Research report

The collagenase model of intracerebral hemorrhage in awake, freely moving animals: The effects of isoflurane

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HIGHLIGHTS

● We present a method for inducing ICH in awake animals.
● We used telemetry to examine the effect of isoflurane on physiology after ICH.
● Physiological confounds induced by isoflurane resolve within minutes to hours.
● Post-ICH pain levels were comparable in anesthetized and non-anesthetized models.

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ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating stroke often modelled in rats. Isoflurane anesthetic, commonly used in preclinical research, affects general physiology (e.g., blood pressure) and electrophysiology (e.g., burst suppression) in many ways. These physiological changes may detract from the clinical relevance of the model. Here, we revised the standard collagenase model to produce an ICH in rats without anesthetic. Guide cannulas were implanted stereotaxically under anesthetic. After 3 days of recovery, collagenase was infused through an internal cannula into the striatum of animals randomly assigned to the non-anesthetized or isoflurane group. We assessed whether isoflurane affected hematoma volume, core temperature, movement activity, pain, blood pressure, and seizure activity. With a small ICH, there was a hematoma volume increased from 8.6 (± 3.3, 95% confidence interval) µL in anesthetized rats to 13.2 (± 3.1) µL in non-anesthetized rats (P = 0.008), but with a larger ICH, hematoma volumes were similar. Isoflurane decreased temperature by 1.3 °C (± 0.16 °C, P < 0.001) for 2 h and caused a 35.1 (± 1.7) mmHg group difference in blood pressure (P < 0.007) for 12 m. Blood glucose increased twofold after isoflurane procedures (P < 0.001). Pain, as assessed with the rat grimace scale, did not differ between groups. Seizure incidence rate (62.5%) in non-anesthetized ICH rats was similar to historic amounts (61.3%). In conclusion, isoflurane appears to have some significant and injury size-dependent effects on the collagenase model. Thus, when anesthetic effects are a known concern, the use of the standardized cannula infusion approach is scientifically and ethically acceptable.

1. Introduction

Intracerebral hemorrhage (ICH) has a mortality rate of approximately 40%, and accounts for 10–20% of all strokes (Feigin et al., 2009). Despite improvements in patient care and risk factor management, incidence and mortality rates have not changed in the past three decades (van Asch et al., 2010). In order to enhance the chance of translational success and replicability, preclinical animal models must be as accurate to clinical presentation as possible. Currently, a major discrepancy of many preclinical ICH studies is the
presence of volatile (e.g., isoflurane, sevoflurane, and desflurane) or injectable anesthetics (e.g., pentobarbital, ketamine and xylazine) shortly before or during the hemorrhage (MacLellan et al., 2012). These anesthetics are often necessary, but introduce a confound not present in ICH patients. Thus, understanding the effect of anesthetics in pre-clinical stroke models is vital, especially considering their influences on physiology and neuroprotection (Archer et al., 2017; Jiang et al., 2017; Statler et al., 2006; Tsurugizawa et al., 2016). For example, a photothrombosis model in awake animals has been developed to carefully examine the effects of isoflurane after focal ischemia (Seto et al., 2014), but effects may differ after ICH.

The fluorinated ether anesthetic family, including isoflurane, sevo-flurane, and desflurane, are believed to work by inhibiting pre-synaptic excitatory activity and enhancing inhibitory post-synaptic GABA receptors activity (Ramachandra et al., 1989). Isoflurane, in particular, is commonly used in preclinical laboratories due to its affordability and low solubility coefficient in blood, which allows for quicker anesthetic induction and recovery (Ramachandra et al., 1989). Isoflurane anesthesia is commonly coupled with nitrous oxide gas as this reduces the blood solubility coefficient, increasing uptake rate (Xie et al., 1993). Adding nitrous oxide to isoflurane also causes heightened cardiovascular activity, including increased blood pressure and heart rate compared to isoflurane alone (Duke et al., 2006). In animals, isoflurane is eliminated from the brain according to the two-compartment model, with half-lives of 26 and 174 min (Chen et al., 1992). Thus, while they regain consciousness quickly there is still isoflurane present at the time of the bleed (MacLellan et al., 2008).

Concerningly, isoflurane alters physiology in ways that may impact stroke outcome. Notably, isoflurane can be neuroprotective when administered during pre, post, or peri-ischemic periods (Matchett et al., 2009; Wang et al., 2016). This neuroprotective effect arises from several mechanisms including limiting excitotoxicity, pro-apoptotic signaling, and the expression of pro-inflammatory factors; improving intracellular Ca\(^{2+}\) homeostasis; and causing vasodilation, thereby improving cerebral blood flow (Matchett et al., 2009; Wang et al., 2016). Both pre- and post-conditioning with isoflurane can reduce cell death, edema, and behavioural deficits following ICH, presumably through similar mechanisms (Gigante et al., 2011; Khatibi et al., 2011).

Therefore, the effects of anesthetics must be carefully considered in preclinical research, including when testing neuroprotective agents (Seto et al., 2014).

Despite the promising neuroprotective potential of isoflurane, it may also impact stroke outcome negatively, especially during or following ICH. As mentioned, isoflurane is a potent vasodilator that increases cerebral blood flow (Constantinides and Murphy, 2016; Sicard et al., 2003). During a hemorrhage, an increase in cerebral blood flow could increase the size of the hematoma; however, this may be compensated for, as isoflurane also reduces blood pressure (BP) and heart rate (Yang et al., 2014). In addition to these hemodynamic factors, temperature is an important component in hemostasis (Wolberg et al., 2004). Volatile anesthetics can lower temperature by several degrees in patients (Khurram et al., 2014) and animals (Colbourne et al., 1993) during and transiently after surgical procedures. This is potentially problematic, as mild reductions in temperature can inhibit clotting enzymes and can cause platelet dysfunction (Wolberg et al., 2004). Temperature reductions also impact other mechanisms of injury, such as inflammation and apoptosis (Moore et al., 2011). Thus, establishing whether anesthetic influences these physiological factors in the collagenase model of ICH is vital, as the predictive accuracy of our preclinical model could be affected.

As with spontaneous ICH in patients, rats experience seizures in the days following a collagenase-induced ICH (Klahr et al., 2016, 2014). Electroencephalograms (EEG) show burst suppression under isoflurane anesthesia, a pattern in which cortical activity alternates between silence and high amplitude slow or sharp waves (Amzica, 2009). Isoflurane also disrupts cortical network connectivity (Hentschke et al., 2017), a feature that has also been associated with seizures (Vega-Zelaya et al., 2015). However, isoflurane also depresses glutamate transmission, which is believed to play a role in post-ICH injury (Castillo et al., 2002). How these alterations influence seizure activity following the collagenase ICH model is unclear; therefore, establishing whether isoflurane is interacting with the injury to produce seizures, or whether this is an intrinsic property of the ICH model is important. Post-stroke seizure activity may be notably different in frequency and intensity in animals that are not given anesthetics.

Thus, in order to assess isoflurane’s impact on the collagenase ICH...
model, we utilized a common drug delivery approach to infuse collagenase into awake animals. Rats were randomized to either the isoflurane (ISO) or the no isoflurane (NO-ISO) group. In the first experiment, we assessed the impact of isoflurane on blood glucose as well as hematoma volume, temperature, activity, and pain acutely following a small ICH (Fig. 1). In the second experiment, based on these results, we again assessed hematoma volume, blood glucose, and pain after a large ICH to determine if our findings were dependent on ICH size. In the third experiment, we compared the historic characteristics, including rate, duration, and severity of post-stroke seizures commonly observed following an anesthetized ICH procedure with that seen here in conscious animals to determine the effect of isoflurane on seizure activity. In the fourth experiment, we compared the BP and hematoma volume observed during and following the anesthetized and conscious ICH models to investigate one mechanism by which isoflurane may be affecting hematoma volume.

2. Results

2.1. Exclusions and mortality

Five animals were completely excluded from experiment 2, and 1 additional animal was excluded from RGS analysis. One animal in the ISO group died spontaneously after ICH, and 1 animal in the NO-ISO group was not given an ICH due to a blocked cannula. In the ISO group, 1 animal was excluded from RGS analysis because he was sleeping for the duration of the video. Three animals were excluded based on insufficient hematoma volume, 2 from the ISO group and 1 from the NO-ISO group. In experiment 3, one animal spontaneously died ~42 h after ICH, but was still included in assessment. Two animals’ BP data in experiment 4 were excluded from the ISO group due to blocked catheter. No animals were excluded or prematurely euthanized for animal welfare concerns.

2.2. Experiment 1: The effects of isoflurane after a small ICH

2.2.1. Blood glucose

Blood glucose was measured before and after the telemetry probe and cannula implantation surgeries, in which both groups were exposed to isoflurane. There was a significant interaction between group and time on blood glucose after the telemetry probe and cannula implantation surgeries (Fig. 2c, \( P = 0.046 \), interaction effect). There were no group differences at either time of measurement (Fig. 2c, \( P = 0.489 \), before surgery; \( P = 0.300 \), after surgery). Blood glucose was increased by the end of the surgery in both groups (Fig. 2c, ISO group \( P < 0.001 \), Cohen’s \( d = 1.91 \); NO-ISO group \( P < 0.001 \), Cohen’s \( d = 2.83 \)).

2.2.2. Hematoma volume

Hematoma volume was significantly larger in the NO-ISO group (Fig. 2a, \( P = 0.042 \), Cohen’s \( d = 0.60 \), moderate effect). Data was not distributed normally in either group (both \( P < 0.05 \), Shapiro-Wilk’s test). When assessed with a Mann-Whitney U test, the NO-ISO group still had a significantly larger hematoma volume than the ISO group (Fig. 2, \( P = 0.008 \)).

2.2.3. Temperature and activity

Average core temperature during the 24 h baseline did not differ between the groups (\( P = 0.088 \), data not shown). There was a significant interaction between time and group on temperature post-ICH (Fig. 3a, \( P < 0.001 \), interaction effect). Isoflurane significantly reduced temperature for the first 2 h after surgery (Fig. 3a, \( P < 0.001 \) immediately post-ICH, Cohen’s \( d = 3.62 \); \( P = 0.037 \) at 1 h post-ICH, Cohen’s \( d = 1.10 \)). Similarly, there was a significant interaction between time and group on activity (Fig. 3b, \( P = 0.020 \), interaction effect). The NO-ISO rats were less active at 10 h post-ICH only (Fig. 3b, \( P = 0.037 \), Cohen’s \( d = 1.17 \)). As this difference only occurred at one time comparison, it is likely due to chance.

2.2.4. Rodent grimace scale

There was no group difference in observed pain after ICH at either time (Fig. 4a, \( P = 0.577 \), 6 h; \( P = 0.529 \), 23 h). Pain scores did not differ between 6 and 23 h post-ICH (Fig. 4a, \( P = 0.333 \)). There were no differences in pain between groups on any of the four subscales (all \( P > 0.454 \)). The median score of 0.28 shows the majority of rats are below the proposed analgesic intervention threshold of 0.67 (Oliver et al., 2014). In the ISO group, 1 rat was above this threshold, and in the NO ISO group there were 3 rats above this threshold (\( P = 0.609 \)).

2.2.5. Weight loss

There was no difference in weight loss between the groups (\( P = 0.602 \), interaction effect; \( P = 0.685 \), group main effect) and there was no significant weight loss after the ICH (\( P = 0.659 \), time main effect, data not shown).

2.3. Experiment 2: The effects of isoflurane after a large ICH

2.3.1. Blood glucose

Blood glucose levels were significantly increased after cannula implantation surgery (Fig. 2d, \( P < 0.001 \), time main effect, partial \( \eta^2 = 0.79 \)). There was no difference in blood glucose between the ISO and NO-ISO groups (Fig. 2d, \( P = 0.824 \), group main effect; \( P = 0.807 \), interaction effect), who were both given isoflurane for that procedure, which was three days prior to the ICH induction.

2.3.2. Hematoma volume

Isoflurane did not significantly impact hematoma volume in this experiment (Fig. 2b, \( P = 0.169 \)). Animals in the NO-ISO group had significantly less variability in hematoma sizes as compared to the ISO group (Fig. 2b, \( P = 0.009 \)). Our conclusion did not change when analysis was redone with Welch’s correction (\( P = 0.183 \)).

2.3.3. Rodent grimace scale

There was no effect of isoflurane on rat grimace scale scores after ICH (Fig. 4b, \( P = 0.358 \), 6 h; \( P = 0.936 \), 23 h). There were no group differences in pain on any of the four subscales (all \( P > 0.151 \)). With the larger ICH, the median score of 0.80 is slightly higher than the analgesic intervention score of 0.67, indicating that approximately half of the animals were experiencing pain (Oliver et al., 2014). The median score seen here is similar to what is seen in other injuries, such as spinal cord injury (Schneider et al., 2016), and lower than a previous ICH study (Saine et al., 2016). The grimace scale scores did not improve or worsen by 23 h post-ICH (Fig. 4b, \( P = 0.333 \)).

2.3.4. Weight loss

There was significant weight loss after the ICH (\( P < 0.001 \), time main effect), with animals losing an average of 5.7% of their body weight. However, there was no difference in weight loss between the groups (\( P = 0.991 \), interaction effect; \( P = 0.537 \), group main effect, data not shown).

2.4. Experiment 3: Seizure activity after non-anesthetized ICH

2.4.1. Baseline EEG in the collagenase group

When we compared the RMS of slow wave sleep of the day prior to stroke with days 1 and 2 post-ICH in those rats with epileptiform activity, we found a significant effect for day (\( P = 0.013 \)) but no interaction with hemisphere (\( P = 0.266 \), Fig. 5a). This shows that the fluctuations in non-epileptiform EEG traces after ICH were higher on day 1 compared to day 2 in both hemispheres.

2.4.2. Seizures after collagenase

Five out of eight rats (62.5%) had seizures and interictal spikes
(Figs. 5–7) within the first two days after the stroke, with the earliest occurring after ~7.5 h and the latest occurring after ~45 h. Seizures ranged in duration from ~5 to 45 s (Table 1, Fig. 6). All rats with seizures had extended periods of abnormal interictal activity, as seen previously (Klahr et al., 2016, 2014) Table 2. Most seizures were bilateral, but there were instances in which seizures occurred only ipsilaterally or contralaterally (Fig. 6). Surprisingly, in animals that spent the least amount of time in seizure activity, slow wave sleep RMS was reduced compared to baseline slow wave sleep RMS, indicating that the fluctuations had decreased in amplitude. Increases in power were seen at almost all frequencies for rat 3 (Table 1), which had the highest RMS and the longest seizures. However, for rat 1, which had a low RMS, it had an overall decrease in power, with those frequencies mainly affected being those below 11 Hz.

For coherence calculations, we concentrated on increases in coherence at the frequencies in which we noted a change in power. Coherence values range from 0 to 1; values of 0 indicating that signal-specific frequencies between the two channels are completely unrelated, whereas values of 1 indicate that they are completely related. We found that for animal 1 there was an overall increase in coherence. Even though rat 3 had power increases along most frequencies of interest, the seizures showed mostly decreases in coherence in frequencies ranging from 0.12 to 1 Hz and 6–13 Hz, and 20–25 Hz and increases in frequencies between 40 and 50 Hz.

2.4.3. Lesion volume

The mean lesion volume in this experiment was 47.1 mm$^3$ (± 31.8 mm$^3$, 95% CI). We did not observe a relationship between lesion volume and number of seizures (Fig. 5b, R$^2$ = 0.170, P = 0.310) or the total duration of seizures (R$^2$ = 0.103, P = 0.438).

2.5. Experiment 4: Effect of isoflurane on blood pressure and hematoma volume

2.5.1. Blood pressure

During the ICH procedure, there was a significant interaction between group and time on BP (Fig. 8a, P = 0.003), but these changes were transient. The animals in the NO-ISO group had significantly elevated BP as compared to the ISO group from minutes 2–12 of the ICH procedure (Fig. 8a, all P < 0.007, partial η$^2$ = 0.43). Note that animals in the ISO group were kept under isoflurane for 25 m, but animals in the NO-ISO group were typically done their infusion procedure within 10 m (infusion plus handling time).

In the 24 h following the ICH procedure, there was no effect of group on hourly averaged BP (Fig. 8b, P = 0.492, group main effect; P = 0.152, interaction effect). There was a significant effect of time on BP (Fig. 8b, P < 0.001), likely a circadian rhythm effect.

2.5.2. Hematoma volume

There was no group difference in hematoma volume (P = 0.955,
The size of the hematoma was not predicted by peak BP ($R^2 = 0.097$, $P = 0.382$), average BP ($R^2 = 0.244$, $P = 0.147$), average BP during the ICH procedure ($R^2 = 0.103$, $P = 0.366$), or average BP during the first 6 h post-ICH ($R^2 = 0.252$, $P = 0.139$).

### 3. Discussion

Models of ICH typically require anesthetics that often markedly affect physiology (Tsurugizawa et al., 2016). Here, we used a cannula infusion system to investigate a model of collagenase ICH in non-anesthetized animals in order to assess the effects of isoflurane on outcome after ICH. Non-anesthetized animals had similar pain levels after ICH, indicating that this is an ethically acceptable way to induce an ICH and avoid anesthetic confounds. Isoflurane slightly decreased BP, decreased temperature, and increased blood glucose; however, these effects often resolved within minutes to hours. The effects of isoflurane lead to an increase in bleeding when the ICH was small, but did not significantly increase bleeding when the ICH was large. It is possible that other anesthetics or a longer duration of anesthetic may have greater effects, although Esposito and colleagues found that higher doses of isoflurane did not impact collagenase induced ICH (Esposito et al., 2013). This cannula infusion model is beneficial in cases where anesthetic effects are being tested and when anesthetic interactions with other therapies are a concern.

We used the RGS to determine if the lack of isoflurane during the ICH procedure resulted in additional pain. We found that the RGS scores both 6 and 23 h after ICH did not differ between groups, indicating that conducting the procedure under isoflurane anesthesia does not reduce post-ICH pain and is an ethical method of ICH induction. Further, animals experienced approximately the same weight loss in the day after ICH, providing further evidence that the non-anesthetized procedure was not causing significant additional stress. This also suggests that most of the pain experienced after collagenase-induced ICH is due to the injury itself and not the surgical procedure. After a large ICH, the majority of rats in both groups were above the analgesic

Fig 3. Experiment 1. Isoflurane rats had (a) significantly lower core temperature (°C) for 2 h ($P < 0.001$ at 1 h, $p = 0.0373$ at 2 h) and (b) lower activity at 11 h (AU = arbitrary units of animal movement detected and recorded by a telemetry receiver placed under the animal (Colbourne et al., 1998), $p = 0.0372$) post-ICH induction. There was a group by time interaction for both (a) temperature ($P < 0.01$) and (b) activity ($P = 0.020$). Sample size was 12 per group.

Fig 4. Experiments 1 and 2. Rat grimace scale (RGS) scores were not significantly different between isoflurane and awake rats after (a) a small ICH ($P = 0.369$) or (b) a moderate to large ICH ($P = 0.402$). Pain was also not significantly different between 6 and 23 h post-ICH induction for either experiment 1 ($P = 0.421$) or experiment 2 ($P = 0.435$). Sample size was 24 per group for experiment 1 and 18 per group for experiment 2.

ISO = 12.61 ± 11.7, NO-ISO = 13.05 ± 15.69, data not shown). The size of the hematoma was not predicted by peak BP ($R^2 = 0.097$, $P = 0.382$), average BP ($R^2 = 0.244$, $P = 0.147$), average BP during the ICH procedure ($R^2 = 0.103$, $P = 0.366$), or average BP during the first 6 h post-ICH ($R^2 = 0.252$, $P = 0.139$).
intervention threshold (Oliver et al., 2014). In this case, we did not treat the pain in these rats, as selectively treating animals with analgesics that influence injury can confound studies (Ferland et al., 2007; Saine et al., 2016). Others may choose to treat post-stroke pain, and further research is needed to determine the impact of those analgesics on bleeding and brain injury.

As hypothesized, isoflurane affected all of the physiological variables that were tested, and would have affected many others that were not tested. Despite that, we found only little differences between models, likely because these effects were short lasting or minor in nature. Temperature was decreased for the first 2 h post-surgery in the isoflurane group, and was increased above baseline in the conscious group. However, in this case, such a transient and modest temperature difference only led to a significant difference in hematoma volume after a small ICH. In the NO-ISO group, BP increased for the duration of the ICH procedure, and quickly returned to baseline once the dummy cannula was replaced. Anesthetized animals had their BP gradually drop throughout the duration of the surgical procedure, which returned to baseline within the hour. We expected that fluctuations in BP would result in large differences in hematoma volume. The lack of effect may be due to the collagenase enzyme causing prolonged bleeding over several hours (MacLellan et al., 2008), whereas the observed BP changes only lasted for up to 25 m. Additionally, BP increases may not have equated to increases in cerebral perfusion pressure. Isoflurane increases intracranial pressure (Scheller et al., 1988), and after large ICH, intracranial pressure increases even further (Hiploylee and Colbourne, 2014; Silasi et al., 2009). When intracranial pressure increases and arterial BP decreases by a similar amount, cerebral perfusion pressure stays the same (Su et al., 2008), which may be one possible explanation for why we only see an increase in hematoma volume after a small ICH. The observed BP changes may have effects that we did not measure. For example, stress responses, such as increased corticosterone, may also affect the course of injury and recovery (DeVries et al., 2001). Clinically, ICH patients are commonly hypertensive, and BP is often elevated during the course of injury (Shah et al., 2007), although this is typically for longer and to a much greater extent than what we see in rats. Therefore, the more accurate model of BP during an ICH would likely be increased BP.

Isoflurane increased blood glucose by nearly twofold by the end of

Fig 5. Experiment 3. (a) RMS in the rats that had seizures was higher for day 1 compared to day 2 in SWS EEG after collagenase-induced ICH. This indicates that there were more fluctuations in the EEG traces during the first day after the stroke. There were no statistical differences between sides. Sample size was n = 5. There was no relationship between the number of seizures and the lesion volume in all rats (b, N = 8, P = 0.310). Representative section from the middle of the lesion is embedded into graph b (lesion volume = 40.40 mm³ as indicated by arrow).

Fig 6. Experiment 3. Seizures occurring after NO-ISO collagenase-induced ICH. (a) Seizure of rat 1, which had a decreased RMS and power compared to baseline SWS (see Table). (b) Seizure of rat 3 with high power and high RMS. (c) Example of contralateral seizure occurring in rat 1.
the surgical procedure. We could not determine how long blood glucose was elevated for, as blood sampling required anesthetizing the animals or additional handling stress. However, previous research shows that in mice, isoflurane-induced glucose elevations resolve within an hour (Durand et al., 2009). In patients, higher blood glucose at admission was associated with mortality (Fogelholm et al., 2005). In a rat model of focal ischemia, the harmful effects of hyperglycemia are well known (Kagansky et al., 2001). It is possible that the non-anesthetized rats experience a stress induced hyperglycemic response during the ICH procedure, as indicated by increased blood-pressure (Márquez et al., 2004). As many preclinical ICH studies use a similar anesthetic protocol as we use here, we believe that a substantial portion of ICH research would be technically difficult to do. Although the physiological impacts of isoflurane after ICH are likely similar, our conclusions are limited to the collagenase model of ICH. Here, we did not visually assess seizure activity, as we did not video record any of the seizure events. Therefore, we cannot provide any conclusive information regarding the behavioural manifestations of this epileptiform activity.

Our findings suggest brief use of isoflurane anesthesia is an appropriate model. However, we recommend the use of non-anesthetized ICH in specific cases. For example, isoflurane may interact with many drugs (Glass et al., 1997; Wood, 1991), including analgesics (e.g., opiates) and potential neuroprotectants, especially those given before or at the time of surgery. Validating findings in a model without anesthesia would be beneficial to ensure effects are not dependent on or interacting with isoflurane. Further, avoiding anesthetic would be ideal in situations where researchers are specifically examining the effects of

**Fig 7.** Experiment 3. Left- Example of seizure in rat 1 displayed lower power (top) but increased coherence (bottom) than baseline SWS EEG, meaning the magnitude of brain activity was decreased while coordination between hemispheres was increased. Right- Example of seizure in rat 3 displayed higher power and lower coherence, signifying that the magnitude of epileptiform activity was increased, and activity in each hemisphere was less coordinated than baseline levels. The dashed lines in the power spectrum represent the 95% CI and the solid lines represent the mean values for the spectrum. For the coherence, any increase in coherence above the CI limit of the dashed lines is associated with mortality (Fogelholm et al., 2005). In a rat model of focal ischemia, the harmful effects of hyperglycemia are well known (Kagansky et al., 2001). It is possible that the non-anesthetized rats experience a stress induced hyperglycemic response during the ICH procedure, as indicated by increased blood-pressure (Márquez et al., 2004). As many preclinical ICH studies use a similar anesthetic protocol as we use here, we believe that a substantial portion of ICH research would be technically difficult to do. Although the physiological impacts of isoflurane after ICH are likely similar, our conclusions are limited to the collagenase model of ICH. Here, we did not visually assess seizure activity, as we did not video record any of the seizure events. Therefore, we cannot provide any conclusive information regarding the behavioural manifestations of this epileptiform activity.

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Table 1

| Rat Incidence | Laterality | Total duration (Mean ± CI, Seconds) | Time | RMS Ratio (Mean ± CI) | Power Change (mV^2, Mean ± CI) | Coherence change (Mean ± CI) |
|---------------|------------|-----------------------------------|------|-----------------------|-------------------------------|-----------------------------|
| 60.23 (20.08 ± 4.89) | First: 9 h 12 m | 0.73 ± 0.22 | N/A | 1.10-48.00 | 0.00030 | N/A |
| 136.20 (9.73 ± 1.03) | Last: 34 h 6 m | 1.61 ± 0.27 | N/A | 1.10-50.05 | 0.056 | N/A |
| 92.35 (30.78 ± 17.63) | First: 12 h 17 m | 4.31 ± 0.78 | N/A | 0.12-0.98 | 0.52 ± 0.019 | N/A |
| 45.63 (15.21 ± 7.56) | Last: 31 h 36 m | 0.76 ± 0.16 | N/A | N/A | N/A | N/A |
| 19.64 | Last: 37 h 37 m | 0.48 | N/A | N/A | N/A | N/A |

Rats were randomized to receive an ICH under ISO or NO-ISO to examine the effect of isoflurane on collagenase-induced ICH (Fig. 1). Animals were either randomized to receive an ICH under ISO or NO-ISO to examine the effect of isoflurane on ICH. In experiment 1, we assessed whether isoflurane affected temperature and activity for the first 24 h after a small collagenase-induced ICH. Blood glucose was measured at the beginning and end of anesthetic procedures, and pain was measured at 6 and 23 h post-ICH. Hematoma volume was assessed at 24 h post-ICH. Rats were randomized to ISO (n = 12) or NO-ISO (n = 12) conditions. As the variability was greater than expected, this experiment was repeated with an additional 12 animals per group to increase statistical power and the results were pooled for a total sample of n = 24 per group.

Temperature and activity were only assessed in the first 24 animals (n = 12 per group). In experiment 2, we assessed the impact of isoflurane on blood glucose before and after surgical procedures, pain at 6 and 23 h post-ICH, and hematoma volume at 24 h after a large collagenase ICH. We used a larger insult in this experiment to test whether the effects of isoflurane were dependent on bleed size. Here, we had 18 animals per group. In experiment 3, we determined whether isoflurane influenced seizure activity after stroke. Here, we assigned 8 animals to the NO-ISO group. After 48 h of EEG activity measurements post-ICH, anesthetics. Previously, most studies looking at the effects of anesthetics on stroke simply compared anesthetics to each other, or compared different doses of the same anesthetic, without having a no anesthetic control group (Bhardwaj et al., 2001; Zausinger et al., 2002).

By using a non-anesthetized ICH, researchers can compare findings to a no anesthetic control group, increasing the validity of their findings. Although we only assessed isoflurane in this study, a variety of other anesthetics are commonly used in ICH research (e.g., chloral hydrate and sodium pentobarbital, MacLellan et al., 2012) and should be similarly assessed for their effects on ICH outcome.

In conclusion, we demonstrate that isoflurane can have small hematoma-size-dependent impacts on the ICH model. The physiological impacts of isoflurane are often transient, but should still be considered a potential confound. The non-anesthetized ICH is an important tool for researchers when anesthetic effects are a concern, especially in neuroprotectant studies and those with a short survival time.

4. Experimental procedure

4.1. Subjects and exclusion criteria

All procedures were done according to the Canadian Council on Animal Care Guidelines and were approved (protocol AUP960) by the Biosciences Animal Care and Use Committee at the University of Alberta. We obtained 104 male Sprague Dawley rats (275–600 g, approximately 2–4 months old) from Charles River (Saint Constant, Quebec). Animals were kept in a temperature and humidity-controlled room with lights on from 7:00 am to 7:00 pm, and all procedures were done during the light phase. Animals were single-housed during experiments with food and water provided ad libitum. All animals were handled for a total of 30 m over days prior to the collagenase infusion to decrease stress during handling. Rats were handled gently including repeatedly touching the dummy cannula to give rats exposure to having the device manipulated and to increase comfort level with experimenter handling.

We established a priori exclusion criteria of a hematoma volume < 5 μL for experiment 2, thus excluding any animals that did not receive a moderate-sized ICH.

4.2. Experimental design

Animals were randomly assigned to groups using random.org and data was analyzed in a blinded manner for all experiments. Group sizes were calculated a priori using a power analysis for a desired 80% power to detect a 33% difference in the primary endpoint, hematoma volume. In this study, we conducted 4 experiments to examine the effect of isoflurane on collagenase-induced ICH (Fig. 1). Animals were either randomized to receive an ICH under ISO or NO-ISO to examine the effect of isoflurane on ICH. In experiment 1, we assessed whether isoflurane affected temperature and activity for the first 24 h after a small collagenase-induced ICH. Blood glucose was measured at the beginning and end of anesthetic procedures, and pain was measured at 6 and 23 h post-ICH. Hematoma volume was assessed at 24 h post-ICH. Rats were randomized to ISO (n = 12) or NO-ISO (n = 12) conditions. As the variability was greater than expected, this experiment was repeated with an additional 12 animals per group to increase statistical power and the results were pooled for a total sample of n = 24 per group.

Temperature and activity were only assessed in the first 24 animals (n = 12 per group). In experiment 2, we assessed the impact of isoflurane on blood glucose before and after surgical procedures, pain at 6 and 23 h post-ICH, and hematoma volume at 24 h after a large collagenase ICH. We used a larger insult in this experiment to test whether the effects of isoflurane were dependent on bleed size. Here, we had 18 animals per group. In experiment 3, we determined whether isoflurane influenced seizure activity after stroke. Here, we assigned 8 animals to the NO-ISO group. After 48 h of EEG activity measurements post-ICH,
we collected brain tissue for lesion volume assessment. We determined post-ICH seizure incidence rate, which is ~61.3% after anesthetized collagenase-induced ICH (Klahr et al., 2016, 2014). In experiment 4, we determined the effects of isoflurane and the no-anesthetic procedure on BP and hematoma volume after ICH. Animals were randomly assigned to ISO (n = 6) or NO-ISO (n = 6).

4.3. Telemetry probe implantation

Rats were anesthetized with isoflurane (4% induction, 2–2.25% maintenance, 60% N2O, and remainder O2). In animals used for core temperature measurements, a sterile calibrated probe was inserted into the peritoneal cavity (Model TA10TA-F40, Data Sciences International, St. Paul, MN, accurate to ± 0.2 °C). In animals used for EEG analysis, an EEG telemetry probe (F40EET, Data Sciences International, sampled at 500 Hz and low-pass filtered at 100 Hz) was inserted into the peritoneal cavity as previously described (Klahr et al., 2014). Leads were channeled under the skin and attached to screws (B000FMWBA0, Small Parts) and secured with dental cement. The screws were placed ipsilateral (AP−4, ML −4) and contralateral (AP-4, ML 4) to Bregma to avoid interfering with the cannula. In animals used for BP measurements, a calibrated PA-C10 probe’s catheter was inserted into the left femoral artery, and the probe (Data Sciences International, accurate to ± 3 mmHg) was implanted subcutaneously, as previously described (Hiploylee and Colbourne, 2014).

A guide cannula was implanted (see Cannula Implantation and St. Paul, MN, accurate to ± 0.2 °C). In animals used for EEG analysis, an EEG telemetry probe (F40EET, Data Sciences International, sampled at 500 Hz and low-pass filtered at 100 Hz) was inserted into the peritoneal cavity as previously described (Klahr et al., 2014). Leads were channeled under the skin and attached to screws (B000FMWBA0, Small Parts) and secured with dental cement. The screws were placed ipsilateral (AP−4, ML −4) and contralateral (AP-4, ML 4) to Bregma to avoid interfering with the cannula. In animals used for BP measurements, a calibrated PA-C10 probe’s catheter was inserted into the left femoral artery, and the probe (Data Sciences International, accurate to ± 3 mmHg) was implanted subcutaneously, as previously described (Hiploylee and Colbourne, 2014).

A guide cannula was implanted (see Cannula Implantation and

Table 2

| Study          | Seizure Incidence Rate | ID | Number of Seizures (Ipsilateral or Bilateral) | Total Duration (s) | Time of onset after ICH |
|----------------|------------------------|----|-----------------------------------------------|--------------------|-------------------------|
| Klahr et al. 2014 | 6/9 (Collagenase Group Only) | 1  | 1                                             | 14                 | 11 h 52 m               |
|                |                        | 2  | 102                                           | 18 h 50 m          |                         |
|                |                        | 3  | 163                                           | 22 h 7 m           |                         |
|                |                        | 4  | 108                                           | 16 h 25 m          |                         |
|                |                        | 5  | 173                                           | 11 h 37 m          |                         |
|                |                        | 6  | 616                                           | 9 h 57 m           |                         |
| Klahr et al. 2016 | 13/22 (Control Group Only) | 1  | 34                                            | 481                | 6 h 27 m                |
|                |                        | 2  | 375                                           | 9 h 3 m            |                         |
|                |                        | 3  | 1455                                          | 8 h 12 m           |                         |
|                |                        | 4  | 607                                           | 7 h                |                         |
|                |                        | 5  | 718                                           | 4 h 48 m           |                         |
|                |                        | 6  | 495                                           | 6 h 36 m           |                         |
|                |                        | 7  | 18                                            | 9 h 44 m           |                         |
|                |                        | 8  | 31                                            | 19 h 32 m          |                         |
|                |                        | 9  | 4138                                          | 7 h 23 m           |                         |
|                |                        | 10 | 21                                            | 17 h 18 m          |                         |
|                |                        | 11 | 537                                           | 3 h 53 m           |                         |
|                |                        | 12 | 1831                                          | 11 h 32 m          |                         |
|                |                        | 13 | 45                                            | 8 h 58 m           |                         |

Fig 8. Experiment 4. There was a significant group by time interaction on (a) BP during the ICH procedure (mmHg; averaged per minute, P = 0.003). Awake animals had significantly higher BP from 2 to 12 m of the ICH surgery when compared to the isoflurane group (P < 0.007). Isoflurane animals were kept under anesthesia for 25 m, while the infusion procedure was typically completed in awake animals within 10 m. No group effect was found on (b) BP 24 h after ICH induction (mmHg; averaged hourly, P = 0.4924, interaction effect P = 0.1524). There was a significant effect of time on BP (P < 0.001). Sample size was 4 in the isoflurane group and 6 in the awake group.
Collagenase Infusion) following implantation procedures. Meloxicam (0.2 mg/kg SC) and bupivacaine hydrochloride (0.5 mg/kg SC) were administered for analgesia, with the exception of EEG implantation where only bupivacaine hydrochloride was administered. This was to avoid excessive analgesia and suture removal by animals, which otherwise would be more problematic with this procedure.

Baseline measurements were taken for 24 h prior to ICH induction. Data measurements were taken every 30 s prior to and after the ICH. Post-ICH core temperature and activity readings were corrected hourly to baseline values in order to account for temperature changes due to circadian rhythm. In our past experience, we do not see spontaneous seizure activity in this rat strain (Klahr et al., 2016, 2014). Nonetheless, baseline EEG data was assessed to ensure there was no pre-ICH seizure activity. For BP measurements, baseline data was taken for 3 h on the day prior to ICH and averaged. All data were corrected for probe offset readings taken prior to implantation.

4.4. Cannula implantation and collagenase infusion

Animals were anesthetized with isoflurane and temperature was maintained at 37 °C using a rectal temperature probe and heating pad placed under the animal. Meloxicam and bupivacaine hydrochloride were administered at the start of surgical procedures as an analgesic. A hole was drilled into the skull at 0.5 mm anterior, 3.5 mm lateral (left side) to Bregma (Paxinos and Watson, 2014). The dura mater was punctured to minimize possible pain during the non-anesthetized procedure. A guide cannula (C316G/SPC guide with 1 mm below pedestal, Inviv01, Roanoke, VA) was placed onto the hole and secured in place using 3 anchoring screws and dental cement. Dummy cannula was placed on the guide cannula to prevent pathogen entry and to maintain patency during recovery.

Rats recovered for 3 days following the cannula implant procedure. Then, an internal cannula (C3161/SPC, 5.5 mm extension from guide, inviv01, Roanoke, VA) was inserted 6.5 mm into the striatum (the depth used in previous research). Bacterial collagenase (Type IV-S, Sigma, 0.6 U/μL in sterile saline) was infused through PE tubing into the internal cannula and striatum. Either 1.0 μL (experiment 1) or 3.0 μL (experiments 2–4) of collagenase solution was infused over 2.5 m. The internal cannula was kept in place for 5 m to prevent backflow before being slowly removed, and the dummy cannula reinserted. Animals in the NO-ISO group were awake and lightly restrained by a scorer blinded to group identity. Each subscale was scored from 0 to 2. Animal’s scores were the average of the scores on the 10 images. A second rater scored a subset of animals to check for inter-rater reliability, which was high (Spearman’s rho of 0.839, P = 0.004). Four naïve animals served as a negative control, and these animals received an average score of 0.162.

4.6. Grimace scale

Pain was assessed using the rat grimace scale (RGS) to test whether the conscious ICH procedure caused additional pain post-ICH (Sotocinal et al., 2011). Animals were video recorded for 10 m at both 6 h and 23 h post-ICH. We did not assess pain during the procedure, as collagenase-induced bleeding occurs over hours (MacLellan et al., 2008). Ten images were selected from each video at approximately 1 m intervals (to allow full view of face at each time). Each image was scored on orbital tightening, nose/cheek flattening, ear changes, and whisker changes by a scorer blinded to group identity. Each subscale was scored from 0 to 2, and the combined score was added for a total score ranging from 0 to 8, with zero meaning no pain. Group differences between both subscales and total scores were assessed. For ease of comparison with previous research, total scores were averaged by subscale for a score ranging from 0 to 2. Animal’s scores were the average of the scores on the 10 images. A second rater scored a subset of animals to check for inter-rater reliability, which was high (Spearman’s rho of 0.839, P = 0.004). Four naïve animals served as a negative control, and these animals received an average score of 0.162.

4.7. Hemoglobin assay

The amount of hemoglobin in each hemisphere was determined using a spectroscopic assay based upon a standard curve (MacLellan et al., 2008). Hematoma volume was calculated as ipsilateral blood volume minus contralateral blood volume. This accounts for the blood in the hemisphere that is not attributed to the hematoma (blood in the vasculature).

4.8. Electroencephalogram analysis

Baseline and post-infusion EEG traces were visualized with Dataquest A.R.T software (v. 2.3, Data Sciences International). Two to five-minute long epochs of slow wave sleep (SWS) activity prior, and day 1 and 2 after collagenase injection as well as putative epileptiform traces were exported and analyzed using custom code written in MATLAB (R2012a, Mathworks, Natick, MA). We computed the root mean square (RMS), a measure of the fluctuation in the EEG signal, for all traces of interest. Baseline SWS data was the average of a 6 min epoch of slow wave sleep prior to collagenase injection (control) and putative epileptiform traces were exported and analyzed using custom code written in MATLAB for detection of seizures. The code identified epileptiform peaks that were above 4 standard deviations from the mean of the control traces, and considered 10 peak clusters occurring within 1 s apart as seizures (Klahr et al., 2016, 2014). Seizures detected by the MATLAB code were also visually verified for further accuracy, and any artefacts were excluded. Power spectral density using Welch’s averaged modified periodogram method (6 s window, 2 s overlap) as well as dual channel coherence for ipsilateral and contralateral recordings (3 s window; 1 s overlap) were computed for those epileptiform traces longer than 25 s. Power spectra and coherence were compared to non-epileptic control traces similar in duration to determine which frequencies had significant changes in power during seizure activity (i.e., outside 95% confidence interval, CI). A randomized coherence distribution based on a series of sequential time-shifted (and also time-reversed) coherence computations from these actual traces was computed to calculate the coherence significance level. During epileptiform activity, we determine cross-hemispheric coupling changes by subtracting the coherence values of normal activity from those of epileptic traces and considered that any increases equal to or larger than the confidence limit for that trace to be significant.

4.9. Histological analysis

At 48 h post-ICH, animals from the seizure experiment were injected with 100 mg/kg IP of sodium pentobarbital and perfused with 0.9% saline followed by 10% neutral-buffered formalin. Prior to cryostat sectioning, brains were placed in a 20% sucrose solution for cryoprotection. Coronal sections (20-μm thick) were taken every 200 μm and
analyzed every 400 μm. Sections were stained with cresyl-violet as done previously (MacLellan et al., 2006). Due to edema confounds and distortion caused by the hematoma itself at 48 h post-ICH, we were not able to calculate lesion volume by comparing hemisphere volumes (Williamson and Colbourne, 2017). Instead, the lesion was calculated as: (average area of damage × interval between sections × number of sections).

4.10. Statistical analysis

Data were analyzed using GraphPad Prism (v 6.0, GraphPad Software Inc., La Jolla, CA). Data are presented as mean ± 95% CI. T-tests were used for 2 group comparisons. Welch’s correction was applied when group variances were not equal, as determined with an F test to compare variances. Blood glucose levels and RMS were analyzed using a 2-way repeated measures ANOVA with Sidak’s multiple comparisons test. Rat grimace scale scores were assessed using a Mann-Whitney U test, and group contingencies were assessed using a Fisher’s exact test. Relationship between variables was assessed with Pearson’s correlation coefficient. All P values below 0.05 were considered statistically significant.

Author’s contributions

CMW, ACJK, and FC designed the experiment. CMW, ACJK, TK, DRM, and ACK collected the data. CMW, DRM, ACK, and CTD analyzed the data. CMW, ACJK, TK, and ACK wrote the manuscript and all authors edited the manuscript.

Availability of data

All electronic data from this study are available on Mendeley data.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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