Isoflurane Preconditioning Affords Functional Neuroprotection in a Murine Model of Intracerebral Hemorrhage

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Abstract *Introduction*: Exposure to isoflurane gas prior to neurological injury, known as anesthetic preconditioning, has been shown to provide neuroprotective benefits in animal models of ischemic stroke. Given the common mediators of cellular injury in ischemic and hemorrhagic stroke, we hypothesize that isoflurane preconditioning will provide neurological protection in intracerebral hemorrhage (ICH).

Methods: 24 h prior to intracerebral hemorrhage, C57BL/6J mice were preconditioned with a 4-h exposure to 1% isoflurane gas or room air. Intracerebral hemorrhage was performed using a double infusion of 30-μL autologous whole blood. Neurological function was evaluated at 24, 48 and 72 h using the 28-point test. Mice were sacrificed at 72 h, and brain edema was measured.

Results: Mice preconditioned with isoflurane performed better than control mice on 28-point testing at 24 h, but not at 48 or 72 h. There was no significant difference in ipsilateral hemispheric edema between mice preconditioned with isoflurane and control mice.

Conclusion: These results demonstrate the early functional neuroprotective effects of anesthetic preconditioning in ICH and suggest that methods of preconditioning that afford protection in ischemia may also provide protection in ICH.

Keywords Intracerebral hemorrhage · Preconditioning · Isoflurane

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Introduction

Intracerebral hemorrhage (ICH) is associated with the highest fatality rate of any stroke subtype, yet remains the cerebrovascular disease with perhaps the least impactful treatment options [1]. Strategies designed to prevent hematoma expansion have been minimally effective, and no therapy currently exists to mitigate injury to parenchyma surrounding the hematoma [2]. Spontaneous intracerebral hemorrhage is thought to impose mechanical stress on the surrounding parenchyma with consequent dysfunctional cellular and mitochondrial activity, as well as abnormal neurotransmission [3, 4]. Within hours of ictus, hemoglobin, cellular degradation products and coagulation factors induce vasogenic and cytotoxic edema by disruption of the blood-brain barrier and initiation of apoptosis. Peri-hematoma histology is characterized by edema, neuronal damage, macrophages and neutrophils and nests of intact neural tissue within and surrounding the hematoma [5]. Evidence also suggests that local mass effect may result in reduced cerebral blood flow to the hematoma periphery, placing intact neural tissue at particularly high risk for infarction [6]. Given evidence of multiple mechanisms involving post-hemorrhagic tissue injury, an area of at-risk tissue adjacent to the hematoma may exist at which novel therapeutic interventions can be directed.

Isoflurane, one of the most commonly utilized inhaled anesthetics, has been studied as a neuroprotective agent in ischemia [7]. Pretreatment with volatile anesthetics has been shown to induce prolonged neuroprotection in vitro and in vivo primarily by decreasing apoptosis and excitatory stress and increasing neuronal stability [8]. Studies in a rodent model of middle cerebral artery occlusion demonstrated significant reduction in infarct volume after pretreatment with isoflurane [7] and highlight the potential for preconditioning in reducing ischemic injury. Given the mechanisms of peri-hematoma cellular injury in ICH may reflect those found in ischemic injury and apoptosis, we hypothesized that isoflurane preconditioning would mitigate neurological injury in our murine model of intracerebral hemorrhage.

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Materials and Methods

Isoflurane Preconditioning

Adult male C57BL/6J rats (8–10 weeks, 25–30 g body weight) (Jackson Laboratory, Bar Harbor, ME) were housed in the Barrier Facility of the Columbia University Medical Center, maintained at 22 ± 2°C with constant humidity under a 12:12 h light:dark cycle, and were allowed free access to water and laboratory chow. Experiments were carried out in accordance with the guidelines set forth by the IACUC Guide for the Care and Use of Laboratory Animals. Isoflurane preconditioning was carried out by placing mice in an air-tight anesthesia induction chamber while delivering 1% isoflurane in room air. Body temperature was controlled as needed using a feedback-regulated heat lamp. Mice were preconditioned with 1% isoflurane or room air alone for 4 h. Twenty-four hours after preconditioning, mice subsequently underwent experimental ICH ($n = 16$) according to our established model of double-injection of autologous blood [9].

Experimental Intracerebral Hemorrhage

Preconditioned adult male C57BL/6J mice were subjected to experimental intracerebral hemorrhage (ICH) using autologous blood infusion via our model as previous described [9]. Mice were anesthetized with ketamine/xylazine (1 mg/kg) throughout the duration of surgery. Body temperature was regulated using a rectal probe connected to a feedback-controlled heat lamp. Once experimental ICH was achieved, mice were allowed to fully recover from anesthesia in an intensive care unit set at 37°C with constant humidity for 1 h. Once the mice were fully recovered from anesthesia, they were placed in a clean cage with access to food and water in our satellite facility.

Assessment of Neurological Deficit

Neurological deficit was determined using a 28-point test as outlined by Clark et al. [10]. At 24, 48 and 72 h post-ICH, all animals were assessed according to the 28-point test, evaluating body symmetry, gait, climbing, circling behavior, front-limb symmetry, compulsory circling and sensory response.

Preparation of Brain Samples

Three days after experimental ICH, mice were anesthetized with ketamine/xylazine (1 mg/kg) and were transcardially perfused with cold saline followed by removal of the brain for albumin Western blots ($n = 16$).

Albumin Western Blot

Protein samples were loaded onto a NuPAGE 4–12% Bis-Tris Gel, and electrophoresis was performed in MOPS buffer at room temperature. Samples were then transferred to a 0.2-μm pore size nitrocellulose membrane, which was then blocked in 1.5% gelatin, 100 mM NaCl and 25 mM Tris pH 7.5 for 1 h at room temperature. Membranes were then washed and incubated with anti-albumin horseradish peroxidase conjugated antibody (A90-134P; Bethyl Laboratories) in 1% gelatin, 100 mM NaCl, 25 mM Tris pH 7.5 and 0.1% Tween-20 for 1 h at room temperature. Antibodies were then visualized using chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). Densitometric analysis was performed using ImageJ software (NIH, http://rsb.info.nih.gov/ij), and results were normalized to beta-actin expression.

Quantitative Assessment and Statistical Analysis

All data in this study are presented as mean ± standard error. Data were analyzed with a Student's *t*-test, and significance levels were measured at $p < 0.05$.

Results

Functional Performance

Two cohorts of mice underwent functional testing following experimental ICH. Isoflurane-preconditioned mice ($n = 8$) were administered 1% isoflurane in an air-tight chamber prior to ICH, and room air-preconditioned mice ($n = 8$) were administered room air in the same preconditioning chamber prior to ICH. Functional 28-point testing was performed at 24, 48 and 72 h after ICH. Mice preconditioned with isoflurane performed significantly better than room air-preconditioned mice at 24 h (10.44 ± 0.87 vs. 14.1 ± 0.57 $p = 0.009$). Though a continued trend was seen towards improved performance in mice preconditioned with isoflurane, the difference was not significant at 48 h (8.11 ± 1.39 vs. 11.30 ± 1.14, $p = 0.27$) and 72 h (6.22 ± 0.66 vs. 8.10 ± 0.92, $p = 0.33$) (Fig. 1). As demonstrated in previous experiments with our murine autologous double injection ICH model, the likelihood of observing significant functional deficits by 72 h diminishes considerably because all animals show a gradual recovery of function by 72 h after ICH [9].

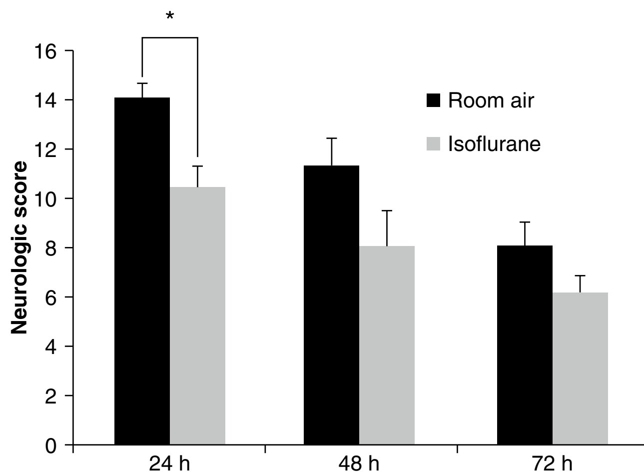

Fig. 1 Neurological score as assessed by 28-point testing in room air- and isoflurane-preconditioned mice at 24, 48 and 72 h. Values are expressed as mean ± SEM. An *asterisk* represents significant difference by Student's T-testing ($p < 0.05$)

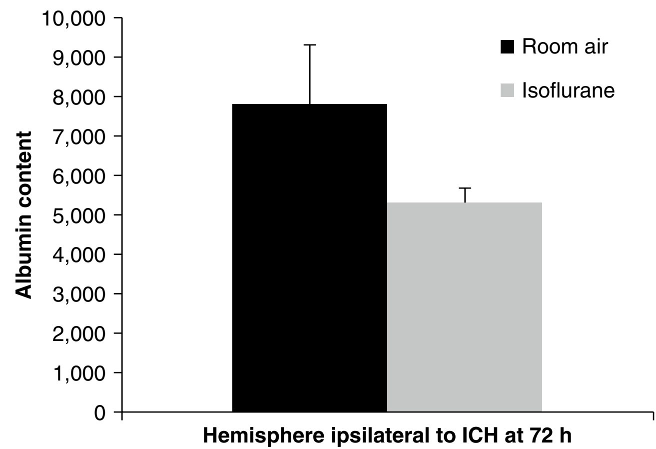

Fig. 2 Edema in the hemisphere ipsilateral to the ICH as measured by albumin content, assessed by Western blot from brains sacrificed at 72 h. Values are expressed as mean ± SEM

Brain Edema

Perihematomal brain edema was assessed by albumin content in the basal ganglia and cortex ipsilateral to the ICH ($n = 12$). Brain edema was not significantly less in mice preconditioned with isoflurane compared to control mice (5357.39 ± 341.58 vs. 7782.52 ± 1582.13, $p = 0.165$) (Fig. 2).

Discussion

In light of recent evidence demonstrating the neuroprotective effects of preconditioning with volatile anesthetics in animal models of cerebral ischemia [11], we hypothesized that anesthetic preconditioning would also afford neurological protection in ICH, a condition that subjects neurological tissue to multiple, complex mechanisms of cellular injury [5, 12]. Cell death and ischemia may occur in salvageable perihematomal tissue following ICH, as microvasculature is subjected to mass effect, mechanical deformation, edema and inflammation. Although the relevance of "penumbral" ischemia in ICH remains controversial, and the precise mechanisms of neuroprotection in ischemic preconditioning are yet undefined, a historical overlap of neuroprotective interventions successful in both ischemic injury and ICH suggests a significant overlap in their contributory mechanisms of injury [13].

Our study is the first report of anesthetic preconditioning-induced neuroprotection in ICH and demonstrates that isoflurane pretreatment affords functional neuroprotection at 24 h after ICH, as assessed by 28-point testing. The only other report of preconditioning-induced protective effects we identified in the ICH literature was a report of reduced perihematomal edema after hyperbaric oxygen preconditioning in rats [14]. That functional protection occurred in isoflurane-preconditioned mice at 24 h but not at 48 and 72 h may be a reflection of the tendency for functional deficits to improve by 2–3 days in our particular ICH model. Whether a reflection of the animal model alone or a true failure of anesthetic preconditioning to provide sustained neuroprotection in ICH, improved early recovery may still have important clinical implications.

This study did not demonstrate a significant reduction in ipsilateral hemispheric edema in preconditioned mice. Though in human ICH the contribution of brain edema to functional outcome remains controversial [5], we evaluated edema as an outcome measure because previous rodent ICH studies have suggested an association between reduced edema and improved functional performance, despite no direct evidence of a causal relationship [15–17]. Furthermore, the only other ICH preconditioning study, with hyperbaric oxygen pretreatment, demonstrated a significant reduction in perihematomal edema [14, 18]. However, our results support the notion that functional protection can be achieved in the absence of significant edema reduction, which both reflects human ICH studies demonstrating that absolute edema volume is not an independent predictor of outcome [19, 20] and points toward other mechanisms of neuronal injury modulation with experimental anesthetic preconditioning. At present, the downstream molecular effects of isoflurane preconditioning are incompletely understood [21]. A majority of the data showing neuroprotection with volatile anesthetics comes from animal models of ischemia, and has been hypothesized to involve the enactment of gene upregulation and long-term neuronal tolerance to ischemic insults [21–24]. Immediate early genes, ubiquitous transcription factors induced by a stress response, are thought to be the main component of tolerance when preconditioned hours to

days before an insult [7, 22]. The inducible nitric oxide synthase (iNOS) gene, whose downstream effects include modulation of glutamate signaling and the vasodilatory response, has been heavily implicated as one of these mediators in anesthetic preconditioning [7, 25, 26].

A limitation of the present study is a lack of comprehensive investigation of the potential mechanisms of protection, including assessment of the role of the iNOS and other candidate genes, and histological evaluation of peri-hematomal inflammatory cell infiltration and apoptotic cell death. Further studies are critical to determine the molecular and cellular processes of protection, which will ultimately lead us toward the optimal therapeutic targets for mitigation of injury in human ICH.

Conflict of interest statement We declare that we have no conflict of interest.

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