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Hydrogen inhalation ameliorated mast cell mediated brain injury after ICH in mice

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Abstract

OBJECTIVE—Hydrogen inhalation was neuroprotective in several brain injury models. Its mechanisms are believed to be related to anti-oxidative stress. We investigated the potential neurovascular protective effect of hydrogen inhalation especially effect on mast cell activation in a mouse model of intracerebral hemorrhage (ICH).

DESIGN—Controlled in vivo laboratory study.

SETTING—Animal research laboratory

SUBJECTS—171, 8 weeks old male CD-1 mice were used.

INTERVENTIONS—Collagenase-induced ICH model in 8 weeks old, male, CD-1 mice was used. Hydrogen was administrated via spontaneous inhalation. The blood-brain barrier (BBB) permeability and neurological deficits were investigated at 24 and 72 hours after ICH. Mast cell activation was evaluated by Western blot and immuno-staining. The effects of hydrogen inhalation on mast cell activation were confirmed in an autologous blood injection model ICH.

MEASUREMENT AND MAIN RESULTS—At 24 and 72 hours post-ICH, animals showed BBB disruption, brain edema, neurological deficits, accompanied with phosphorylation of Lyn kinase and release of tryptase, indicating mast cell activation. Hydrogen treatment diminished phosphorylation of Lyn kinase and release of tryptase, decreased accumulation and degranulation of mast cells, attenuated BBB disruption and improved neurobehavioral function.

CONCLUSION—Activation of mast cells following ICH contributed to increase of BBB permeability and brain edema. Hydrogen inhalation preserved BBB disruption by prevention of mast cell activation after ICH.

Keywords

Intracerebral hemorrhage; brain edema; mast cells; hydrogen; ROS; neuroprotection

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INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for 10–15% of strokes with a mortality rate greater than 40%. Many survivors are permanently disabled(1). In experimental ICH animal models, neurological deficits were associated with an increase in production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)(2), which damage the BBB and increase brain edema(3, 4). Hydrogen therapy neutralized ROS and diminished oxidative stress in several brain injury models(5–7). However, the mechanism underlying the beneficial effects of hydrogen therapy remain poorly understood.

Mast cells are resident inflammatory cells that are present in various tissues including the central nervous system(8). They contain granules with vasoactive substances such as histamine, proteolytic enzymes (tryptase), and anticoagulants (heparin). Mast cell activation and degranulation, which contribute to BBB disruption and increased bleeding(8) following brain injury, have been observed in several experimental models. In an autologous blood injection ICH model, mast cell stabilization reduced brain edema and improved neurological deficits(9). Similarly, mast-cell deficient animals had shown significantly less brain edema than wild type after ICH(9). Mast cell activation was accompanied by increased ROS(10) and RNS(11) production. Hydrogen suppressed FcεR-mediated signal transduction and prevented degranulation of mast cells (12).

Interestingly, activation of mast cells was accompanied by increased production of ROS(10) and RNS(11), and hydrogen suppressed FcεR-mediated signal transduction and prevented degranulation of mast cells (12). Therefore, we tested the hypothesis that firstly ICH produced mast cell activation by increased phosphorylation of Lyn kinase in the brain tissues. Activated mast cells degranulated, enhanced tryptase release, and augmented redox stress. Secondly, hydrogen inhalation diminished the phosphorylation of Lyn, which in turn decreased mast cell activation and redox stress. Thirdly, hydrogen inhalation preserved BBB integrity, decreased brain edema, and ameliorated neurological deficits.

MATERIAL AND METHODS

Experimental animals

All procedures and methods for these studies were approved by the Animal Care and Use Committee at Loma Linda University and complied with the Guide for the Care and Use of Laboratory Animals (animals protocol number 8100024). A total of 171 male CD-1, 8 weeks old mice were used.

Animals group

Details were listed in Supplementary Text (S.Text).

Intracerebral hemorrhage induction

Experimental ICH was induced by intrastriatal injection of either bacterial collagenase or autologous blood as we previously described (13, 14). Details were listed in S.Text.

Hydrogen inhalation treatment

One hour after operation, animals were placed in chamber connected to a hydrogen gas tank. The hydrogen treatment chamber was continually flushed with gas mix 2.9% hydrogen and 21% oxygen balanced with nitrogen, flow rate 8 l/minute. Hydrogen concentration was controlled at outlet using a Hydrogen-meter (H₂ Scan, Valencia, CA, USA). Chamber was warmed by using warming path (37°C). No hydrogen toxicity was observed.

Vehicle (medical air inhalation) and sham (needle trauma, medical air inhalation) animals were placed in a chamber. Chamber was warmed and continually flushed with medical air (flow rate 8 l/minute).

3-amino-1,2,4-triazole treatment

3-amino-1,2,4-triazole (AT) was purchased by Sigma Aldrich (St. Louis, MO, USA). AT was dissolved in saline and administrate intraperitoneally five hours before ICH induction.

Evaluation of brain edema and neurological deficits

The brain water content and neurological deficits were evaluated as previously described (13). Details were listed in S.Text.

Evaluation of BBB permeability (Evans Blue Assay) and hematoma volume (hemoglobin assay)

BBB permeability and hematoma volume were evaluated as previously described (15) Details were listed in S.Text.

Western blot analysis

Mice were perfused transcardially with 40 ml of cold PBS. Hemispheres were isolated and stored at -80°C until analysis. Protein extraction and Western blots were performed according to the manufacture recommendation as described before(16). Details were listed in S.Text.

Histological study

Details were listed in S.Text.

Immunohistochemistry study

Immunohistochemistry was performed as described previously(17) using the following primary antibodies: rabbit anti-tryptase 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Evaluation of hydrogen peroxide production

For evaluation of hydrogen peroxide (H_2O_2), commercial available Amplex[®] Red Hydrogen Peroxide Assay Kit (Life Technologies, NY, USA) was used. Details were listed in S.Text.

Statistical analysis

Quantitative data are expressed as the mean \pm SEM. Analysis were performed by independent statistician. Statistical significance was verified by analysis of variance (ANOVA) (Tukey test). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Hydrogen Inhalation on Brain Edema and Neurological Deficits at 24 hrs and 72 hrs

Animals were divided into sham operated and ICH groups. ICH animals were subsequently divided into vehicle, one, and two hours of hydrogen inhalation therapy. At 24 hrs post-ICH, collagenase treated animals had significantly increased edema in the ipsilateral basal ganglia than sham operated animals (83.34 ± 0.38 vs. 77.75 ± 0.15 , $p < 0.05$, Fig. 1A). One hour of hydrogen inhalation treatment significantly decreased brain water content vs. medical-air treated animals (81.76 ± 0.31 vs. 83.34 ± 0.38 , $p < 0.05$), while two hours of hydrogen

inhalation treatment tended to decrease edema (82.25 ± 0.48 vs. 83.34 ± 0.38 , $p=0.16$ Fig. 1A).

Statistically significant neurological deficits were evident in all ICH vs. sham-animals at 23 hours after collagenase injection as tested by the modified Garcia test (Fig. 1B), wire hang, and beam balance tests (Fig. 1C). A statistically significant improvement was seen in all three neurobehavioral assessments after one hour of hydrogen inhalation. Two hours of hydrogen inhalation led to statistically significant neurofunctional improvement in the wire hang and beam balance tests and showed a tendency to ameliorate neurological deficits in the Garcia Test.

Since one hour of hydrogen inhalation was effective at 24 hours after ICH, the effect of a single hydrogen treatment (starting one hour after ICH) was compared with multiple treatments (one hour duration daily for 72 hours) and evaluated at 72 hours after ICH. At 72 hours post-ICH, brain water content remained significantly increased in the ipsilateral basal ganglia of collagenase treated animals vs. sham (83.60 ± 0.56 vs. 77.75 ± 0.15 , $p<0.05$, Fig. 1D). Single hydrogen treatment tended to decrease brain water content (82.79 ± 0.404 vs. 83.60 ± 0.56 , $p=0.49$). Statistically significant neurological deficits were observed in all ICH vs. sham animals (Fig 1 E-F). Single hydrogen treatment tended to ameliorate neurological deficits at 72 hrs after ICH. Multiple hydrogen treatments were ineffective.

Effect of Hydrogen on the Hematoma Volume-hemoglobin assay

One hour of hydrogen inhalation had no effect on hemorrhage volume at 24 or 72 hours (data not shown).

Effect of Hydrogen on Blood-Brain Barrier permeability-Evans Blue assay

Significant accumulation of Evans Blue stain was observed in the ipsilateral hemisphere of ICH compared to sham animals (9.11 ± 0.69 vs. 4.09 ± 0.14 μg of Evans Blue/g of tissue, $p<0.05$, Fig 2A). One hour of hydrogen inhalation significantly decreased amount of the stain in the ipsilateral hemisphere of hydrogen-treated compared to vehicle at 24 hour (7.20 ± 0.45 vs. 9.11 ± 0.69 , $p<0.05$, Fig 2A). However, one hour of hydrogen treatment demonstrated a non significant tendency to preserve BBB at 72 hour (7.55 ± 0.76 vs. 6.50 ± 0.6 , $p=0.30$, Fig. 2B).

Effect of ICH and Hydrogen on LYN kinase expression and phosphorylation

Lyn expression and phosphorylation was evaluated 6 hours after ICH in both hydrogen treated and untreated groups. Neither ICH nor hydrogen had an effect on LYN kinase expression (Fig. 3A). However, ICH significantly induced Lyn phosphorylation in brain of ICH compared to sham animals. Hydrogen inhalation significantly reduced ICH induced Lyn phosphorylation (Fig. 3B). Catalase inhibition had no effect on Lyn expression (Fig 3A, last column), but increased Lyn phosphorylation (Fig. 3B, last column).

Effect of ICH and Hydrogen Inhalation on the Release of Mast Cells Specific Proteins, Mast Cells Accumulation and Degranulation

Significant tryptase release was observed six hours after ICH by Western blot analysis. One hour of hydrogen treatment significantly decreased tryptase release (Fig. 4). Catalase inhibition reversed effect of hydrogen on tryptase release (Fig. 4, last column). Similarly, ICH increased amount of tryptase positive cells in the brains of ICH compared to sham animals, visualized by immunostaining against tryptase. Hydrogen inhalation ameliorated this effect (Fig 5).

Additionally, ICH increased mast cell accumulation at 6 hours after ICH, evaluated by Toluidine Blue (Fig 6). There were more Toluidine Blue positive cells in peri-hematoma region of ICH compared to the same brain region of sham-operated animals (Fig 6B compared to 6A). Hydrogen inhalation decreased the number of Toluidine Blue positive cells (Fig 6C). Moreover most of the mast cells detected in brain of ICH animals showed the clear signs of degranulation with less intensive Toluidine Blue staining compared to granulated intensive stained cells in brain of sham operated animals (Fig 6A, white arrow). Mast cells after ICH had the appearance of “ghost” cells (Fig 6B, white arrow, black outline). Hydrogen inhalation decreased the number of such cells and increased the amount of granulated mast cells (Fig 6C, white arrow).

We also tested if the effect on mast cell activation was caused by collagenase or by collagenase-induced intracerebral bleeding. To this end we compared collagenase model with direct injection of autologous blood into the basal ganglia. Hematoma caused by collagenase or autologous blood injection induced the release of the proteins tryptase and chymase by mast cells. This release was evaluated by Western blot at 6 hours after ICH and demonstrated no significant difference between the two models (Fig 7).

Effect of ICH and Hydrogen Inhalation on the H₂O₂ Production

We evaluated the effect of hydrogen inhalation on H₂O₂ production after ICH. ICH significantly increased H₂O₂ production as early as two hours after ICH insult. One hour of hydrogen inhalation significantly decreased ICH-induced H₂O₂ production (Fig 8). Catalase inhibition four hours before hydrogen inhalation resulted in H₂O₂ production augmentation compared to hydrogen treated animals (Fig 8, last column).

Effect of Hydrogen on Nitrotyrosine Accumulation

Western blot examination demonstrated a significant increase of nitrotyrosine in brain tissue of ICH mice at 24 hours. One hour of hydrogen inhalation reduced ICH-induced accumulation of nitrotyrosine. The hydrogen inhalation tended to decrease nitrotyrosine accumulation at 72 hours (Fig 9).

Effect of Catalase Inhibitor, 3-amino-1,2,4-triazole on the Effects Induced by Hydrogen Treatment

Inhibition of catalase four hours before hydrogen inhalation resulted in H₂O₂ production enhancement compared to hydrogen treated animals (Fig 8, last column). The enhancement of H₂O₂ production had no effect on Lyn expression (Fig 3A, last column) but resulted in increase of Lyn phosphorylation (Fig 3B, last column) and reversed effect of hydrogen on tryptase release (Fig 4, last column).

DISCUSSION

We investigated hydrogen inhalation therapy's ability to ameliorate secondary brain injury after ICH in mice. ICH induced mast cell accumulation and degranulation, which increased tryptase release and redox stress in the brain. Those molecular changes were accompanied with BBB disruption, brain edema, and neurological deficits. Hydrogen inhalation diminished ICH-induced mast cell activation, which reduced redox stress, enhanced BBB preservation, and diminished neurological deficits. This is the first study that investigated hydrogen inhalation therapy's effects on brain mast cell activation and brain injury after ICH.

Hydrogen selectively scavenges hydroxyl radicals (7), which are the dominant reactive oxygen species causing DNA fragmentation, lipid oxidation, and protein nitrosylation.

Several hemorrhagic stroke models investigated the neuroprotective effects of hydrogen therapy (5, 18, 19). Those studies demonstrated that hydrogen therapy reduced ROS levels and postulated that its beneficial effects are linked to its anti-oxidative properties. However, pathways leading to diminished oxidative stress following hydrogen treatment remain to be elucidated. After ICH, ROS causes peroxidation of lipid-rich structures of BBB, resulting in life-threatening BBB disruption and vasogenic brain edema (20–22). It has been recently established that hydrogen, along with its anti-oxidant property, is a biologically active, gaseous signaling molecule (12). Iton et al. demonstrated that hydrogen treatment attenuated mast cell activation (23).

Mast cells are resident cells of several types of tissues including central nervous tissue. They are located perivascularly close to neurons (24). Mast cells include granules that contain substances such as histamine, heparin, tryptase, and TNF- α (25). Release of these substances may increase initial bleeding after ICH and contribute to ICH-induced BBB disruption. Mast cell activation is mediated by the Fc ϵ RI receptor (26). Fc ϵ RI receptor stimulation results in downstream phosphorylation of Lyn (27), a Src family kinase that regulates mast cell activation (23). Itoh et al. demonstrated that hydrogen had no effect on Fc ϵ RI expression, but hydrogen compromised the initial steps of mast cell activation by reducing Lyn phosphorylation (12). In the present study, we used a mixture of oxygen (21%), hydrogen (2.9%), and nitrogen, which is inflammable and safe to use. The neuroprotective effects of this hydrogen concentration after brain injury was demonstrated by others (15, 19, 28).

We observed that hydrogen inhalation ameliorated ICH-induced increase of brain water content and neurological deficits at 24 hours. We were unable to demonstrate that hydrogen inhalation ameliorated ICH-induced brain injury and neurological deficits at 72 hours even though a tendency was observed. Additionally to brain water content, we evaluated BBB integrity by measurement of Evan Blue (13, 15, 16, 29). We observed that hydrogen inhalation preserved BBB integrity at 24 hours after ICH and showed a tendency to protect BBB at 72 hours after ICH.

Mast cell activation after brain injury was observed in several experimental animal studies (30–33). Esposito et al. reported that acute stress activated mast cells in the brains of animals without brain injury (34). Acute stress increased BBB permeability in wild-type but not in mast cell deficient animals. Mast cell stabilization ameliorated stress-induced BBB disruption (34). These results indicated that mast cells are critical in increasing BBB permeability. In an ICH model, Strbian et al. demonstrated that mast cell degranulation inhibition by cromoglycate significantly decreased hematoma volume calculated from T2 MRI images at 24 hours post-ICH (9). Furthermore, hematoma volume was significantly decreased in mast-cell deficient compared to wild type animals. Mast cell stabilization decreased brain swelling, although mast cell activation was not studied directly and cromoglycate was used before ICH induction (9). In the present study, we showed that ICH increased mast cell activation. The mast cell activation was evaluated as a ratio of phosphorylated Lyn divided by unphosphorylated Lyn as well as by the release of mast cell specific protein tryptase in the animals' brains (12, 35). We observed that ICH significantly increased LYN phosphorylation and the release of tryptase in the brains of ICH compared to sham animals.

We employed the collagenase model of ICH I in most experiments. One limitation of the model is the inherent inflammatory reaction caused by collagenase. To rule out collagenase as a factor in mast cell activation we employed the autologous blood-injection ICH model and compared the release mast cell specific proteins, between both models. We did not observe a significant difference in the release and postulated that ICH was the causative

factor in mast cell activation. Therefore, it was likely that hydrogen inhalation decreased ICH-induced up-regulation of tryptase, as shown by Western blot and immunostaining of tryptase positive cells. Additionally, hydrogen inhalation decreased ICH-induced mast cell degranulation by Toluidine Blue staining, as described previously (36). In the brain of sham operated and hydrogen treated ICH animals, mast cells could be identified by their metachromatic cytoplasmic granules, whereas in ICH untreated animals most mast cells lost some of the stain and had a "ghost" shape. Moreover, more mast cells were present in the brains of ICH-untreated animals when compared to sham-operated and ICH hydrogen-treated animals. This was in agreement with the other study demonstrating that mast cell stabilization has a beneficial effect after ICH (9).

It has been established that Lyn-mediated signaling pathway activation enhanced NADPH oxidase activity in mast cells (37). NADPH oxidase generates superoxide and is considered a main source of reactive oxygen species in the blood vessels (38, 39). By the use of knock out animals, we previously demonstrated that oxidative stress resulted from NADPH oxidase activation and significantly contributed to ICH induced brain injury (40). Hydrogen attenuated NADPH oxidase activity and consequently reduced H₂O₂ production (12). We evaluated H₂O₂ production by Amplex[®] Red peroxidase assay as described previously (41, 42). Hydrogen inhalation significantly reduced ICH-induced H₂O₂ production. Furthermore there is evidence that rodents' mast cells produce NO (43). Although NO is a weak oxidant, combinations of various NO redox forms induce severe BBB damage (44). Nitrotyrosine, a product of tyrosine nitration, is a common NO-induced cell damage indicator. Nitrotyrosine accumulation after brain injury was reported by others and is regarded as redox stress marker (5, 19, 45). We observed considerable nitrotyrosin accumulation in the brains of ICH-animals that became significantly reduced in hydrogen-treated animals. These findings were in concurrence with prior studies by our laboratory that demonstrated hydrogen inhalation's anti-oxidant effects in other hemorrhagic stroke models (5, 19).

Because oxidative stress leads to BBB disruption, we investigated the effect of hydrogen inhalation on BBB integrity by measuring brain water content and analysis by Evans Blue assay. We demonstrated that decreased mast cell activation *via* hydrogen inhalation resulted in BBB preservation. This observation is similar to that of Strbian et al. who demonstrated that pretreatment with a mast cell degranulation inhibitor ameliorated BBB disruption after ICH (9). It is also similar to Zhan et al. (2011), who demonstrated that hydrogen had a protective effect on BBB after subarachnoid hemorrhage (19). However, we were unable to demonstrate any effect of hydrogen inhalation on hematoma volume (9). The discrepancy in the effects of mast cell stabilization on hematoma volume between this study and the results presented by Strbian et al may be due to the use of different ICH models.

Itoh et al. (12) postulated a feed-forward loop between Lyn phosphorylation and NADPH oxidase activity as well as demonstrated that hydrogen-induced attenuation of Lyn phosphorylation led to inactivation of NADPH oxidase and decreased H₂O₂ production in mast cells. It is presumed that NADPH inhibition and diminished H₂O₂ production attenuated phosphorylation of Lyn and its downstream molecules, and H₂O₂ accumulation counter-acted the effect of hydrogen. To test this hypothesis we used 3-amino-1,2,4-triazole (AT), a catalase inhibitor and extremely potent enzyme that decomposes H₂O₂ into water and oxygen (46), (47, 48). Consistent with previous publications, we found that giving AT three hours before ICH reversed the protective effect of hydrogen treatment and led to accumulation of H₂O₂. AT-induced accumulation of H₂O₂ suppressed phosphorylation of Lyn kinase and increased tryptase release, indicating that mast cells are a potential target for hydrogen therapy.

CONCLUSIONS

We concluded that ICH caused mast cell activation and degranulation. Mast cell activation enhanced the release of mast cell specific proteins and production of reactive oxygen and nitrogen specie, which contributed to BBB disruption and brain edema. Hydrogen inhalation diminished redox stress and BBB disruption by reducing the mast cell activation and degranulation, which ameliorated ICH-induced brain edema increase and neurological deficits in mice.

Hydrogen therapy is easy to use and proven to be neuroprotective. So far, there have been no reported side effects from the therapy. Further studies translating presented results into the clinical setting for ICH management are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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* Significant vs. sham
 # Significant vs. vehicle
 ANOVA (Tukey test $p < 0.05$)

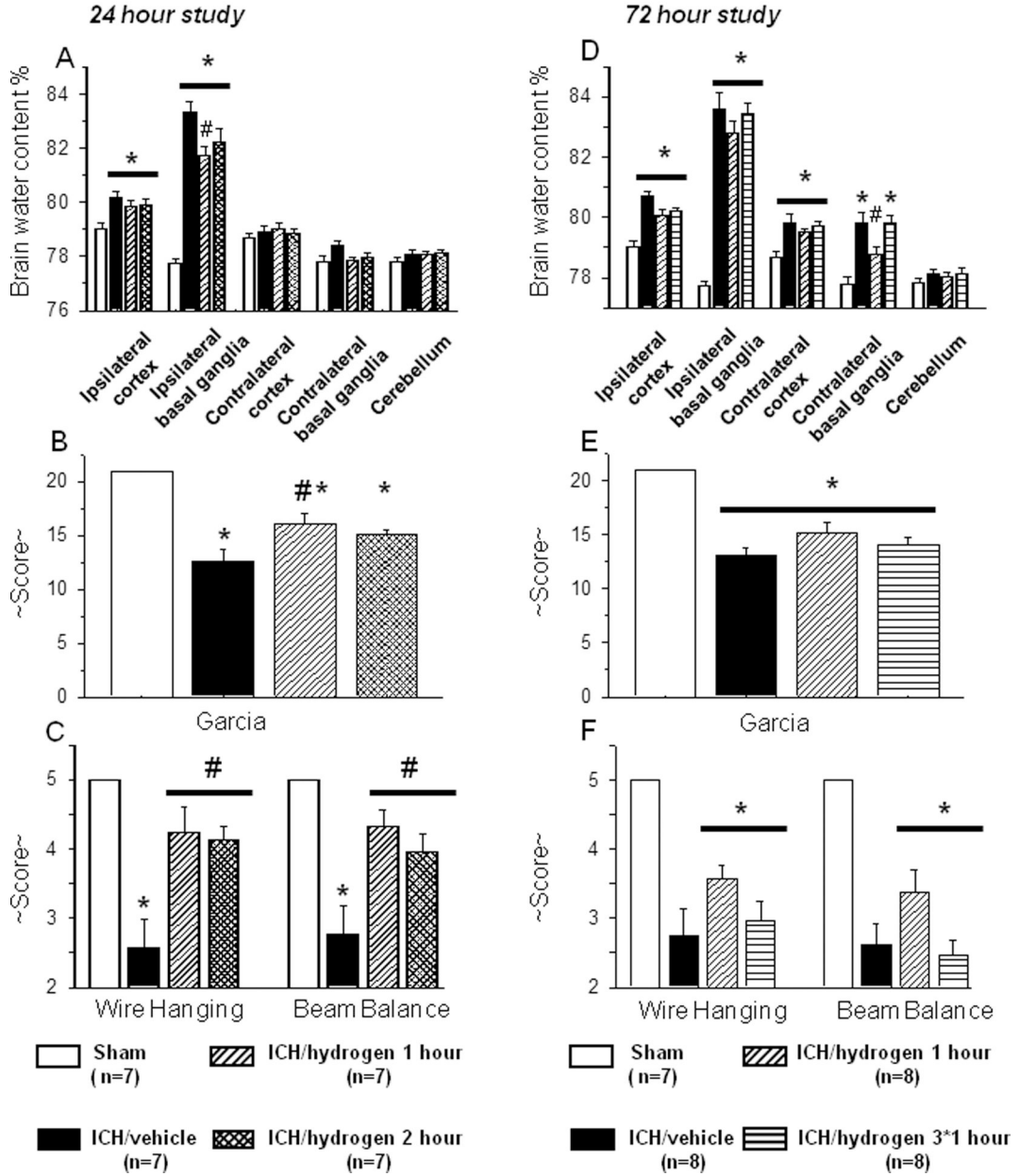


FIGURE 1.

(A) Intracerebral hemorrhage induced a significant increase of brain water content in ipsilateral basal ganglia and cortex (■ N=7) when compared to sham operated (□ N=7) animals. One hour of hydrogen inhalation starting one hour after inducing of intracerebral hemorrhage (▨ N=7) significantly diminished brain edema. Two hours of hydrogen inhalation starting one hour after inducing of intracerebral hemorrhage (▩ N=7) showed a strong tendency to ameliorate intracerebral hemorrhage induced increase of brain water content. (B–C) All animals after intracerebral hemorrhage demonstrated significant neurological deficit according to performance on the modified Garcia (B), wire hanging and

beam balance (C) tests. One hour of hydrogen treatment ameliorated neurological deficits in all three tests. Two hours of hydrogen treatment significantly decreased the neurological deficits evaluated by wire hanging and beam balance tests and showed a tendency to improvement in the Garcia test (B–C). * Significant vs. sham, # significant vs. vehicle, $p < 0.05$ ANOVA, Tukey test.

At 72 hours after inducing of intracerebral hemorrhage, brain water content in ipsilateral basal ganglia and cortex of ICH (■ N=8) remained elevated compared to that of sham operated (□ N=7) animals. Both a single one hour treatment with hydrogen starting one hour after inducing of intracerebral hemorrhage (▨ N=8) and multiple treatments (one hour daily for 3 days ▩ N=8) showed a tendency to decrease brain water content (D) and to ameliorate neurological deficits (E-F). * significant vs. sham, $p < 0.05$ ANOVA, Tukey test.

* Significant vs. sham

Significant vs. vehicle

ANOVA (Tukey test $p < 0.05$)

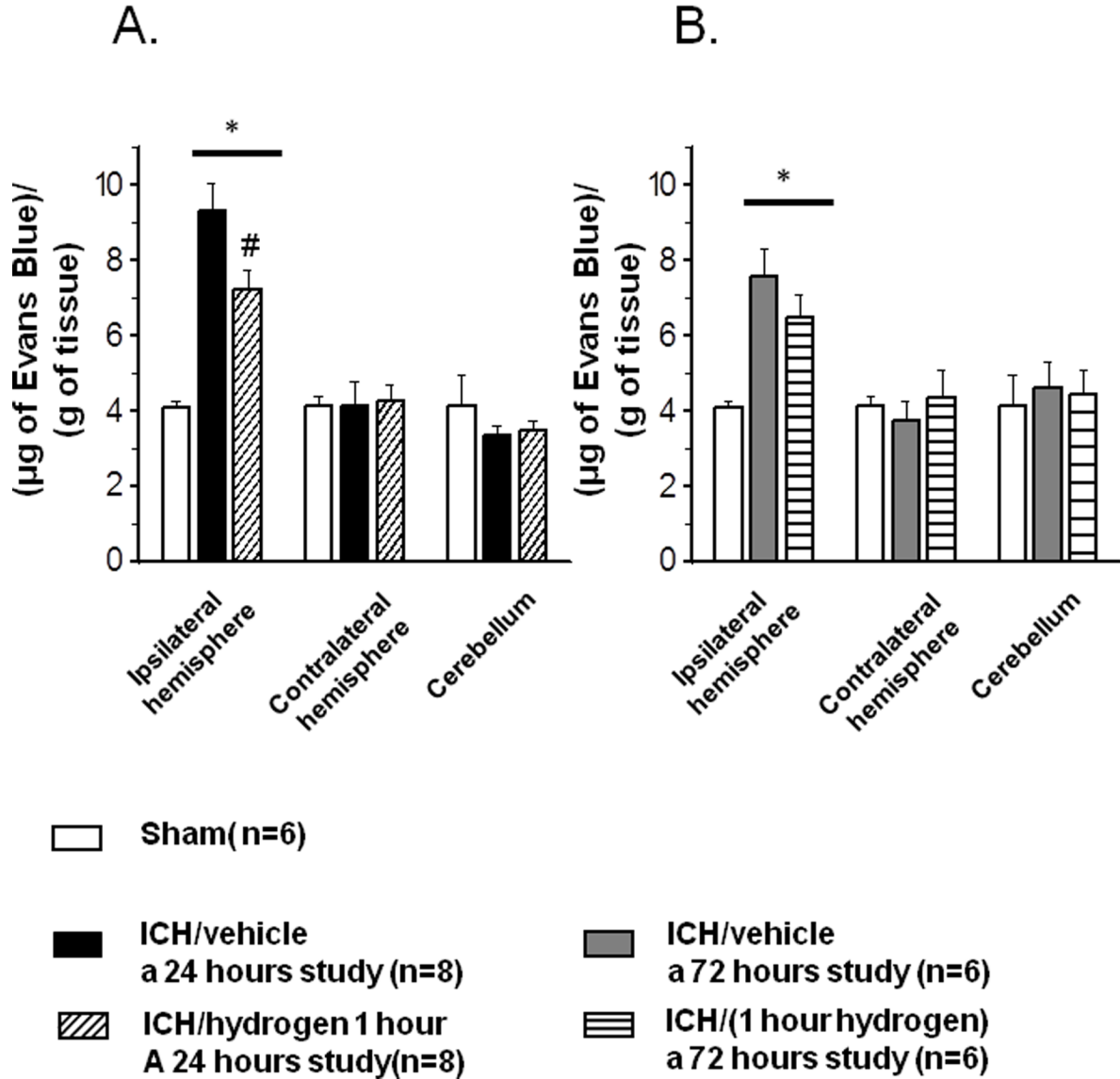


FIGURE 2.

(A) Intracerebral hemorrhage caused significant disruption of blood-brain barrier at 24 hours (□ sham operated animals N=6, ■ vehicle-treated animals N=8). One hour of hydrogen therapy (▨ N=8) significantly reduced intracerebral hemorrhage induced disruption of blood-brain barrier at 24 hrs. (B) One hour hydrogen therapy showed a tendency to preserve intracerebral hemorrhage induced disruption of blood-brain barrier at 72 hours (□ Sham operated animals N=6, ■ vehicle treated animals N=6, ▨ hydrogen treated animals N=6). * significant vs. sham, $p < 0.05$ ANOVA, Tukey test.

* Significant vs. sham
Significant vs. vehicle

ANOVA (Tukey test $p < 0.05$)

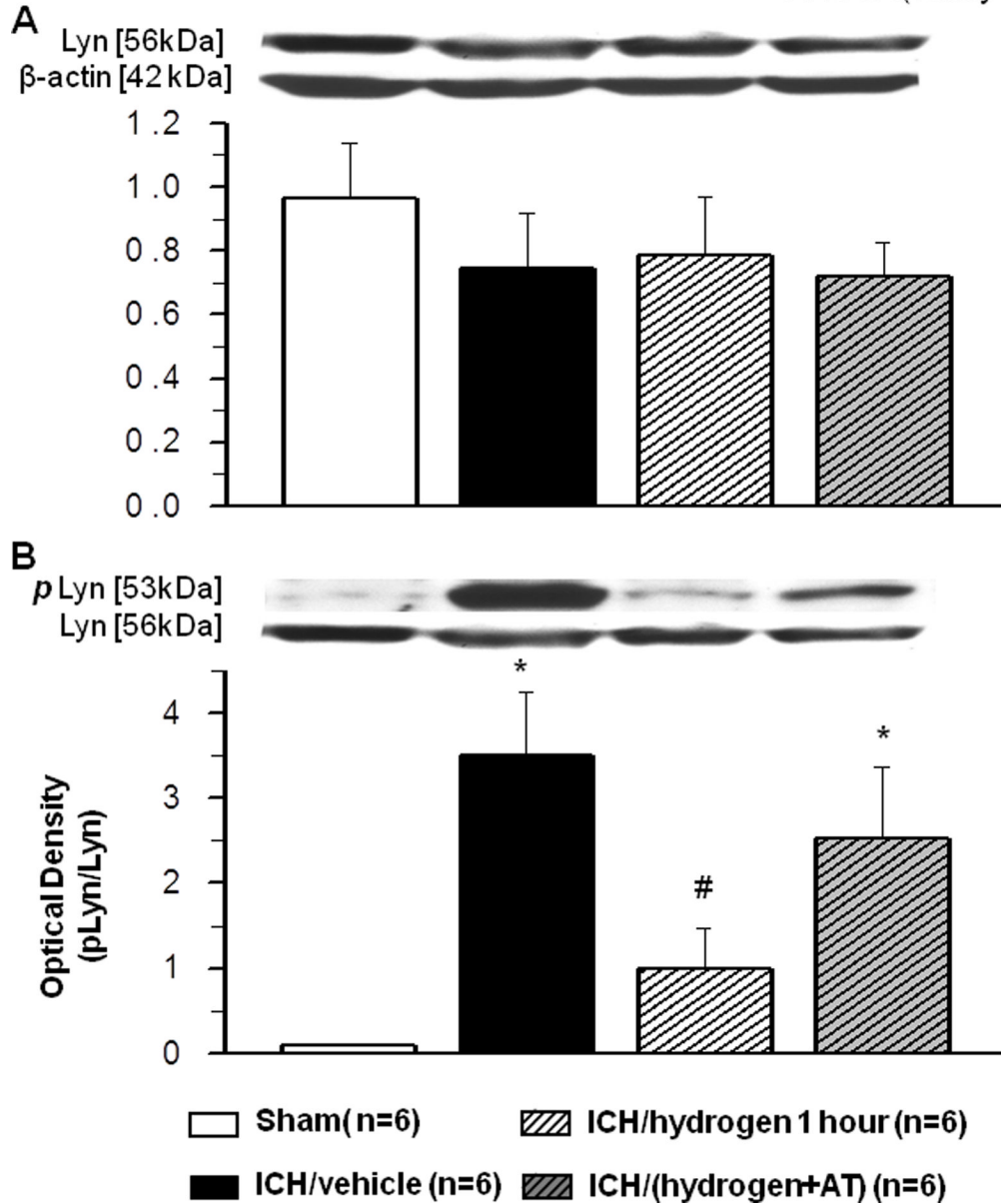


FIGURE 3.

(A) Neither intracerebral hemorrhage nor hydrogen treatment effected *expression* of Lyn kinase at 6 hours after inducing of intracerebral hemorrhage. (B) Intracerebral hemorrhage significantly increased *phosphorylation* of Lyn kinase at 6 hours after inducing of intracerebral hemorrhage (□ Sham operated animals N=6, ■ Vehicle N=6). One hour of hydrogen treatment (▨ N=6) significant diminished Lyn kinase phosphorylation when compared to intracerebral hemorrhage animals treated with vehicle. Pretreatment with catalase inhibitor at three hours before intracerebral hemorrhage (▩ N=6) prevented effect

of hydrogen on Lyn kinase phosphorylation. * significant vs. sham, # significant vs. vehicle, $p < 0.05$ ANOVA, Tukey test.



* Significant vs. sham
 # Significant vs vehicle
 ANOVA (Tukey test p<0.05)

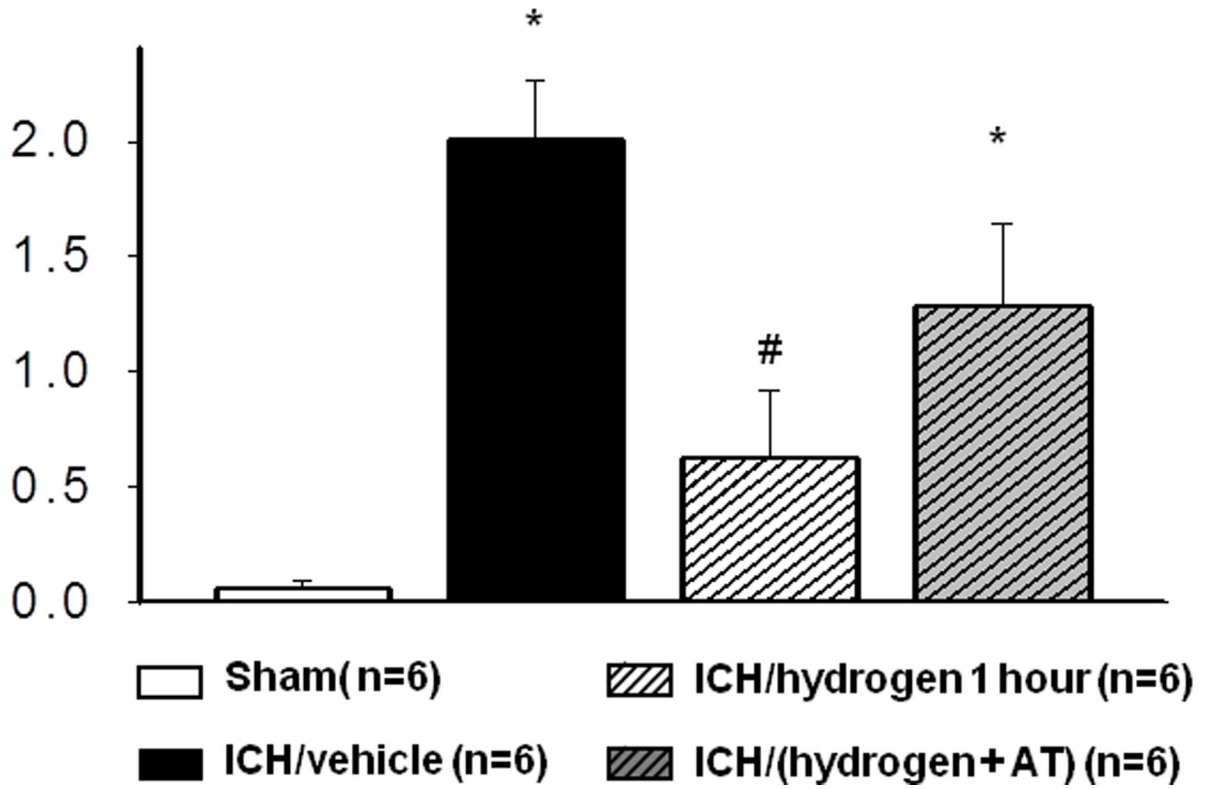


FIGURE 4. Intracerebral hemorrhage increased the release of tryptase at 6 hours after induction of intracerebral hemorrhage (□ Sham operated animals N=6, ■ (Vehicle N=6). One hour of hydrogen treatment (▨ N=6) significant diminished intracerebral hemorrhage induced release of tryptase. Pretreatment with catalase inhibitor at three hours before induction of intracerebral hemorrhage (▩ N=6) reversed effect of hydrogen on the release of tryptase. * significant vs. sham, # significant vs. vehicle, p<0.05 ANOVA, Tukey test.

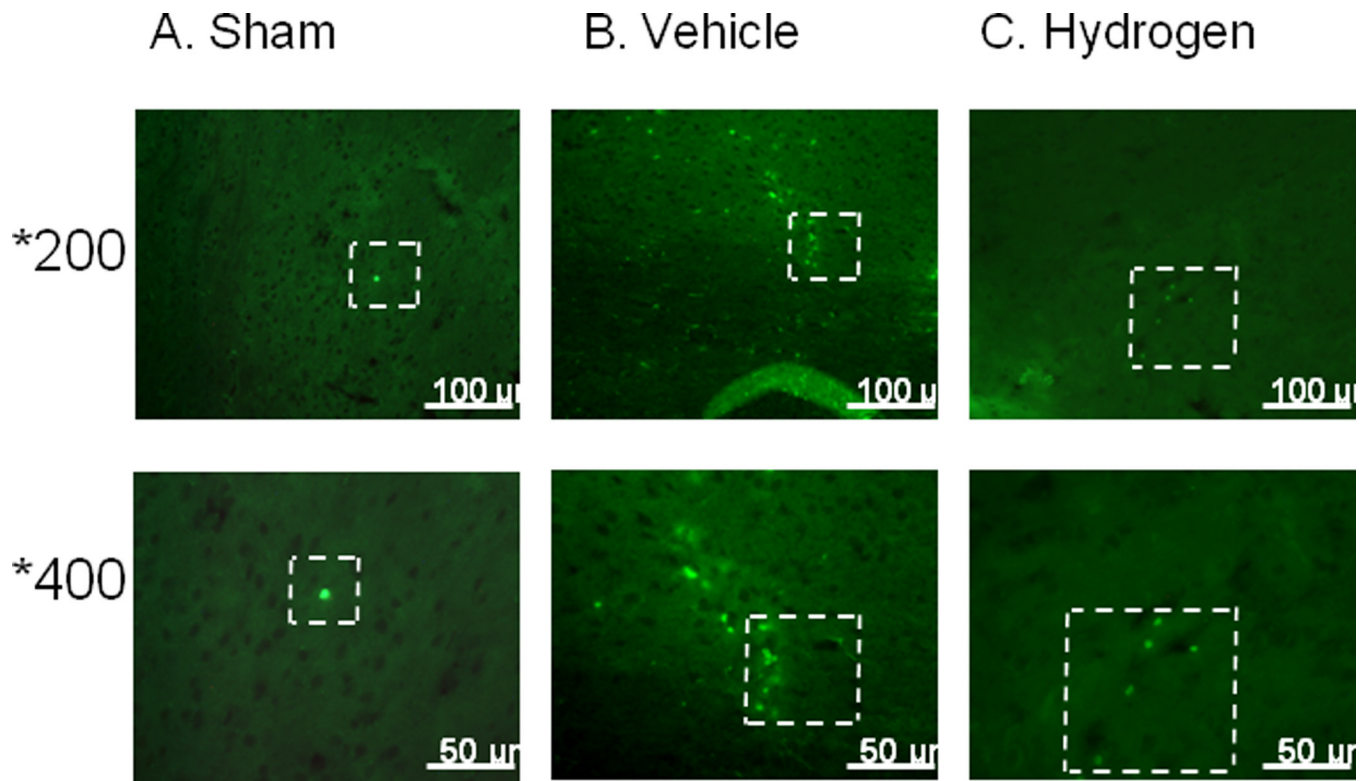


FIGURE 5. 6 hours after intracerebral hemorrhage induction, there were more tryptase positive cells in brain of vehicle (B) animals compared to sham-operated animals (A). One hour of hydrogen treatment (C) diminished number of tryptase positive cells.

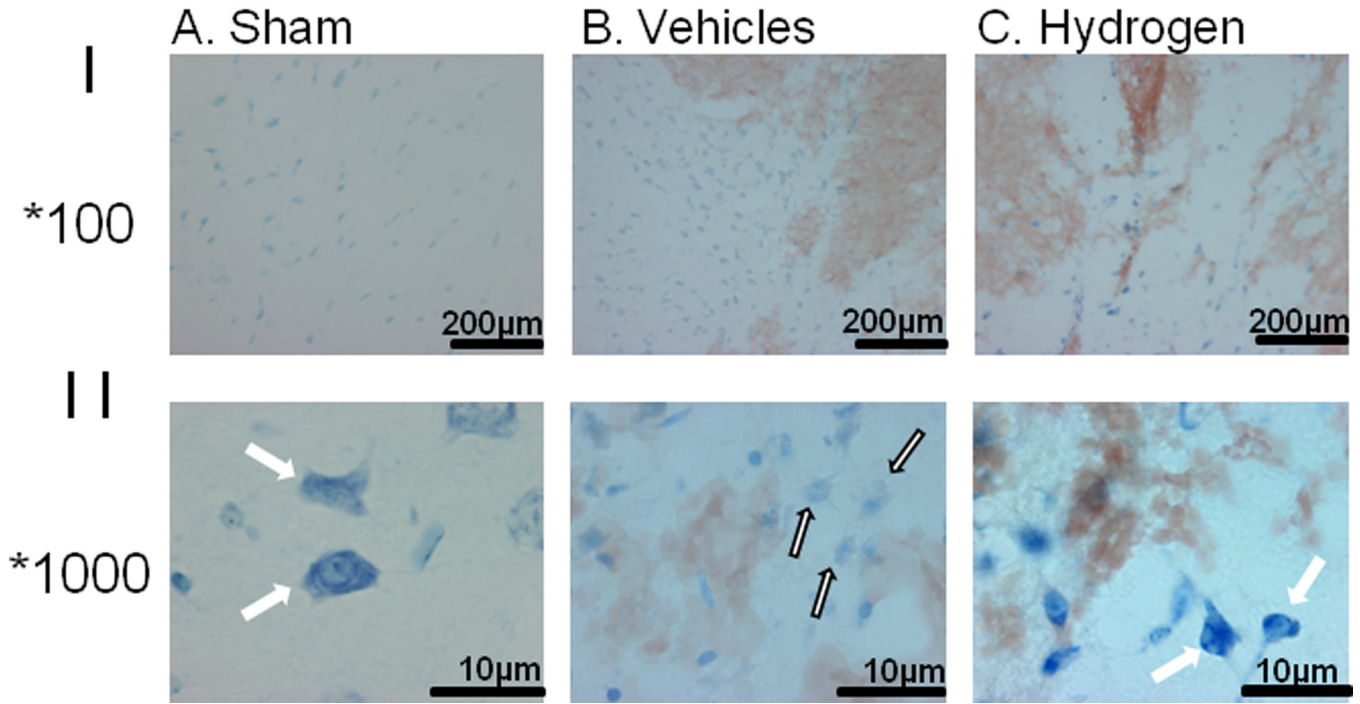
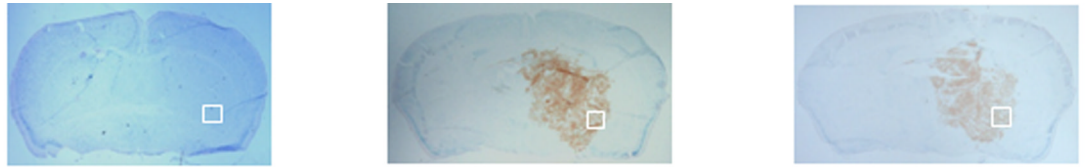
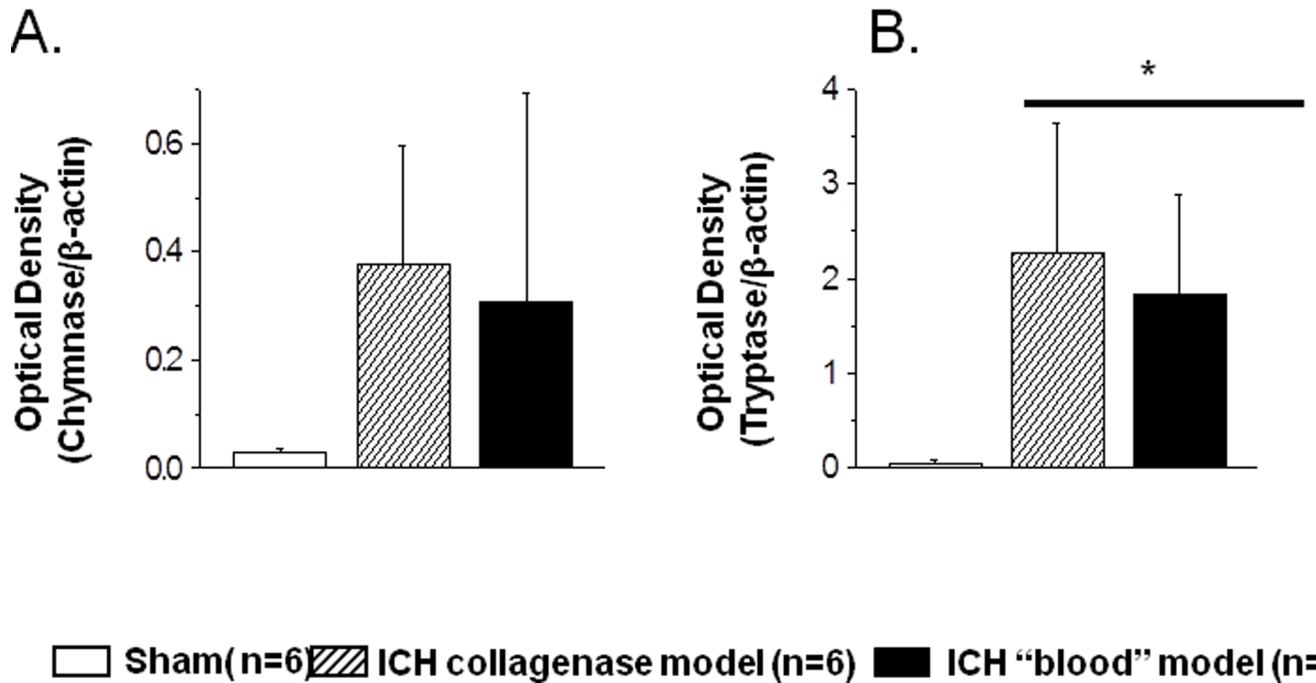
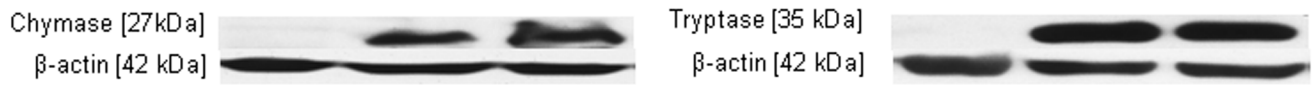


FIGURE 6. Effect of intracerebral hemorrhage and hydrogen treatment on mast cells proliferation and activation was investigated by Toluidine Blue staining at 6 hours after inducing of intracerebral hemorrhage. More mast cells were observed in brain of vehicle animals (Panel I-B) compared to sham-operated animals (Panel I-A). One hour of hydrogen treatment reduced the number of mast cells (Panel I-C). Additionally if compared to granulated intensive stained cells in brain of sham operated animals (Panel II-A white arrow), most of the mast cells detected in brain of animals after intracerebral hemorrhage showed the clear signs of degranulation with less intensive Toluidine Blue staining and the appearance of 'ghost' cells (Panel II-B white arrow, black outline). Hydrogen inhalation decreased the number of such cells and increased the amount of granulated mast cells (Fig 7C, white arrow).

* Significant vs. sham
ANOVA (Tukey test $p < 0.05$)



□ Sham (n=6) ▨ ICH collagenase model (n=6) ■ ICH "blood" model (n=6)

FIGURE 7. Collagenase-induced intracerebral hemorrhage and intracerebral hemorrhage induced by direct injection of autologous blood demonstrated comparable release of mast cell proteins. No differences between these two models were detected. * significant vs. sham, $p < 0.05$ ANOVA, Tukey test.

* Significant vs. sham
 # Significant vs vehicle
 ANOVA (Tukey test $p < 0.05$)

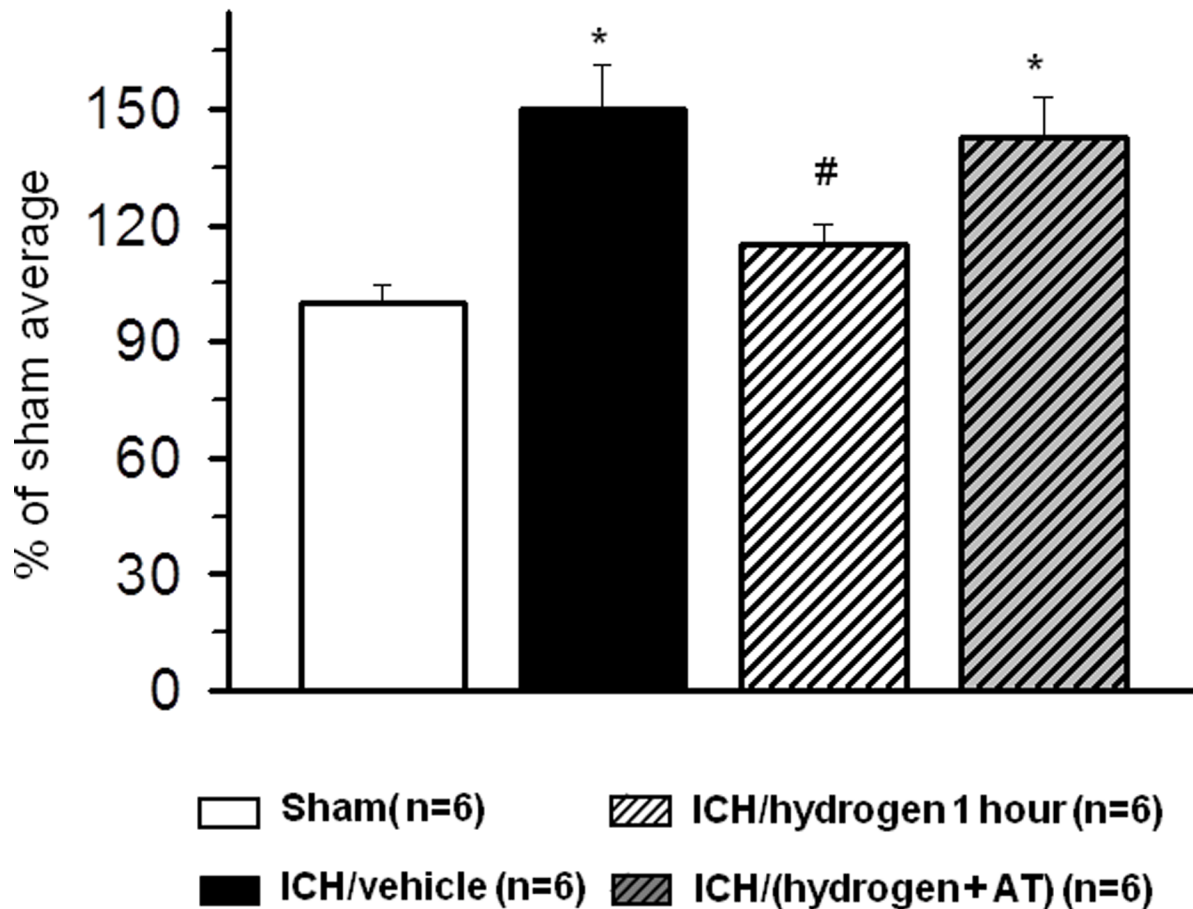


FIGURE 8. Intracerebral hemorrhage (■ Vehicle N=6) significantly increased the production of hydrogen peroxide (H_2O_2) evaluated by Amplex Red peroxidase assay at 2 hours after ICH, when compared to sham operated animals (□ Sham N=6). One hour of hydrogen treatment significantly diminished ICH-induced production of H_2O_2 (▨ N=6). Pretreatment with catalase inhibitor at three hours before inducing of intracerebral hemorrhage (▩ N=6) reversed effect of hydrogen on the H_2O_2 production. * significant vs. sham, # significant vs. vehicle, $p < 0.05$ ANOVA, Tukey test.

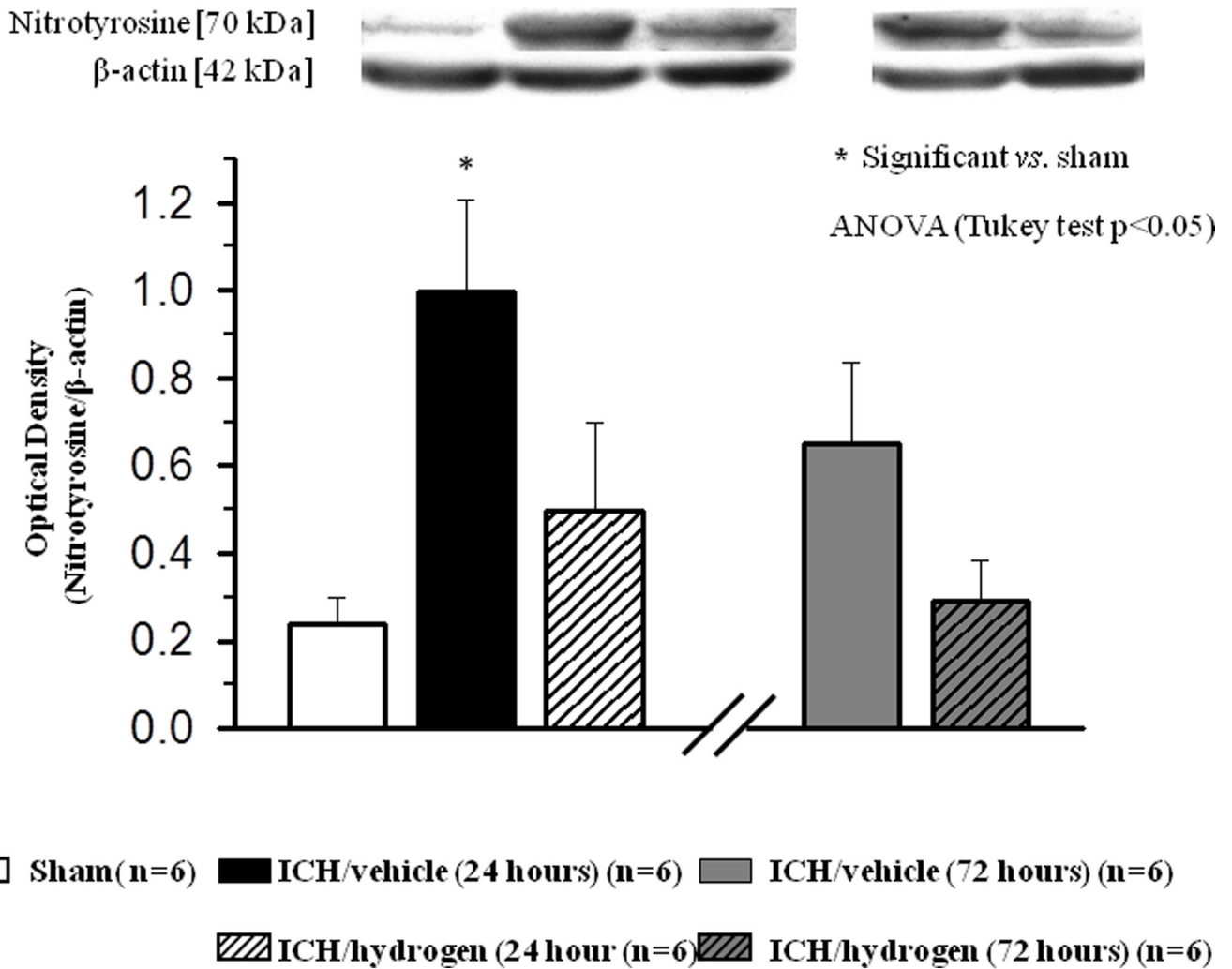


FIGURE 9. At 24 hours after inducing of intracerebral hemorrhage, significant accumulation of nitrotyrosine was observed in brain of animals after intracerebral hemorrhage (■ N=6) compared to sham operated animals (□ N=6). Hydrogen inhalation decreased intracerebral hemorrhage induced accumulation of nitrotyrosine at 24 hours (▨). At 72 hour after inducing of intracerebral hemorrhage, hydrogen treatment showed a strong tendency to diminish nitrotyrosine accumulation in brain of hydrogen treated (▩ N=6) compared to untreated (▒ N=6) animals. * significant vs. sham, # significant vs. vehicle, p<0.05 ANOVA, Tukey test