



## Delayed formation of hematomas with ethanol preconditioning in experimental intracerebral hemorrhage rats

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### ABSTRACT

**Objective:** Spontaneous intracerebral hemorrhage (ICH) accounts for 10%–15% of all strokes and causes high mortality and morbidity. In the previous study, we demonstrated that ethanol could aggravate the severity of brain injury after ICH by increasing neuroinflammation and oxidative stress. In this study, we further investigate the acute effects of ethanol on brain injury within 24 h after ICH. **Materials and Methods:** Totally, 66 male Sprague-Dawley rats were assigned randomly into two groups: saline pretreatment before ICH (saline + ICH), and ethanol pretreatment before ICH (ethanol + ICH). Normal saline (10 mL/kg) or ethanol (3 g/kg, in 10 mL/kg normal saline) was administered intraperitoneally 1 h before induction of experimental ICH. Bacterial collagenase VII-S (0.23 U in 1.0 µL sterile saline) was injected into the right striatum to induce ICH in the rats. We evaluated the hematoma expansion, hemodynamic parameters (heart rate and blood pressure), activated partial thromboplastin time (aPTT), prothrombin time (PT), and striatal matrix metalloproteinase 9 (MMP-9) expressions at 3, 6, 9, and 24 h after ICH. **Results:** The ethanol + ICH group exhibited decreased hematoma at 3 h after ICH; nevertheless, there was a larger hematoma compared with the saline + ICH group at 9 and 24 h after ICH. The ethanol + ICH group had lower blood pressure at 3, 6, and 9 h post-ICH, but both groups maintained similar heart rates after ICH. There was no significant difference in the aPTT and PT between the two groups. Incremental ethanol concentrations had no influence on collagenase VII-S activity at 120 min *in vitro*. MMP-9 expression was upregulated in the right striata of the ethanol + ICH group, especially at 3 and 9 h after ICH. **Conclusion:** Ethanol delayed hematoma formation in the first 3 h due to a hypotensive effect; however, the accelerated growth of hematomas after 9 h may be a sequela of ethanol-induced MMP-9 activation.

**KEYWORDS:** Ethanol, Intracerebral hemorrhage, Matrix metalloproteinase-9

### INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) presenting as bleeding in the brain parenchyma accounts for approximately 10%–15% of all strokes, with an incidence of 4.3 per 10,000 person-years [1]. The high 30-day fatality rate approaches 40% after ICH [2]. The risk factors for ICH include hypertension, alcohol use, current cigarette smoking, and oral anticoagulant and antiplatelet usage [3]. Taylor and Combs-Orme reported binge drinking may enhance all types of strokes among young adults [4]. There is much evidence disclosing how binge drinking can aggravate brain injury [5,6].

In the previous study, we found prior ethanol treatment could aggravate the mortality and severity of ICH-induced brain injury by inducing oxidative stress and neuroinflammation in experimental rat models [7]. However, the acute effects

of ethanol on stroke patients are controversial. Epidemiologic studies suggest that light to moderate ethanol consumption reduces the risk of adverse cerebrovascular events and overall mortality compared with those in abstainers while heavy drinkers (3–4 or more drinks per day) demonstrate increased risks [8,9]. Wang *et al.* even proposed that ethanol preconditioning can ameliorate ischemia/reperfusion-induced brain damage by a mechanism that involves mild reactive oxygen species production through nicotinamide adenine dinucleotide phosphate oxidase [10]. Thus, the acute effects of ethanol on ICH-induced brain injury are still unclear.


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In ICH animal models, elevated matrix metalloproteinase-9 (MMP-9) contributes to blood-brain barrier (BBB) disruption, perihematomal edema, and neuronal cell death [11]. Li *et al.*, found increased MMP-9 levels on admission were associated with poor clinical outcomes at 90 days in human subjects [12]. MMP-9 could be taken as a molecular marker for the prognosis of the severity and secondary injury in ICH. Chronic ethanol exposure increased cerebral MMP-9 activity and resulted in degradation of tight junctions and extracellular matrix in postmortem human brains [13]. Treating brain microvascular endothelial cells with ethanol also promoted MMP-9 activity at 2–48 h *in vitro* [14].

To investigate the acute effect of ethanol on ICH, we injected ethanol intraperitoneally (IP) before induction of ICH. The hemodynamic parameters and coagulative function were monitored in free-moving and awake ICH rats. The hematoma volume was evaluated by serial brain slices.

## MATERIALS AND METHODS

### Animals

All experimental protocols were approved by the Animal Care and Use Committee of Tzu Chi University, Hualien, Taiwan (Approval no. 101-34), in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed under a 12-h light/dark cycle with free access to food and water. All efforts were made to minimize suffering and the number of animals used.

### Grouping

Totally, 66 male Sprague-Dawley rats were used for our study. Normal saline (10 mL/kg) or ethanol (3 g/kg, in 10 mL/kg normal saline) was administered IP in assigned groups before ICH. A total of 48 rats were distributed to saline pretreatment before ICH (saline + ICH,  $n = 6$ ), or ethanol pretreatment before ICH (ethanol + ICH,  $n = 6$ ) for the evaluation of hematoma expansion and western blotting at four separate time points after ICH. Another six rats were sacrificed for brain tissues analysis before experimental ICH as the normal controls. We used another 12 rats for the investigation of hemodynamics and coagulative parameters in the saline + ICH ( $n = 6$ ) and ethanol + ICH ( $n = 6$ ) groups for 24-h consecutive monitoring after ICH.

### Intracerebral hemorrhage induction

Male Sprague-Dawley rats (300–350 g) were anesthetized with pentobarbital 50 mg/kg IP. Bacterial collagenase VII-S (0.23 U in 1.0  $\mu$ L sterile saline) was infused through a 2 mm diameter burr hole into the right striatum (0.0 mm posterior, 3.0 mm right, 5.0 mm ventral to the bregma at the skull surface) of the rat over a period of 10 min [15]. The syringe needle was kept in place for another 10 min to prevent backflow. The burr hole was sealed with bone wax, and the rats were allowed to recover in separate cages equipped with a heating pad (CMA-150, CMA Microdialysis, Stockholm, Sweden) kept at 37°C.

### Evaluation of hematoma expansion

Morphometric measurement of hematomas was conducted 3, 6, 9, and 24 h after ICH [7]. Briefly, rats were decapitated under deep anesthesia and the brains were rapidly removed. The

brains were sliced coronally through the needle entry plane, and then serially sliced into 2-mm thickness. Images were taken by a digital camera. Digital photographs of serial slices were quantified with Image J (NIH, Bethesda, MD, USA). The sliced tissues were also subjected to western blot analysis as indicated below.

### Evaluation of blood pressure, platelet count, activated partial thromboplastin time, and prothrombin time

Twelve rats were randomly assigned into the saline + ICH ( $n = 6$ ) and ethanol + ICH ( $n = 6$ ) groups for evaluation of mean arterial blood pressure (MABP), heart rate (HR), platelet count, activated partial thromboplastin time (aPTT), and prothrombin time (PT). Under isoflurane anesthesia (initial: 5%, maintain: 2%), the femoral arteries of all rats were cannulated with a PE-50 polyethylene tube for monitoring of arterial blood pressure and heart rate. Femoral veins were cannulated for blood withdrawal for platelet counts, and aPTT and PT assays. After the operations, isoflurane was withdrawn to let all rats recover from anesthesia. The hemodynamic signals were transduced to an amplifier (MP35, BIOPAC System, Inc., Goleta, CA, USA) and collected 10 min before (baseline), and 3, 6, 9, and 24 h, after ICH in these conscious rats.

### Collagenase assay

An EnzChek® Gelatinase/Collagenase assay kit was used to measure the effect of ethanol on collagenase activity according to the manufacturer's instruction (E-12055, Molecular Probe, Eugene, OR, USA). Briefly, 0%, 2.5%, 5%, 10%, 20%, 30, and 40% (w/v) of ethanol was mixed with 0.2 U/mL collagenase and incubated for 2 h in DQ collagen solution (100  $\mu$ g/mL). Fluorescence intensity was measured at 0, 15, 30, 60, 90, and 120 min using a microplate reader set for excitation at 495 nm and emission detection at 515 nm.

### Western blotting

Ipsilateral and contralateral striata were dissected from the slices for western blot analysis at 0, 3, 6, 9, and 24 h after ICH insult. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A total of 50  $\mu$ g of total protein from each sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to an Immobilon®-P polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% nonfat milk in 0.05% Tween-Tris-buffered saline. The membranes were probed with various primary antibodies and subsequently with appropriate secondary antibodies. The primary antibodies were anti-MMP 9 antibody (Abcam, Cambridge, MA, USA) and anti- $\beta$  actin (Becton Dickinson, Franklin Lakes, NJ, USA). The antigen-antibody complexes were visualized with an electrochemiluminescence system (Amersham Bioscience, Buckinghamshire, UK) and exposed to Kodak X-OMAT film (GE Healthcare Limited, Buckinghamshire, UK). The intensity of each band was quantified with a GS-800 calibrated densitometer (Bio-Rad) and calculated as the (optical density)/(fix area of band).

### Statistical analysis

Data are presented as mean  $\pm$  standard deviation. Statistical analysis was performed using independent Student's *t*-test for hematoma volume and MMP-9 expression, and two-way

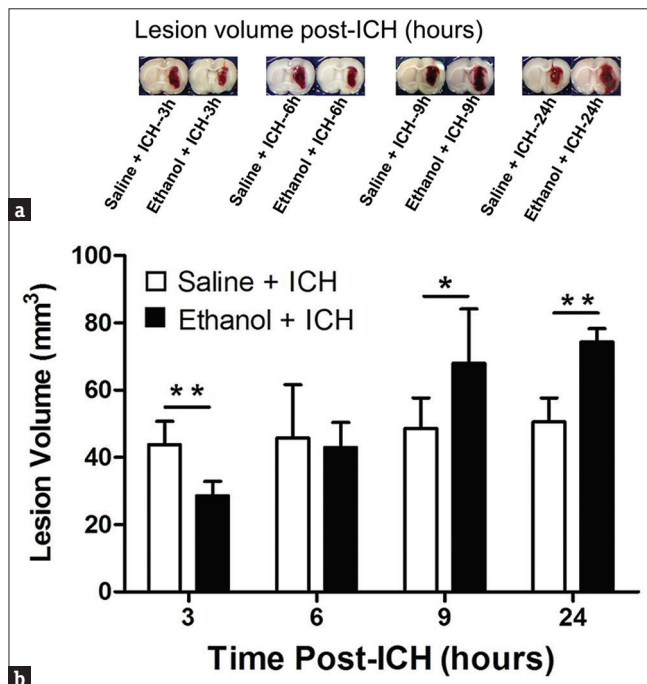
analysis of variance for hemodynamic and coagulative parameters with Prism Graph 5.0 (GraphPad Software Inc., La Jolla, CA, USA). In all instances, *n* refers to the number of animals in a particular group. *P* < 0.05 is considered statistically significant.

## RESULTS

During the hyperacute phase, i.e., 3 h post-ICH insult, the ethanol + ICH rats demonstrated a lower hematoma volume ( $28.6 \pm 4.3 \text{ mm}^3$ ) than the saline + ICH rats ( $43.8 \pm 6.9 \text{ mm}^3$ ). The hematoma volume of the ethanol + ICH group increased significantly at 9 h ( $67.9 \pm 16.2 \text{ mm}^3$ ) and 24 h ( $74.4 \pm 3.9 \text{ mm}^3$ ) while the hematoma volumes of the saline + ICH group were  $48.6 \pm 9.1 \text{ mm}^3$  at 9 h and  $50.6 \pm 7.2 \text{ mm}^3$  at 24 h [Figure 1].

No significant differences in the baseline MABP, HR, platelet count, PT, and aPTT were found in either group [Table 1]. However, ethanol did cause a significant decrease in the MABP (20–30 mmHg) almost immediately after injection. This decrease was sustained up to 9 h and returned to baseline at 24 h [Table 1]. The platelet count, PT, and aPTT demonstrated no differences between the ethanol + ICH and saline + ICH groups at any time point.

To rule out the possible inhibitory effect of ethanol on collagenase activity, various ethanol concentrations (0%, 2.5%, 5%, 10%, 20%, 30%, and 40%, w/v) were tested. There was no inhibition of collagenase activity as revealed by changes in substrate concentration (fluorescence intensity) measured at 0, 15, 30, 60, 90, and 120 min [Figure 2].



**Figure 1:** Morphometric measurements of intrastriatal hematoma volume. (a) Representative images are shown for saline- and ethanol-pretreated animals at 3, 6, 9, and 24 h after ICH. (b) Quantitative analysis of hematomas at 3, 6, 9, and 24 h after ICH. Data are presented as mean  $\pm$  SD. \**P* < 0.05 and \*\**P* < 0.01 versus saline + ICH (*n* = 6 for each group). ICH: Intracerebral hemorrhage, SD: Standard deviation

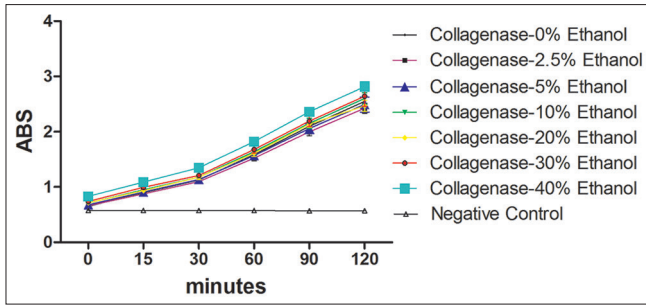
Compared with the saline + ICH rats, the striatal MMP-9 expression of the ethanol + ICH rats significantly increased at 3 and 9 h after ICH [Figure 3].

## DISCUSSION

To study the consequences of hematoma growth after ICH insult, we adopted the collagenase injection model instead of the single blood injection model because the latter only results in a constant hematoma volume [15]. In the collagenase injection model, initial bleeding can occur as early as 10 min after induction. The volume of the hematoma progressed over 1–4 h [16,17]. Our study showed similar hematoma growth in the saline + ICH rats: the hematoma volume stabilized at 3 h post-ICH. In contrast, the hematoma in the striata of the ethanol + ICH rats increased gradually throughout the 24 h observation period after ICH, especially after the first 3 h. To exclude a possible inhibitory effect of ethanol on collagenase, we used the EnzChek Gelatinase/Collagenase assay kit to determine the dose-dependent influence of ethanol on collagenase activity. As shown in Figure 2, there was no direct effect of ethanol on collagenase activity.

In this study, we demonstrated pretreatment with ethanol decreased the hematoma volume at 3 h post-ICH, but aggravated hematoma formation at 9 h post-ICH. The ethanol + ICH rats had persistent hypotension until the end-point of hemodynamic monitoring, 24 h post-ICH. The ethanol did not affect the heart rate or any coagulation function tests in the rats. The concentration of ethanol (up to 40%, w/v) had no influence on the enzyme activity of the collagenase that was used to induce ICH. However, the MMP-9 in the striata of the ethanol + ICH rats significantly increased at 3 and 9 h post-ICH compared with that in the saline + ICH group.

We noted the ethanol + ICH rats exhibited less hematoma expansion than the saline + ICH rats the first 3 h after ICH [Figure 1]. Simultaneously, the ethanol-treated rats showed profound hypotension without changes in cardiac rates [Table 1]. Similar findings were mentioned by Phelan *et al.*, who reported alcohol-intoxicated rats had significantly lower basal mean arterial pressure than controls at baseline [18]. The vasodilation induced by alcohol might contribute to the hypotensive effect after ethanol intake [19,20]. Abdel-Rahman *et al.*, reported ethanol could inhibit baroreflex sensitivity in conscious rats [21]. In normal rats, decreased MABP may induce tachycardia for compensation. Abdel-Rahman also reported ethanol produced a dose-related negative chronotropic effect in both Wistar rats and spontaneously hypertensive rats (SHRs), and was of longer duration in the SHR, particularly at a dose of 1 g/kg [22]. All this evidence suggests why the MABP decreased in ethanol + ICH rats without influencing their heart rates. To the best of our knowledge, the major intermediate metabolite of ethanol is acetaldehyde. Hellström and Tottmar reported only a slight decrease in mean blood pressure was seen at high blood acetaldehyde level (150–250  $\mu\text{M}$ ) after intravenous administration of acetaldehyde (0.5M) [23]. No effect on blood pressure was seen when the concentration of blood acetaldehyde level was lower than 50  $\mu\text{M}$  in the same study. Thus, the hypotension



**Figure 2:** Kinetics of the EnzChek collagenase reaction with various ethanol concentrations after incubation for 0, 15, 30, 60, 90, and 120 min. ABS: Absorbance

**Table 1: Hemodynamic parameters, platelet counts, prothrombin time, and activated partial thromboplastin time of rats with intracerebral hemorrhage**

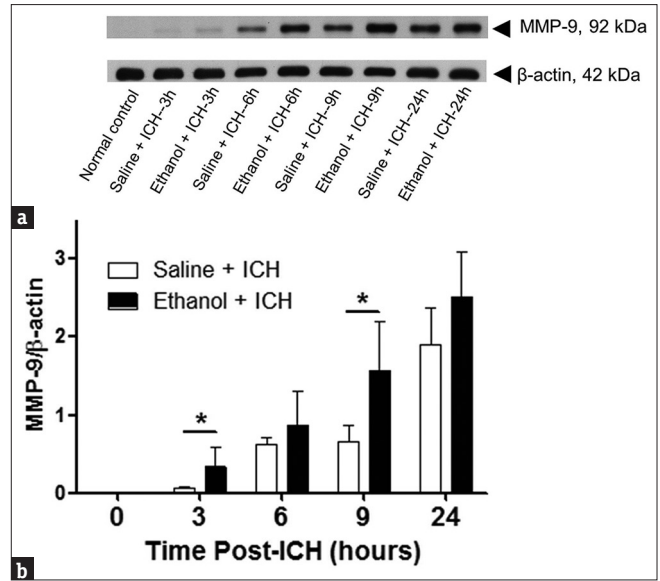
	Baseline	Post-ICH			
		3 h	6 h	9 h	24 h
MABP (mmHg)					
Saline + ICH	107±3	102±6	100±4	105±8	102±4
Ethanol + ICH	113±10	79±12**	78±17*	68±20**	89±6
HR (beats/min)					
Saline + ICH	427±29	425±39	430±37	417±34	414±61
Ethanol + ICH	433±8	437±6	436±7	438±8	435±5
Platelets (10 <sup>9</sup> /L)					
Saline + ICH	289±130	331±102	311±148	320±87	365±53
Ethanol + ICH	300±71	311±68	384±51	353±57	346±67
PT (s)					
Saline + ICH	10.4±0.2	10.6±0.6	10.5±0.1	10.7±1.0	11.5±1.1
Ethanol + ICH	10.6±0.2	11.3±4.4	10.4±1.9	11.2±2.9	12.4±2.3
aPTT (s)					
Saline + ICH	29.1±9.2	22.9±8.6	17.3±3.9	14.7±1.6	15.2±0.1
Ethanol + ICH	26.9±3.0	24.1±6.3	17.7±7.0	17.9±4.9	19.7±4.2

\* $P < 0.05$ , \*\* $P < 0.01$ . Data are presented as mean±SD. aPTT: Activated partial thromboplastin time, HR: Heart rate, ICH: Intracerebral hemorrhage, MABP: Mean arterial blood pressure, PT: Prothrombin time, SD: Standard deviation

in our experimental ICH rats was probably due to the pharmacological effect of ethanol, but not its metabolites. As a consequence, ethanol-induced hypotension might prevent hematoma volume progression in the early stage of ICH.

Larger intrastriatal hematomas emerged at 9 h, with sustainable hypotension after ICH in ethanol-treated rats. The rapid progression of hematoma enlargement might be due to coagulopathy. Further experiments revealed ethanol did not disturb coagulative functions including platelet count, PT, and aPTT. Several studies have shown ethanol intoxication did not affect fibrinolytic activity in healthy men or rats [24,25]. Erstad *et al.* also demonstrated that recent ethanol exposure was not associated with significant changes in transfusion requirements or coagulation parameters in major trauma patients [26]. It is thus becoming a consensus that acute ethanol administration causes no coagulopathy or impaired hemostasis.

The ethanol + ICH rats produced more MMP-9 at 3 and 9 h post-ICH [Figure 3] than the saline + ICH rats. Interestingly, the hematomas in the ethanol + ICH animals were significantly smaller than those in the saline + ICH 3 h after ICH. Delayed hematoma expansion was demonstrated in ethanol-pretreated



**Figure 3:** MMP-9 protein expression (a) representative changes in MMP-9 and  $\beta$ -actin at 3, 6, 9, and 24 h post-ICH insult. (b) Quantitative densitometry analysis showing relative expression of MMP-9 protein expression normalized with  $\beta$ -actin. Data are presented as mean  $\pm$  SD \* $P < 0.05$  versus saline + ICH ( $n = 6$  for each group). MMP-9: Matrix metalloproteinase-9, ICH: Intracerebral hemorrhage, SD: Standard deviation

rats in our study. MMPs are important executors in extracellular matrix remodeling. They comprise of 8 subgroups named after their substrates: matrilysins, collagenases, stromelysins, and gelatinases [27]. MMPs can be activated by multiple pathways in the brain after ICH, for instance, hemoglobin and its derivatives [28], oxygen, or nitrogen free radicals [29], and neuroinflammatory factors [30]. On the other hand, ethanol and its metabolite, acetaldehyde, have been proven to activate MMPs through protein tyrosine kinase signaling in brain microvascular endothelial cells [14]. These activated MMPs might cause secondary brain injury, such as disruption of the BBB, brain edema, and massive neuronal death. Among these MMPs, MMP-9 is crucial for degrading basal lamina surrounding cerebral blood vessels and tight junctions of the BBB [31]. A human brain magnetic resonance imaging study revealed high BBB permeability surrounding ICH correlated to large hematomas and edema formation [32]. Hence, we proposed the ethanol-induced increment of MMPs might be responsible for the aggravated hematoma expansion after 3 h post-ICH.

## CONCLUSION

Brott *et al.* demonstrated that 26% patients with acute ICH had hemorrhagic expansion within the 1<sup>st</sup> h, and an additional 12% of patients had hematoma growth within 1–20 h [33]. The hematoma expansion could be predicted by a systolic BP >160 mmHg at 1.5 h after admission [34]. Recently, Rodriguez-Luna *et al.* also mentioned that a systolic BP >180 mmHg in the 24 h after ICH elevated the odds ratio of hematoma growth [35]. The intensive blood pressure reduction in acute cerebral hemorrhage trial proved rapid intensive lowering of blood pressure could achieve 2–4 mL absolute attenuation of hematoma growth [36]. This suggests that aggressive blood pressure control in the early stage of ICH might improve patients' clinical outcomes.

The smaller hematoma volume could be a result of sustained hypotension in the early phase (0–3 h) while increases of ethanol-induced MMP-9 might cause rapid progression of hematoma growth in the later period. The underlying mechanism between ethanol and MMP-9 activation in ICH rats still needs further investigation.

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#### Conflicts of interest

There are no conflicts of interest.

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