Research Report

Forced exercise does not improve recovery after hemorrhagic stroke in rats

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ABSTRACT

Exercise can improve recovery following ischemia and intracerebral hemorrhage (ICH) in rodents. We tested whether forced exercise (EX; running wheel) prior to and/or following ICH in rats would reduce lesion volume and improve functional outcome (walking, skilled reaching, spontaneous paw usage) at 7 weeks post-ICH. A striatal hemorrhage was produced by infusing collagenase. First, we compared animals that received EX (2 weeks; 1 h/day) ending two days prior to ICH and/or starting two weeks following ICH. EX did not improve functional recovery or affect lesion size. Doubling the amount of EX given per day (two 1-h sessions) both prior to and following ICH did not alter lesion volume, but worsened recovery. We then determined if EX (1 h/day) prior to and following ICH would affect outcome after a somewhat milder insult. There were no differences between the groups in lesion volume or recovery. Finally, we used a hemoglobin assay at 12 h following ICH to determine if pre-stroke EX (2 weeks; 1 h/day) aggravated bleeding. It did not. These observations suggest that EX does not improve outcome when given prior to and/or when delayed following ICH. Effective rehabilitation for ICH will likely require more complex interventions than forced running.

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1. Introduction

Stroke is one of the leading causes of death and disability in North America. Cerebral bleeding, including intracerebral hemorrhage (ICH), accounts for approximately 15% of all strokes and frequently causes severe disability or death (Mayo et al., 1982). Most experimental and clinical stroke studies focus on finding effective hemostatic or cytoprotective (neuroprotective) therapies. Furthermore, most studies target cerebral ischemia and not hemorrhagic stroke. Given the differences in pathophysiology (Lipton et al., 1999; Xi et al., 2006), such as the nature of cell death and the extent and location of injury, it is important to test therapies in ICH models as it is possible that treatments that work in ischemia may fail in ICH. This is not only an issue with cytoprotectants, but rehabilitation therapies may also differ in efficacy between ischemic and hemorrhagic events.

Various rehabilitation interventions promote recovery after ischemic stroke in rats (e.g., environmental enrichment). Interestingly, even simple exercise (EX) treatments such as forced running promote recovery after ischemic stroke, and they also reduce cell death in some situations. For example, Ding and colleagues (2004) found that forced running...
EX prior to temporary middle cerebral artery occlusion (MCAO) reduced infarct volume and promoted neurological recovery, which was associated with increased expression of brain derived neurotrophic factor (BDNF) and nerve growth factor. In another study, pre-stroke forced EX reduced edema and lesion volume measured at 24 h after temporary MCAO (Wang et al., 2001). Reductions in lesion volume and improved behavioral recovery also occur when EX is started following ischemia. For instance, forced EX initiated within 24 h of temporary MCAO reduced functional deficits and lesion volume (Yang et al., 2003).

Rehabilitation also benefits rats suffering an ICH. DeBow and colleagues (2003) showed that constraint-induced movement therapy (CIMT) initiated one week after ICH improved behavioral recovery. A reduction in the total volume of tissue lost also occurred. The CIMT therapy, which lasted 7 days, included a combination of ipsilateral limb restraint (8 h/day) with 1 h/day of EX (e.g., skilled reaching training, walking). The modest EX treatment alone did not improve outcome. However, others have shown that greater amounts of forced EX (running) starting 1 day following ICH reduces lesion volume, caspase-3 expression, and the number of degenerating cells (Lee et al., 2003; Lee et al., 2005). This raises the possibility that greater amounts of EX may improve recovery and reduce injury when given after a more clinically realistic treatment delay (e.g., 2 weeks).

In this study, we examined the effects of forced EX on outcome following ICH in rats. The collagenase model of ICH, developed by Rosenberg and colleagues (1993), was used because it results in consistent hemorrhaging within the striatum and well-characterized behavioral deficits (Maclellan et al., 2005a). Forced EX, via motorized running wheels, was used because of its demonstrated efficacy in ischemia models and because an exact amount of EX can be easily administered. Effective pre-clinical testing requires a comprehensive assessment of long-term recovery and not just use of short-term endpoints (Corbett and Nurse, 1998). Thus, we used a 7-week survival time and gauged skilled reaching, spontaneous paw usage, and walking ability by the staircase, forelimb asymmetry, and horizontal ladder tests, respectively, which are all sensitive to striatal ICH (Hua et al., 2002; Maclellan et al., 2005a). In our first experiment, we examined the effects of EX given before and/or after ICH. We hypothesized that EX either before or following ICH would be beneficial and expected the combination to be superior. The second experiment doubled the amount of daily EX given both before and following ICH. We hypothesized that this EX regimen would lead to greater benefit. In Experiment 3, we reduced the lesion size to determine the effects of EX given both before and after ICH as in Experiment 1. We anticipated somewhat greater effects in treating this smaller lesion than that found in Experiment 1. Lastly, in Experiment 4 we quantified hemorrhage volume at 12 h after ICH to determine if forced EX prior to ICH aggravated bleeding. Given the known angiogenic effects of EX (Black et al., 1990; Kleim et al., 2002; Swain et al., 2003), we predicted that pre-ICH EX treatment might aggravate bleeding and thereby counteract beneficial effects of EX therapy.

2. Results

2.1. Weight data

In all experiments, body weight was similar among all groups at the beginning of the experiment, on the first and last day of PRE and POST EX treatments, surgery, post-surgery, and euthanasia (p ≥ 0.066).

2.2. Experiment 1

No animals in the POST-1 group died, whereas 1 in the CONT-1 group, 4 animals in the PRE&POST-1 group, and 1 animal from the PRE-1 group died following surgery (p = 0.095). The cause of death was presumed to be due to insult severity; however, this was not verified.

The lesion volumes at 7 weeks after ICH are given in Fig. 1A. Injection of collagenase caused significant tissue loss in the striatum as well as damage to thalamus, globus pallidus, and the corpus callosum (Fig. 1D). The main effects (PRE: p = 0.412; POST: p = 0.212) and interaction (p = 0.419) were not significant.

All groups performed similarly during staircase training (data not shown). There was a significant Day effect (p = 0.001; Fig. 2A) because all groups improved over time (contralateral limb reaching success). However, the PRE (p = 0.820) and POST (p = 0.440) main effects and the interaction (p = 0.095) were not significant. Likewise, the main effects (PRE: p = 0.586; POST: p = 0.391) and interaction (p = 0.136) were not significant for the contralateral forelimb slip rate in the ladder test (Fig. 3A). Analysis of contralateral limb use in the asymmetry test revealed a significant POST main effect (p = 0.039; Fig. 4A) and a significant PRE×POST interaction (p = 0.033), but the PRE factor was not significant (p = 0.075). However, Scheffé post hoc tests revealed no significant differences between any of the groups (p ≥ 0.065) in a one-factor ANOVA. The composite score revealed that neither the main effects (PRE: p = 0.811; POST: p = 0.178) nor interaction (p = 0.868) were significant. The CONT-1, PRE-1, POST-1, and PRE&POST-1 groups had mean composite scores (lower reflects better performance) of 35.1±12.7, 34.9±11.7, 31.6±12.6, and 30.5±8.6, respectively.

2.3. Experiment 2

Two animals in the PRE&POST-2 group died after ICH whereas no animal died in the CONT-2 group (p = 0.176). Mortality was assumed to be due to the ICH.

Histological data for one animal in the CONT-2 group was lost before analysis. The one-factor ANOVA revealed no significant difference in lesion size between the PRE&POST-2 and CONT-2 groups (p = 0.465; Fig. 1B).

There was no significant difference in contralateral reaching ability between the two groups (p = 0.052) and the Day effect was not significant (p = 0.090; Fig. 2B). As shown in Fig. 3B, the PRE&POST-2 group made significantly more foot slips with their contralateral forelimb than the CONT-2 group (p = 0.032). Analysis of limb use asymmetry revealed no significant difference between the groups (p = 0.395; Fig. 4B).
There was a significant difference in the composite behavioral scores between the PRE&POST-2 and CONT-2 groups ($p=0.010$), with mean rank scores of $12.6\pm1.0$ and $8.4\pm1.0$, respectively. Thus, the PRE&POST-2 group performed worse on average.

2.4. Experiment 3

One animal in the CONT-3 group did not survive ICH surgery; no other animals died ($p=0.356$). This death was probably due to anesthesia.

The lesion volumes at 7 weeks post-ICH were not different between the PRE&POST-3 and CONT-3 groups ($p=0.179$; Fig. 1C).

In an attempt to reduce the number of animals used, rats that failed to reach criterion performance were excluded from the staircase analysis and remained in the rest of the study. Ten animals were excluded from the CONT-3 and 6 were excluded from the PRE&POST-3 leaving group sizes for skilled reaching of 9 and 13 animals, respectively. There was no significant difference between the groups in contralateral forelimb reaching ability ($p=0.700$; Fig. 2C). Reaching improved over trials as revealed in the significant Day effect ($p<0.001$). The horizontal ladder test showed no difference between the two groups in contralateral forelimb slip rate ($p=0.076$; Fig. 3C). The Day effect for slip rate in the ladder was significant ($p=0.021$). In the forelimb asymmetry test, animals performed significantly better at week 7 as compared to week 2 ($p=0.008$). However, there was no difference between the PRE&POST-3 and CONT-3 groups ($p=0.722$; Fig. 4C). The composite score was based upon the mean rank from the final test point in limb use asymmetry and horizontal ladder and average staircase performance for all 5 testing days. The CONT-3 group had an overall mean rank of $18.8\pm1.6$, and the PRE&POST-3 group had a mean rank of $16.7\pm1.7$ ($p=0.363$).

2.5. Experiment 4

No premature deaths occurred in this experiment. The volumes of cerebral blood at 12 h after ICH were $40.9\pm5.5\, \mu\text{l}$ in the ICH-4 group, $46.1\, \mu\text{l}\pm4.7$ in the CONT-4 group, and $47.7\, \mu\text{l}\pm3.9$ in the PRE-4 group ($p=0.606$). Thus, giving behavioral training and/or EX prior to ICH does not apparently affect bleeding after collagenase infusion.

3. Discussion

Our primary finding is that EX (running) does not improve outcome after ICH in rats. The first experiment showed that a moderate amount of forced EX did not improve recovery when administered either before and/or after ICH. The second experiment showed that doubling the EX not only failed to improve recovery, but actually worsened it. The third experiment suggests that this failure was not simply due to insult severity because forced EX did not improve recovery after a somewhat milder insult. Finally, the last experiment showed...
that a moderate amount of pre-stroke EX did not affect bleeding volume. Accordingly, and in contrast to ischemia studies that report improvements, our forced EX regimen does not improve recovery nor affect lesion size after ICH.

The timing, duration, intensity, and type of EX (e.g., voluntary vs. forced) are all critical factors modulating the efficacy of EX rehabilitation (Kleim et al., 2003). Thus, whereas our results show that our EX regimen does not benefit ICH, alternative forced EX regimens may be of therapeutic value. For instance, an earlier intervention may have improved recovery in our model as others have shown that EX starting 24 h after ICH reduced lesion volume (Lee et al., 2003; Lee et al., 2005). We delayed treatment for two weeks because rats receiving a severe ICH are usually too impaired to run much earlier (unpublished observation), plus the delay better reflects the clinical situation. Interestingly, other therapies such as CIMT (DeBow et al., 2003; Macellani et al., 2005b) and skilled reach training (Biernaskie and Corbett, 2001) can improve recovery when delayed a week or more after a stroke in rats. Modifying the duration or intensity of EX training may also have altered the outcome. For instance, two weeks of pre-stroke EX benefits a temporary MCAO insult (Wang et al., 2003), whereas a 12-week treatment, and not shorter durations, was required prior to permanent MCAO to reduce lesion volume (Ang et al., 2003). Similarly, one may argue that extending the length of EX training following ICH may have improved outcome. However, several studies show that one week of EX is effective in reducing lesion and functional deficits following ischemia (Yang et al., 2003), and two studies show decreased lesion volume with 10 days of EX following ICH (Lee et al., 2003; Lee et al., 2005). It is difficult to compare intensities of EX treatments across studies because of differences in duration, running rate, and method of running (e.g., wheel vs. treadmill). We used one or two 60-min EX sessions per day at a maximum speed of 11 m/min. Whereas several studies have used much higher amounts (Ang et al., 2003; Wang et al., 2003; Yang et al., 2003), the rate we used is in line with other studies (Lee et al., 2003; Lee et al., 2005) and was appropriate for the ICH rats; that is, it could be maintained for 1 h sessions, and did not appear overly stressful.

Besides differences in EX regimens, it should be noted that there are key differences in pathophysiology between ischemic and hemorrhagic insults (e.g., amount of inflammation; Xi et al., 2006) that may contribute to the ineffectiveness of forced EX, or any therapy, after ICH. Location and severity of damage also confound comparisons among studies. These issues along with potential differences in EX-induced recovery mechanisms highlight the need to investigate therapies specifically in ICH models. Notably, EX increases blood vessel density in motor cortex, cerebellum, and striatum (Black et al., 1990; Ding et al., 2004; Kleim et al., 2002; Swain et al., 2003). Exercise also up-regulates endothelial nitric oxide synthase (eNOS) (Endres et al., 2003), and affects the levels of eicosanoids, reducing pro-coagulation factors (i.e., thromboxane) as well as increasing anti-coagulation factors (i.e., prostacyclin) (Chen et al., 1993). Such effects may work to lessen the impact of ischemia by improving reperfusion whereas they could conceivably aggravate hemorrhagic insults. Presently, we found that EX did not aggravate bleeding at 12 h after ICH surgery. The fact that lesion size was not significantly different among groups in each experiment supports this finding as exaggerated bleeding should aggravate lesion size. Likewise, higher mortality might occur in EX groups; however, mortality was not significantly different among groups in any of the 4 experiments. Nonetheless, greater amounts of EX prior to collagenase-induced ICH may worsen outcome through such a mechanism. Experiment 2 shows that greater amounts of forced EX can worsen functional

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**Fig. 2** – Successful skilled reaching on days 42–46 after ICH as a % baseline for the contralateral to stroke limb. Rats in Experiments 1 and 3 showed an improvement in contralateral reaching ability over days. There were no significant group differences in any experiment.
outcome. In this case, the volume of tissue lost was not affected; however, this does not exclude the possibility that some cell death or the rate of cell death was affected.

Undoubtedly, several factors contribute to the beneficial effects of EX on recovery after ischemia. One leading candidate is BDNF, which promotes synaptic plasticity and neuronal survival (Cotman and Berchtold, 2002), and is elevated following EX (Ding et al., 2004; Neeper et al., 1995; Ploughman et al., 2005). Elevations in BDNF after ICH and EX have not been studied, so it is possible that our treatment did not sufficiently affect BDNF levels. However, EX regimens less strenuous than ours have been shown to elevate BDNF levels (Ploughman et al., 2005). Another possibility is that the beneficial effects of forced EX were counteracted by deleterious factors (e.g., corticosterone) that might have a greater influence on ICH than ischemia. Both voluntary and forced EX increases serum corticosterone levels (Ploughman et al., 2005), which on its own reduces the amount of BDNF mRNA in the hippocampus and dentate gyrus (Smith et al., 1995) and aggravates ischemic stroke (Feibel et al., 1977; Olsson et al., 1990). More extensive forced EX, as used in our second experiment, may thus worsen outcome through this mechanism. Clearly, further study is needed to understand the relationship, if any, between ICH, forced EX, corticosterone, and recovery.

Fig. 3 – Contralateral forelimb slip rate (% slips through bars; mean±SEM) on the horizontal ladder. There were no significant group differences in Experiment 1 (A) at 46 days after ICH. In Experiment 2 (day 46 after ICH), EX significantly increased slip rate. At 2 and 7 weeks, there were no group differences in Experiment 3 (C).

Fig. 4 – Forelimb asymmetry expressed as a % contralateral limb use (mean±SEM). There were no group differences at 46 days after ICH in Experiment 1 (A) or 2 (B). There was also no difference in Experiment 3 (C) at 2 and 7 weeks after ICH.
Our study cannot exclude the possibility that cell death was affected by the EX treatments. A reduction in cell death, whereas possible, was either insufficient to influence the total volume of tissue lost at 7 weeks post-ICH or occurred transiently and went undetected. We did not assess cell death or lesion size at earlier times as long-term outcome is the more important clinical endpoint. Neuronal death around the 7-week survival time was not assessed because injury is expected to mature well in advance of this euthanasia time (Gong et al., 2001). Likewise, one could argue that behavioral improvements (treatment effects) occurred and went undetected in these experiments. We used three tests previously shown to be sensitive to ICH-induced striatal injury (Maclellan et al., 2001). Likewise, one could argue that behavioral improvements (treatment effects) occurred and went undetected. We did not assess cell death or lesion size at earlier times as long-term outcome is the more important clinical endpoint. Neuronal death around the 7-week survival time was not assessed because injury is expected to mature well in advance of this euthanasia time (Gong et al., 2001).

In summary, we assessed the effects of forced EX on long-term outcome after ICH. Exercise before and/or starting two weeks following ICH did not improve outcome or reduce lesion size. Furthermore, 2 h/day of EX before and after ICH worsened functional outcome, indicating that one should not assume more is better. The present findings, and that of our previous studies with CIMT (DeBow et al., 2003; MacLellan et al., 2004), clearly indicate that rehabilitation efforts for ICH are not only far from perfect, but they are inferior to those for cerebral ischemia. Further efficacy and mechanistic studies targeting rehabilitation for ICH are needed.

4. Experimental procedures

4.1. Animals

One hundred and eighty-five male, young adult Long–Evans rats (Charles River, Montreal, Quebec, Canada) weighing between 250 and 350 g at the start of the experiment were entered into the experiments. Ten of these animals were excluded due to technical errors (e.g., spectrophotometric assay processing mistake). The animals were individually housed with food and water available ad libitum. All procedures were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Biomedical Sciences Animal Policy and Welfare Committee at the University of Alberta.

In the first experiment (N=79; Fig. 5), animals successfully meeting the criterion for staircase training (see below) were randomly placed into one of four conditions: EX prior to and following ICH (PRE&POST-1; n=20), EX only prior to ICH (PRE-1; n=16), EX only following ICH (POST-1; n=17), and a group receiving no EX (CONT-1; n=18). Eight additional rats were excluded based on poor staircase training performance. The second experiment (N=36) used a greater amount of EX each day and compared rats receiving EX prior to and following ICH (PRE&POST-2; n=12) with those not receiving EX (CONT-2; n=10). Fourteen additional rats failed to meet criterion for staircase training and did not continue in the study. In the third experiment (N=39), rats received a milder ICH insult. We compared rats given EX, which was identical to Experiment 1 (PRE&POST-3; n=19), to those that received no EX (CONT-3; n=20). In an attempt to reduce the number of animals used, rats failing to meet exclusion criterion for staircase continued on in the study and had their staircase data excluded from analysis. In the fourth experiment (N=21), we assessed hemoglobin content 12 h after ICH in three groups of rats. One group received an ICH without any behavioral training or EX therapy (ICH-4, n=7). The second group were trained behaviorally (e.g., staircase test) prior to ICH but did not get EX therapy (CONT-4, n=8). The last group were trained behaviorally and subjected to EX (PRE-4, n=6) identical to Experiment 1.

4.2. Behavior training

4.2.1. Montoya staircase training

Three days prior to the beginning of staircase training, all animals were food deprived to 90% of their free feeding weight adjusted for natural increases in weight with age. This task measures the skilled reaching ability of each forelimb (Montoya et al., 1991). The rats were trained to reach for food reward pellets (45 mg each; Bio-Serv, Frenchtown, NJ, USA). Each rat received two 15-min trials a day separated by 3–5 h, 5 days a week, for 3 weeks. To successfully complete training, an average of 9 of the 21 pellets for each limb must be obtained for 6 consecutive trials.

4.2.2. Horizontal ladder walking test

On the last 2 days of staircase training (i.e., days 3 and 4 prior to EX), each rat was given 3 consecutive trials on a 1-m long horizontal ladder with randomly spaced bars (1–3 cm apart). The trials occurring on the second day of training were...

Fig. 5 – Timeline of procedures for Experiment 1 (days are given relative to ICH surgery). All animals were euthanized on day 49 following ICH. Similar training and testing procedures were used in Experiments 2 and 3 (see Experimental procedures for details).
videotaped and the number of errors (limb slips through the ladder) made with each limb while traversing the middle 0.5 m section was determined (MacLellan et al., 2005a; Metz and Whishaw, 2002).

4.2.3. **Limb-use asymmetry (cylinder) test**

On the last 2 days of staircase training, each rat was placed in a transparent cylinder (height: 45 cm, diameter: 33 cm) and allowed to explore spontaneously for 5 min on each training day. The second cylinder session was video recorded and analyzed for the number of forelimb placements on the wall made with each paw. The percent contralateral paw use was calculated as (contralateral forelimb touches/ipsilateral forelimb touches) × 100, according to established methods (MacLellan et al., 2005a; Tillerson et al., 2001).

4.3. **Pre-ICH EX**

On days 3 and 4 before EX training, all animals were habituated to the motorized wheel (35 cm in diameter; 10 cm wide, bars 1 cm apart) for 15 min at a speed of 2.2 m/min. Training consisted of a 1-h daily session conducted 5 days a week for 2 weeks. The speed was 5.5 m/min for the first 5 min and then 11 m/min for the remaining 55 min. Control animals were placed in a stationary wheel in otherwise identical conditions (e.g., noise, location, etc.). In the second experiment, EX-treated rats ran at the same intensity, but for two 1-h EX sessions per day. These sessions were separated by 3–5 h. All animals were weighed daily during the EX training.

4.4. **ICH surgery**

Rats were subjected to an ICH two days after finishing the last EX session or at the equivalent time in non-EX-treated groups. The rats were anesthetized with isoflurane (4% induction; 1.5–2% maintenance in 70% N2O, 30% O2) and placed in a stereotaxic frame. A rectal temperature probe and electric heating pad were used throughout anesthesia to maintain body temperature at ∼37 °C. Using aseptic technique, a midline scalp incision was made and a small hole was drilled 3.5 mm lateral to bregma, contralateral to the preferred paw (as determined by staircase testing) and at the anteroposterior level of bregma. One microliter of sterile saline containing 0.2 U of collagenase (Type IV-S; Sigma, Oakville, ON, Canada) was injected into the striatum creating a severe ICH (Maclellan et al., 2005a; Rosenberg et al., 1993). The injection was given at the anteroposterior level of bregma, 3.5 mm lateral and to a depth of 5.5 mm.

4.5. **Post-ICH EX**

Two weeks after ICH surgery, half of the rats began post-surgery EX training. Due to the severe motor impairment caused by the ICH insult, the intensity of this EX treatment was reduced compared to pre-ICH EX treatment. The duration of training was the same as the pre-ICH EX, with animals being placed in the motorized wheel for a single 1-h training session per day 5 days a week for 2 weeks (Experiment 2 used two 1-h sessions). For the first 5 days, the intensity was maintained at a pace of 5.5 m/min for the entire EX session, for the remaining 5 days the pace was increased to 11 m/min after the initial 5-min at 5.5 m/min.

4.6. **Behavioral testing**

Rats were food deprived to 90% of their free feeding weight 4 days prior to staircase testing. The staircase test was used to assess skilled reaching on days 42–46 post-ICH (two 15-min trials per day separated by 3–5 h). The results are expressed as a percent of baseline (average of last 3 training days). Each animal was assessed on the horizontal ladder (3 crosses) and forelimb asymmetry tests (one 5-min session) at 46 days after ICH. Rats in Experiment 3 were also tested on the forelimb asymmetry and horizontal ladder tests 11 days after ICH.

4.7. **Histology**

Rats in Experiments 1–3 were euthanized 7 weeks following ICH surgery with an overdose of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, ON, Canada; 80 mg/kg i.p.). They were perfused with saline followed by 10% formalin. Forty-micrometer coronal brain sections were taken every 600 μm with a cryostat and then stained with cresyl violet. The volume of tissue lost was assessed using Scion Image J 4.0 (Scion Corporation, Frederick, MD, U.S.A.) and was calculated by subtracting the remaining volume of injured hemisphere from the remaining volume of normal hemisphere. The volume of each hemisphere was calculated as (average area of complete coronal section of the hemisphere−area of damage−area of ventricle)×interval between sections×number of sections (MacLellan et al., 2005a).

4.8. **Intracerebral blood volume analysis (Experiment 4)**

The volume of blood released into the brain 12 h after collagenase infusion was assessed using a spectrophotometric hemoglobin assay (Auriat et al., 2005; Choudhri et al., 1997). Three groups were compared: CONT-4, PRE-4, and ICH-4. Twelve hours following the induction of ICH, rats were deeply anesthetized with 4% isoflurane and decapitated. The brain was extracted and the olfactory bulbs and cerebellum were discarded. The brain was homogenized (Model 398; Bio-Spect, Racine, WI, USA) in a test tube containing distilled water (total volume 3 ml). This solution was centrifuged (15800×g for
30 min; model OM3590; Thermo Electron Corporation, Waltham, MA, USA) and 4 aliquots of supernatant (100 μl each) were reacted with Drabkin’s reagent (400 μl; Sigma, Oakville, ON, Canada) for 15 min. The absorbance was measured using a spectrophotometer (model RS232; Thermo Electron Corporation, Waltham, MA, USA). The readings from the 4 samples were averaged. Blood volumes were determined from a previously calculated curve that used known blood volumes (MacLellan et al., 2004).

4.9 Statistical analysis

Results are presented as mean±standard error of the mean (SEM). Composite behavioral scores were calculated as an index of overall performance by ranking all animals (best to worst) on each test and taking the average, which was analyzed with ANOVA (MacLellan et al., 2005a). The rest of the data were analyzed with multiple factor ANOVA in the first study and single factor ANOVA in the remaining experiments. In all cases, Scheffé post hoc test was used if needed. Mortality was analyzed with chi-square (SPSS 11.0; SPSS Inc., Chicago, IL, U.S.A.).

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