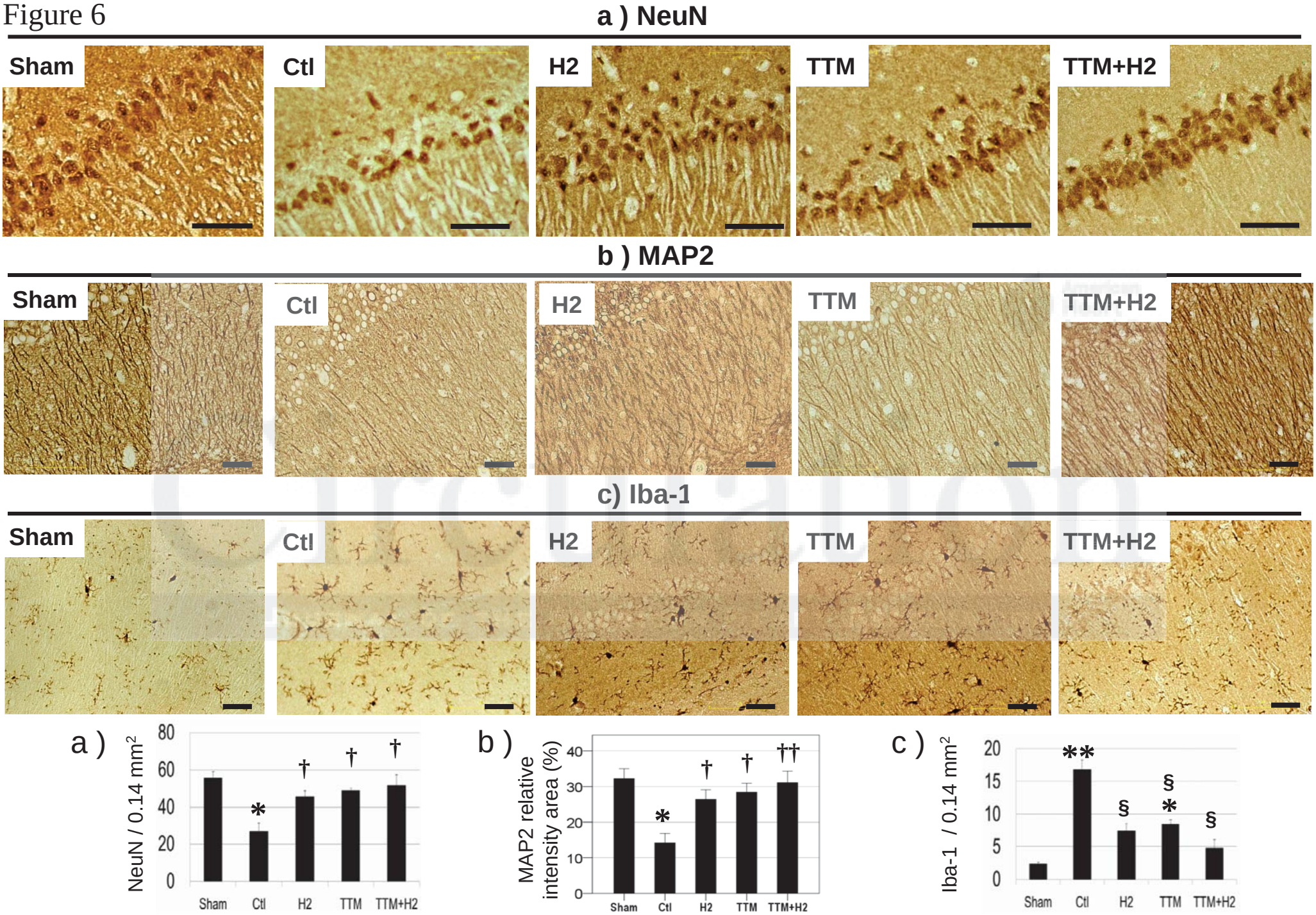
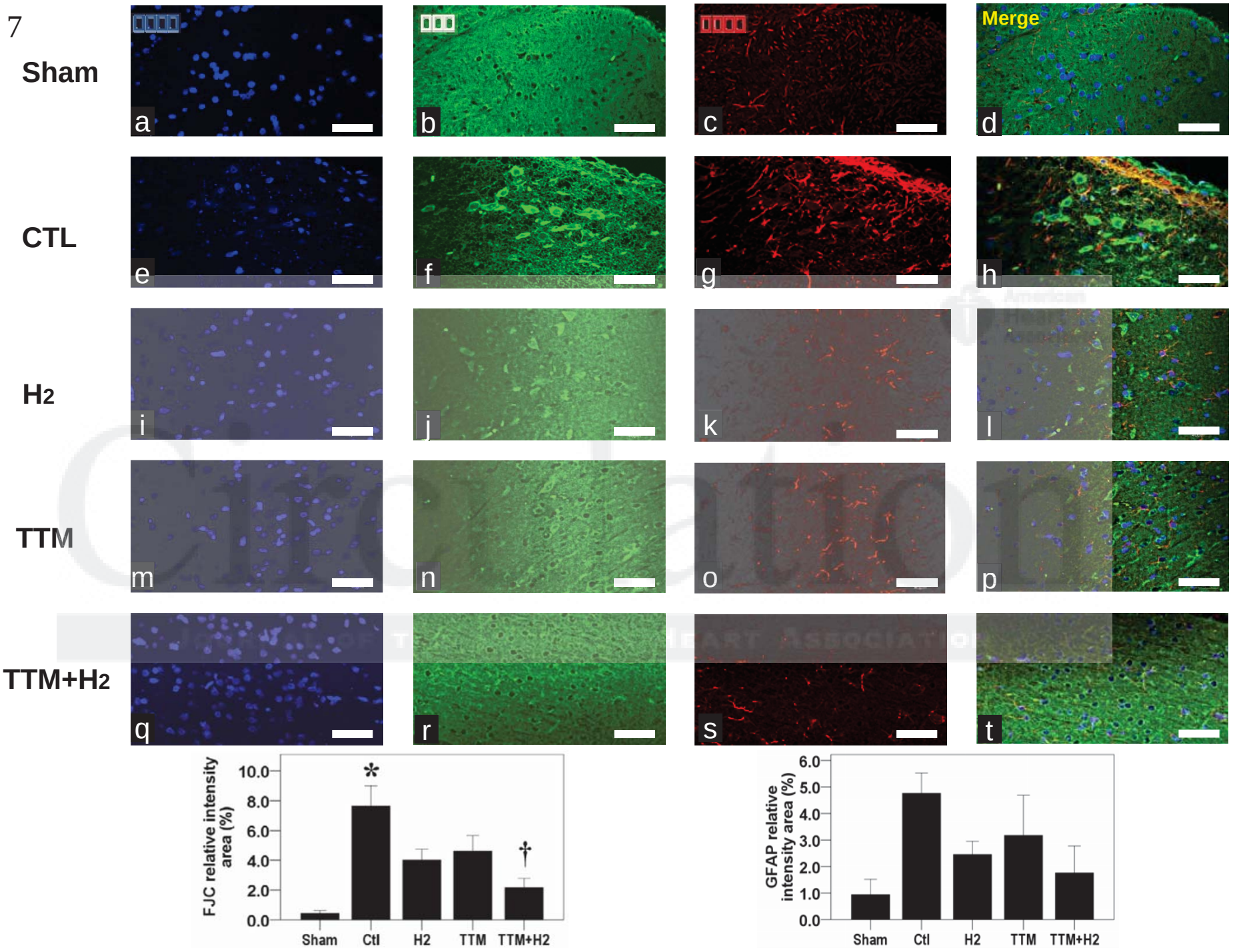


Figure 7



SUPPLEMENTAL MATERIAL

Supplementary Table 1. Variables at baseline and during cardiopulmonary resuscitation

	Ctl (n = 13)	H_2 (n = 7)	TTM (n = 7)	TTM+H_2 (n = 7)
Weight, g	407 ± 3	406 ± 5	406 ± 5	411 ± 4
HR at BL, bpm	381 ± 8	382 ± 6	356 ± 11	395 ± 10
MAP at BL, mmHg	108 ± 4	102 ± 6	94 ± 6	108 ± 7
CPR time to ROSC, s	208 ± 14	192 ± 14	225 ± 22	202 ± 21
Number of gasps during CA, n	26 ± 4	23 ± 6	25 ± 7	20 ± 4
DBP at 60 s after starting CPR, mmHg	50 ± 3	55 ± 7	49 ± 4	59 ± 6
DBP at 150 s after starting CPR, mmHg	41 ± 2	45 ± 7	44 ± 3	42 ± 2
Total administration of defibrillation, n	1.4 ± 0.2	1.3 ± 0.3	1.4 ± 0.3	1.3 ± 0.3

Ctl, control; TTM, targeted temperature management; BL, baseline; HR, heart rate; MAP, mean arterial blood pressure; B.E., base excess; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. CA, cardiac arrest; DBP, diastolic blood pressure; s, second. Values expressed as mean ± SEM.

Supplementary Table 2. Arterial blood gas and chemistries analyses at baseline and during post-CA care after ROSC

	At baseline	At 30 min	At 60 min	At 120 min
pH				
Ctl	7.47 ± 0.01	7.27 ± 0.03	7.33 ± 0.02	7.35 ± 0.02
H_2	7.51 ± 0.03	7.33 ± 0.03	7.37 ± 0.01	7.39 ± 0.02
TTM	7.47 ± 0.02	7.25 ± 0.03	7.32 ± 0.02	7.31 ± 0.02
TTM + H_2	7.46 ± 0.02	7.29 ± 0.03	7.36 ± 0.02	7.36 ± 0.01
PaO_2, torr				
Ctl	80 ± 3	103 ± 7	95 ± 5	103 ± 5
H_2	74 ± 5	99 ± 7	93 ± 4	97 ± 4
TTM	81 ± 4	112 ± 8	111 ± 5	116 ± 6
TTM + H_2	77 ± 3	111 ± 7	114 ± 5	118 ± 5
$PaCO_2$, torr				
Ctl	35 ± 2	36 ± 2	36 ± 3	32 ± 2
H_2	32 ± 2	33 ± 2	33 ± 1	33 ± 1
TTM	34 ± 2	39 ± 1	36 ± 2	39 ± 2
TTM + H_2	36 ± 2	39 ± 3	36 ± 3	37 ± 3
Base excess, mmol/l				
Ctl	2.6 ± 0.5	–9.3 ± 1.9	–6.6 ± 1.4	–7.4 ± 1.2
H_2	2.0 ± 0.8	–7.9 ± 1.5	–5.6 ± 1.0	–4.6 ± 0.8
TTM	1.5 ± 0.4	–9.3 ± 1.5	–6.8 ± 1.1	–5.5 ± 1.1

TTM + H_2	2.5 ± 0.6	–7.0 ± 1.3	–4.4 ± 0.5	–4.4 ± 0.8
Lactate, mmol/l				
Ctl	1.1 ± 0.1	6.3 ± 1.1	4.0 ± 0.7	3.5 ± 0.6
H_2	1.4 ± 0.1	5.2 ± 0.8	3.1 ± 0.4	2.1 ± 0.3
TTM	1.1 ± 0.1	6.1 ± 0.9	3.2 ± 0.6	2.4 ± 0.8
TTM + H_2	0.9 ± 0.0	3.9 ± 0.7	2.2 ± 0.2	2.1 ± 0.6

Ctl, control; TTM, targeted temperature management. Values expressed as mean ± SEM. There were no significant differences for treatment by time among the four groups by mixed-effects model for repeated-measures analyses.

Supplementary Table 3. Neurological Deficit Score of survivors at 7 d after cardiac arrest

	Ctl	H_2	TTM	TTM + H_2
Number of survivors	5	5	5	6
NDS at 7d	193 ± 56	33 ± 19*	59 ± 35*	20 ± 16**

Ctl, control; TTM, targeted temperature management. Values expressed as mean ± SEM.

Significant differences: *$P < 0.05$, ** $P = 0.01$ compared to the Ctl group.