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Remote ischemic conditioning improves outcome independent of anesthetic effects following shockwave-induced traumatic brain injury

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ABSTRACT

Traumatic brain injury due to primary blast exposure is a major cause of ongoing neurological and psychological impairment in soldiers and civilians. Animal and human evidence suggests that low-level blast exposure is capable of inducing white matter injury and behavioural deficits. There are currently no effective therapies to treat the underlying suspected pathophysiology of low-level primary blast or concussion. Remote ischemic conditioning (RIC) has been shown to have cardiac, renal and neuro-protective effects in response to brief cycles of ischemia. Here we examined the effects of RIC in two models of blast injury. We used a model of low-level primary blast in rats to evaluate the effects of RIC neurofilament expression. We subsequently used a model of traumatic brain injury in adult zebrafish using pulsed high intensity focused ultrasound (pHIFU) to evaluate the effects of RIC on behavioural outcome and apoptosis in a post-traumatic setting. In blast exposed rats, RIC pretreatment modulated NF200 expression suggesting an innate biological buffering effect. In zebrafish, behavioural deficits and apoptosis due to pHIFU-induced brain injury were reduced following administration of serum derived from RIC rats. The results in the zebrafish model demonstrate the humoral effects of RIC independent of anesthetic effects that were observed in the rat model of injury. Our results indicate that RIC is effective in improving outcome following modeled brain trauma in pre- and post-injury paradigms. The results suggest a potential role for innate biological systems in the protection against pathophysiological processes associated with impairment following shockwave induced trauma.

Introduction

Brain injury due to primary blast has been extensively investigated over the last decade owing to the large number of casualties of military personnel and civilians exposed to blast associated with military/terrorist conflicts. The effects of low-level primary blast on the brain remains less well understood than the obvious pathology associated with high level blast exposure. Despite the occult nature of low-level blast injury there is mounting evidence that subclinical blast injuries, in which obvious immediate injuries may not be apparent, may have significant implications for ongoing neurological impairment. (Elder et al., 2014) Numerous ongoing studies have described a spectrum of anatomical and physiological changes occurring in the brain with varying degrees of blast force ranging from vascular disruption, inflammatory changes, neurofilament disruption and increased risk of dementia. (Aldag et al., 2017; Elder et al., 2015; Fievisohn et al., 2018; McClelland et al., 2018)

There are currently no effective treatments for traumatic brain injury, regardless of mechanism of injury induction. Remote ischemic conditioning (RIC) has been evaluated in numerous animal models and some clinical trials to demonstrate cardiac, renal and neurological protection in ischemic reperfusion injury. (Basalay et al., 2018; Chen et al., 2018) The underlying premise is that periods of cycled limb ischemia results in the production of blood borne factors in the host that confer protective effects to subsequent ischemic insult or injury. Among these, RIC has been shown to modulate oxidative stress responses through reduction of pro-inflammatory expression of TNF-a, IL-6 (Zheng et al., 2017) and a reduction in oxidative stress in ischemic reperfusion injuries (Chen et al., 2018). Similarly, remote ischemic post-conditioning has been shown to improve outcome in models of

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stroke (Li et al., 2016; Zong et al., 2015) through numerous signaling pathways. To our knowledge, only two recent studies have explored the application or RIC in TBI. These dtudies demonstrated improved cognitive outcome in a mouse model of TBI following the application of post-injury remote ischemic conditioning. (Pandit et al., 2018; Sandweiss et al., 2017) Despite the demonstration of improved cognitive outcome in these studies, a critical role for the effect of prolonged anesthetic exposure was not included in the study design. Given the known protective effects of anesthetic agents, such as isoflurane, we examined the effect of anesthetic exposure on behavioural outcomes in the blast injury model. We first present data describing the results of RIC and anesthesia on TBI outcome in rats using Western blotting and behavioural tests, respectvely. Our behavioural results indicated a significant recovery associated with prolonged anesthetic exposure which limited detecting any potential effect due to RIC. Accordingly, we sought an alternative method to evaluate behavioural outcome using a zebrafish model of injury, previously demonstrated to have outcomes comparable to mammalian responses to TBI.(McCutcheon et al., 2017)

Remote ischemic conditioning modulated the expression of heavy neurofilament after primary blast trauma in rats. Behavioural testing in rats indicated that the anesthetic agent, isoflurane, exerted protective effects in the absence of any RIC treatment. Rat-derived RIC serum administered to adult zebrafish injured using a model of shockwave-induced brain injury demonstrated improved behavioural outcomes and reduced apoptosis. Our results demonstrate that RIC has beneficial effects in pre- and post- injury brain models indicating a possible biological buffering of the pathophysiological neurofilament response to trauma associated with improved behavioural outcome and a reduction in apoptosis.

Methods

All animal procedures were performed in accordance with guidelines established by the Animal Care Committee (ACC) at St. Michael's Hospital in accordance with the standards set by the Canadian Council on Animal Care. The study was designed in accordance with ARRIVE (Animal Research: Reporting and In Vivo Experiments) guidelines. A schematic of the study design is provided in Fig. 1a.

Primary blast injury

Male adult Sprague Dawley rats weighing 250 g were randomized into four groups: sham, blast, RIC + blast and isoflurane controls. Sham rats were subject to a brief period of isoflurane anesthesia, fitted with foam ear inserts and placed on a foam bed in the blast device chamber. Blast rats underwent the same anesthetic and handling regimen as controls with the addition of blast exposure once placed in the blast chamber. RIC + Blast rats were first subject to the RIC procedure under isoflurane anesthesia (40 min), recovered for one hour and subsequently subjected to the blast procedure as described. Isoflurane control rats were subjected to isoflurane exposure for the same duration as RIC rats and were also subject to blast exposure.

The subclinical primary blast model has been previously described by our group (Park et al., 2013, 2011). Briefly, a vertically mounted open-ended shock tube was used to deliver a primary blast shockwave to isoflurane anesthetized rats. The model allows for a titratable level of overpressure exposure. We have previously demonstrated that incident pressure levels of primary blast exposure ranging from 11.5 kPa to 28 kPa result in delayed proteolytic changes in the brain as well as behavioural and electrophysiological deficits in adult rats but minimal cell apoptosis in the brain. In the current study we used a 0.5 mm aluminum bursting diaphragm resulting in incident pressures of approximately 35 kPa which we have also previously shown to reside below the threshold for inducing lung injury. (Park et al., 2011) A subset of rats (n = 3) was exposed to lower blast pressures at 11.5 kPa as previously described (Park et al., 2011) for comparative Western blot assays. Rats that underwent blast procedures were fitted with foam ear inserts to prevent any possible tympanic membrane rupture. Rats were placed off-axis at an angle of approximately 21° from the vertical axis of the shock tube opening at a distance of 19 cm from the nozzle opening to avoid any potential impact from blast wind. Pressure levels at the location of the test subject were verified with a custom designed pressure gauge attached to a DPX101 high-frequency piezoelectric pressure sensor (Omega Engineering, Laval, QC) flush-mounted perpendicular to the length of the aerodynamic probe. The incident waveform at the target location was a complex waveform with a single dominant overpressure peak as previously described for 35 kPa blast exposure. There were no mortalities or obvious signs of neurological impairment following blast procedures, consistent with our previous reports using these parameters with this device.

Remote ischemic conditioning procedure

Rats were anesthetized with 2.0 % isoflurane delivered with lab grade air through a nose cone. Anesthetized rats were placed dorsally on a warming blanket to maintain body temperature during the RIC procedure. A tourniquet was made using a length of nylon string and looped through a 2 cm length of rigid polyethylene tubing. The right hindlimb of RIC treated rats was passed through the looped end of the tourniquet. The RIC procedure was performed by tightening the tourniquet just above the knee to restrict blood flow. The tourniquet was held in place for 5 min by clamping the unlooped ends with a hemostat. The limb was considered ischemic upon discolouration of the footpad to a purple colour. Blood flow was restricted to the hindlimb for 5 min before the hemostat was released to allow normal blood flow. A 5-minute interval was given prior to repeating the RIC procedure. The RIC procedure was repeated 4 times for a total duration of 40 min (5 min ischemia, 5 min normal flow). Isoflurane control rats were subject to 40 min of isoflurane exposure and similar hindlimb manipulations with the omission of tourniquet tightening.

Rat behavioural testing

Rats were evaluated on outcomes previously established using the Baker lab shock tube device (Park et al., 2013). We used the light-dark box anxiety test and open field test to evaluate behaviour in sham, blast exposed rats and blast rats exposed to 40 min of isoflurane. There are numerous lines of evidence that indicate neuroprotective effects of isoflurane exposure in models of TBI (Statler et al., 2006a, 2006b; Statler et al., 2000). Based on these studies we hypothesized that isoflurane would result in some behavioural effect on outcome even in the absence of any RIC treatment. Initial pilot studies indicated that 40 min isoflurane exposure in blast rats resulted in protective effects on behavioural outcome indistinguishable to the isoflurane control group (data not shown). Given the futility of analysis with this treatment group we only evaluated behavioural outcome in sham control, blast and isoflurane control rats to highlight the confounding effects of isoflurane preconditioning on behavioural outcomes. The results from rat behavioural experiments dictated our subsequent zebrafish approach to evaluating *in vivo* behaviour outcomes after TBI and RIC treatment. We used 16 rats per treatment group for rat behavioural tests.

All behavioural tests were performed by two observers blinded to the treatment groups. The light-dark box anxiety test was performed as previously described on days 2, 6 and 9 following primary blast exposure. The test consisted of a two-chamber enclosure. The covered darkened section consistent of an enclosure with dimensions, length 80 cm × width 50 cm × height 50 cm. A 10 cm opening in the dark section led to the open lit compartment. A single compact fluorescent light source was used to illuminate the open section. Rats were placed in the dark portion of the enclosure. The amount of time spent within each compartment was recorded. The number of entrances to the lit area was also recorded. The enclosure was cleaned following every trial

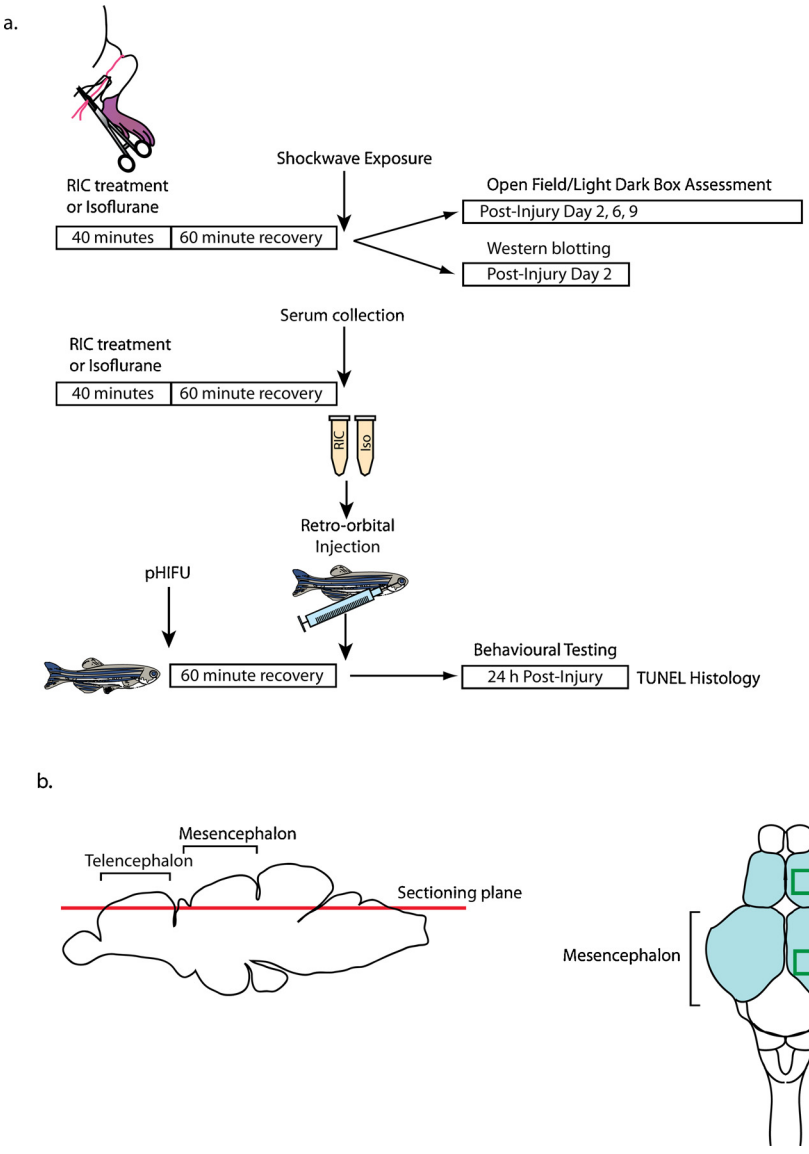

Fig. 1. A schematic timeline of the study design. Rats were subject to either the RIC treatment (including anesthesia) or 40 min of anesthesia alone. Following a 1 h recovery, rats were subject to the primary blast injury. A subset of rats were sacrificed following recovery for serum collection for zebrafish studies. Behavioural testing was performed at 3, 6 and 9 days post-injury. Tissues for Western blot analysis were collected at 48 h post-injury. B) The schematic representation of the zebrafish brain indicates the sectioning plane used for TUNEL histology in adult zebrafish. The boxed areas in the adjacent diagram indicates the sampling regions from the telencephalon and mesencephalon used for TUNEL quantification analysis.

with 70% alcohol. All behavioral assays were run between 13:00 h and 15:00 h.

The open field test was performed in a 100 cm × 80 cm walled enclosure marked with 10 cm × 10 cm grids. Rats were placed one at a time in the enclosure and observed over a 5-minute period. The total number of grid squares that were traversed and number of hindlimb rearings were recorded.

Adult Zebrafish brain injury model

The use of targeted pulsed high intensity focused ultrasound (pHIFU) has previously been described by our group as a method to apply a shockwave-induced brain injury in adult zebrafish (McCutcheon et al., 2017). The model has been shown to produce behavioural deficits analogous to mammalian TBI outcomes. Adult wild-type AB shortfin phenotype male and female zebrafish were used in pHIFU experiments. Holding tanks were kept at a normothermic temperature of 25 °C under a light/dark cycle of 12/12 h and pH 6.8-7.0. Following clove oil anesthesia (150 ppm), fish were placed on the lateral aspect in a custom holder and secured with surgical tape. Fish remained submerged in clove oil containing water during the pHIFU procedure. The pHIFU system consists of an advanced 1–3 piezo-composite high-power ultrasound transducer technology (Imasonic SAS, Voray sur l'Ognon, France) to deliver ultrasound energy into tissues. The acoustic focal pressure and dimensions of the focal zone of the pHIFU transducer produced a pHIFU focal zone previously modeled as an ellipsoid of -6 dB axial and lateral dimensions of 7.5 and 1.2 mm, respectively (Alhamami et al., 2014). The maximum pulsed pressure used in this study was ~9 MPa with a total pulse train duration of 50 ms. We used 12 fish per treatment group for behavioural tests.

Rat serum collection

Two rats were used for serum collection studies which would be administered to zebrafish. One rat was subject to the RIC procedure (including anesthesia) while the other was exposed only to isoflurane anesthetic for the duration of the RIC procedure. Rats were recovered for 1 h and the serum collected by exsanguination of the femoral vein under ketamine anesthetic. Blood was collected in a 10 ml syringe during the exsanguination procedure and aliquoted into 1.5 ml Eppendorf tubes. Blood was clotted and then centrifuged at 2300 G for 15 min at room temperature. The supernatant serum was removed and stored in tubes and stored at - 80 °C until use.

Western blotting

Changes in protein expression in rats following RIC treatment and blast exposure were evaluated at 48 h post-injury. Brain tissue samples were dissected from the ipsilateral injured cortex of sham, injured and RIC-treated rats (n = 6 per treatment group). Samples were homogenized in lysis buffer containing protease inhibitors (50 mM Tris-HCl, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Protein quantification was determined by the modified Lowry method (Peterson et al., 1977). Samples were normalized for equal loading (40 μg/lane) and were electrophoresed on 7.5 % SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk solution for one hour at room temperature.

Our blast model previously demonstrated delayed pathophysiological changes in NF200 expression associated with low-level blast. Changes in heavy neurofilament expression including increased expression is associated with electrophysiological impairment in white matter function following low-level blast exposure. (Park et al., 2013; Park et al., 2011) We examined expression of the heavy neurofilament chain using a mouse monoclonal antibody recognizing both phosphorylated an non-phosphorylated isoforms of NF200 (Sigma N52, Oakville, Ontario). Primary antibodies were used at a dilution of 1:1000 in blocking solution.

Anti-GAPDH antibodies (Cell Signalling) were used as a loading control at a dilution of 1:6000. Each incubation step was followed with three changes of TBST. Secondary antibody incubations with either goat anti-rabbit HRP or goat anti-mouse HRP were performed at a dilution of 1:3000 for 1 h at room temperature. Visualization of signals was performed with an ECL kit and detection using a Gel-dock imaging station (Bio-Rad, Mississauga, Ontario). Western blots were repeated in triplicate in order to verify results.

Retroorbital injections

Rat serum was administered retro-orbitally in injured fish 1 h post-pHIFU injury. This method has previously been shown as an effective method to introduce drugs and cells into the circulation of adult zebrafish with minimal mortality and complications (Pugach et al., 2009). In brief, fish anesthetized with clove oil (200 ppm) were placed laterally onto the left side onto a damp sponge. A 26 G Hamilton microsyringe was backfilled with 4 μl of serum. The needle tip was introduced adjacent to the eye at the 9:00 position. The needle was inserted approximately $1-2$ mm into the retroorbital cavity followed by injection of the serum. Successful injection was confirmed by the protrusion of the eye during the injection procedure. The eye protrusion returned to normal by 12 h. Fish were recovered in normal tank water and returned to their holding tanks in the animal facility. We observed no mortality as a result of the retro-orbital injection procedure in this study. All fish received a retroorbital injection by a technician who was blinded to the contents of the injectable solutions. Sham fish were injected with filtered water, while pHIFU injured fish received RIC serum or serum from rats exposed only to isoflurane anesthesia (Iso).

Adult zebrafish behavioural testing

The novel tank test was performed at 24 h post-injury as previously described [15,34] to evaluate stress, anxiety and exploration activity following modelled brain injury. In brief, a rectangular tank filled with conditioned water at 26 °C was divided into three equal compartments with opaque tank dividers. Fish (n = 12 per treatment group) were individually placed into each compartment and recorded (Canon Vixia HF R500) for a period of 6 min. EthovisionXT software (Noldus, Netherlands) was used to analyze the recorded video. Locomotor activity was assessed by quantifying swim distance, swim velocity, mobility (time spent mobile vs immobile) and meandering (absolute turn angle – representative of the variations in direction of the centre point of the animal divided by the total distance travelled). The time spent in the upper quadrant for each compartment was also calculated to evaluate vertical exploration activity.

Histology and TUNEL assay

Zebrafish brains were collected at 24 h post-injury (n = 6 per treatment group). Briefly, fish were euthanized in water containing clove oil (500 ppm) and then immersed whole in 4 % paraformaldehyde overnight. Brain were dissected and placed into phosphate buffer saline (PBS) containing 30% sucrose for cryoprotection. Brains were sectioned in the horizontal plane on a cryostat at a thickness of 10 μm and thaw mounted onto gelatin subbed slides. Only sections that had full anatomical representation of the telencephalon and mesencephalon were used for quantification. Given the relatively small size of the zebrafish brain, this method of slide selection ensured that sections were taken from roughly the same anatomical region between treatment groups. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed on sections according to the manufacturer's protocol (Roche, Mississauga, Ontario). Sections were counterstained with a Hoechst nuclear stain.

Sections were imaged on a Nikon 90i fluorescent microscope with a 20X objective. A minimum of 2 sections per fish were used for TUNEL analysis. Sampling regions were taken from the telencephalon and two regions of the mesencephalon as indicated in Fig. 1b using a 20x objective. The resulting imaging fields had dimensions 210 μm × 328 μm. The total number of TUNEL positive nucleii was counted for each imaging field for each given brain section. The numbers for each anatomical region was then averaged as a representative number of TUNEL positive cells for that region for each fish.

Statistics

Western blots were quantified using ImageLab software (Bio-Rad). Statistical analysis was performed using a 1-way ANOVA. Protein bands were normalized to GAPDH expression and subsequently expressed relative to sham expression values. Behavioural tests in rats were analyzed using a 2-way repeated measures ANOVA. Post-hoc analysis was performed using the Holm-Sidak test. Behavioural tests in fish were analyzed with a 1-way ANOVA. Post-hoc analysis was performed using Tukey's test for distance and velocity outcomes. A Kruskal-Wallis One Way Analysis of Variance on Ranks was performed for meander and latency data. TUNEL data was analyzed using a 1-Way ANOVA on ranks and a post-hoc Dunn's test. Statistical significance was assumed at P = 0.05. Error bars are represented as mean standard error bars.

Results

Remote ischemic conditioning modulates NF200 expression in response to primary blast

Levels of NF200 expression were significantly different across treatment groups (p = 0.005). There was a reduction in NF200 expression at 48 h in the blast group although values were not statistically significant. Administration of the RIC treatment prior to blast modified the response of NF200 resulting in a marked and significant increase in NF200 expression relative to the blast group (p = 0.004; Fig. 2a & b). The increase in NF200 expression in the RIC treated group was also higher than expression levels in sham animals but not statistically significant (p = 0.06). Interestingly, we observed that anesthetic control animals (ie. Rats that were subject to 40 min of isoflurane anesthesia alone) had a dramatic reduction in NF200 expression relative to control animals (Fig. 2c).

Protein samples from rats subject to 35 kPa overpressure blast were compared to rats that were exposed to 11.5 kPa blast. Consistent

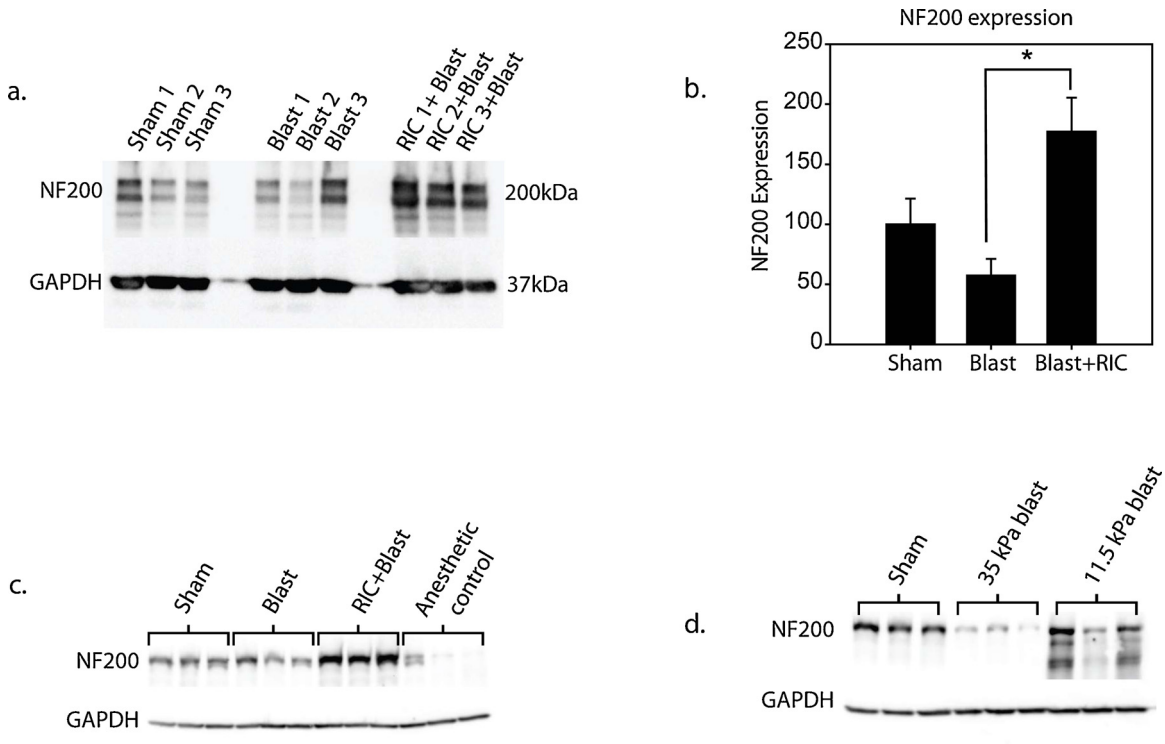

Fig. 2. a & b) Expression of the heavy neurofilament, NF200, was examined in sham, blast and RIC + blast tissues. There was a notable reduction in intact NF200 expression in blast tissues and an increase in NF200 expression in RIC treated rats. Densitometry values between blast and blast + RIC tissues were significantly different ($p = 0.004$). c) Comparison of NF200 expression between treatment groups as well as the inclusion of anesthetic control tissues indicated a marked reduction in N200 in rats subject to anesthetic preconditioning alone. D) Comparison of the NF200 response to two levels of subclinical blast exposure indicates a reduction in overall NF200 expression in the higher blast (35 kPa) group, while the lower level blast injury (11.5 kPa) results in an increase in intact NF200 expression as well as evidence of lower molecular weight breakdown products.

without previous reports, we observed an increase in NF200 expression as well as lower molecular weight breakdown products in the 11.5 kPa blast group whereas in the 35 kPa blast group a reduction in intact NF200 was evident (Fig. 2d).

Isoflurane anesthesia used in a preconditioning paradigm affects behavioural outcome

Open field grid

In the open field test we evaluated two outcomes, grid exploration and number of hindlimb rearings as described in our previous publication. (Park et al., 2013) In grid exploration, there was a significant difference between treatment groups ($p = 0.047$). The mean traversed number of squares travelled at 3 days post injury for sham, blast and isoflurane control rats were 85.3 ± 10.4, 49.0 ± 10.1 and 73.5 ± 10.1, respectively (Fig. 3a). On day 7 mean values for the same groups were 70.5 ± 10.1, 47.9 ± 10.1 and 79.4 ± 10.1 squares traversed. Overall comparisons between sham and blast treatment groups indicated a significant reduction in open field exploration in blast rats ($*p = 0.032$). Similarly, isoflurane exposed rats had significantly increased exploration activity relative to blast rats ($**p = 0.040$) and were not statistically different compared to sham rats.

When evaluating the number of hindlimbs rearings we also observed a similar effect of behavioural modification in response to blast in the isoflurane treated rats, although values were not significantly different (Fig. 3b).

Light-dark Box

Two-way repeated measures ANOVA indicated that treatment groups were significantly different ($p < 0.001$). On day 2 values were not significantly different between treatment groups. On day 6, post-hoc analysis indicated a significant difference between sham vs. blast rats ($*p < 0.001$) with sham rats spending 110.5 ± 20.7 s in the lit section of the test box. By contrast, blast rats only spent 19.3 ± 4.2 s (Fig. 3c) in the lit region. Isoflurane exposed rats spent 53.0 ± 12.8 s lit section ($**p = 0.029$) significantly longer than blast rats. By day 9 values between sham and isoflurane exposed blast rats were not significantly different, whereas blast rats continued to spend significantly less time in the lit section of the test setup relative to sham ($*p = 0.003$) and isoflurane exposed rats ($**p < 0.001$).

Serum derived from RIC rats improves behavioural outcome in an adult zebrafish injury model

It was apparent from our rat behaviour studies that prolonged isoflurane exposure associated with the RIC procedure resulted in improved behavioural outcome in rats even with the omission of the RIC treatment itself. In order to examine the effect of RIC on behavioural outcome and to parse out the effects of RIC in a model that was not sensitive to the effects of isoflurane we used a previously described model of brain trauma in adult zebrafish (McCutcheon et al., 2017). Furthermore, we sought to investigate whether RIC applied in a more therapeutically relevant post-traumatic scenario would still provide beneficial effects. Based on previous studies we assumed that beneficial mechanisms of RIC would be mediated through blood borne molecules produced during the RIC protocol and also that the absence of isoflurane would eliminate any contribution to neuroprotection in test fish. We used rat serum derived from a rat exposed to 40 min of isoflurane anesthetic only as well as serum derived from a rat subject to the RIC protocol (isoflurane + RIC) and randomized administration into injured zebrafish at one hour post-injury.

Distance and velocity outcomes demonstrated results consistent with our previous description of this model. Namely, a reduction in

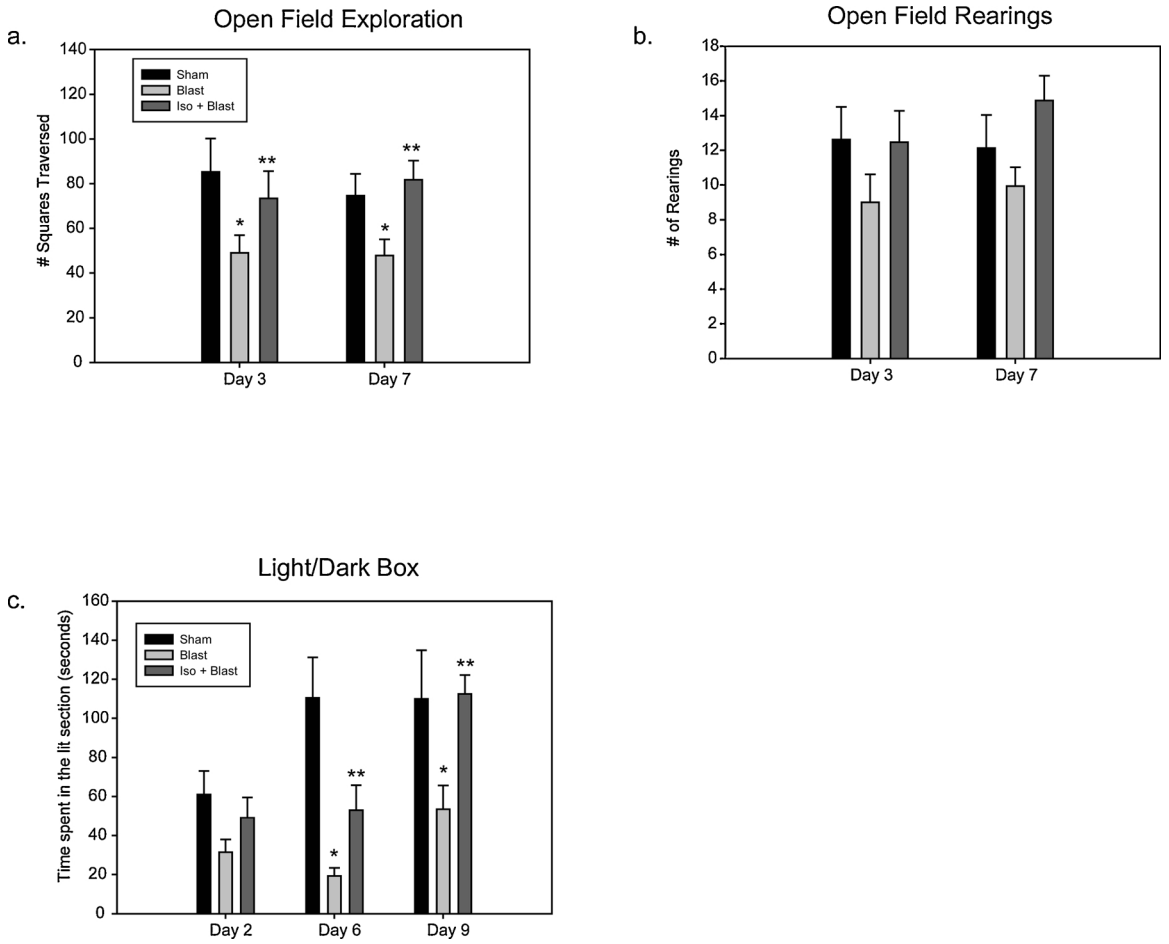

Fig. 3. a & b) Behavioural outcome in the open field test indicated a reduction in both rearings and overall exploration activity in blast rats. Relative to sham controls. Post-hoc p-values between sham and blast rats was *p = 0.032 and ** p = 0.040 between blast and isoflurane control rats. Similarly, although the rearing data suggest a protective effect mediated by isoflurane, values were not statistically significant. c) In the light-dark box test there was a significant reduction in time spent in the lit section in the blast group relative to sham rats (* p < 0.001) and isoflurane exposed rats (**p = 0.029) on day 6. On day 9 blast rats continue to have significantly lower exploration times than sham rats (* p = 0.003). Isoflurane preconditioning resulted in a significant increase in the time spent in the lit section following blast exposure compared to blast alone (** p < 0.001).

swim distance and mean swim velocity following injury. There was also an increase in meander and increased latency in tank exploration in pHIFU injured fish relative to sham fish as previously reported. One-way ANOVA indicated a significant difference between treatment groups in distance ($p < 0.001$) swum by the fish after injury. Pair-wise comparisons indicated a significant improvement in swim distance in pHIFU injured fish treated with RIC serum relative to pHIFU fish receiving the isoflurane control serum (*p = 0.048). The mean swim distance was 897.05 ± 107.24 cm in isoflurane control serum treated fish and 1621.61 ± 118.5 ± 3 cm in RIC serum treated fish (Fig. 4a).

Velocity outcomes were significantly different between treatment groups ($p < 0.001$). Swim velocities were significantly improved in RIC treated pHIFU injured fish (4.81 0.34 cm/s) relative to pHIFU fish receiving the isoflurane control serum (2.60 ± 0.30 cm/s; *p = 0.023; Fig. 4b).

Outcomes measures associated with anxiety included meander and latency activity. Meandering was not statistically significant by 1-way ANOVA. However, the trend in activity suggested an increase in meander activity in pHIFU isoflurane control fish (619.33 ± 148.58 deg/cm) relative to sham controls (460.36 ± 65.95 deg/cm). Treatment with RIC serum (516.32 ± 83.56) reduced the post-injury increase in meander values.

Latency to explore the upper section of the test tank approached statistical significance when mean values were compared between treatment groups ($p = 0.071$). The summarized graphed data suggests an effect from RIC serum treatment. Latency values were 46.93 ± 12.03 s in sham fish, 128.77 ± 34.74 in pHIFU isoflurane control serum treated fish and 47.28 ± 10.76 in RIC serum treated fish.

Ischemic conditioned serum reduces TUNEL in injured zebrafish brains

We quantified the number of TUNEL positive cells in zebrafish brains from 3 sampling regions which included the telencephalon and mesencephalon structures (Fig. 1). We observed TUNEL positivity in all pHIFU injured brain regions suggesting injury due to pHIFU was not isolated to a particular area but rather diffusely distributed throughout the telencephalon and mesencephalon structures (Fig. 5a & b). The total number of TUNEL positive cells in all 3 sampled structures was found to be statistically different among treatment groups ($p < 0.001$). The number of TUNEL cells in pHIFU + isoflurane treated brains was significantly higher relative to sham ($p < 0.001$). Fish administered the RIC serum had significantly reduced number of TUNEL positive cells ($p < 0.001$; Fig. 5c).

Discussion

Primary blast has been implicated in clinical and experimental studies as a contributor to neurologic impairment. However, the effects of subclinical brain trauma due to low-level primary blast remains less

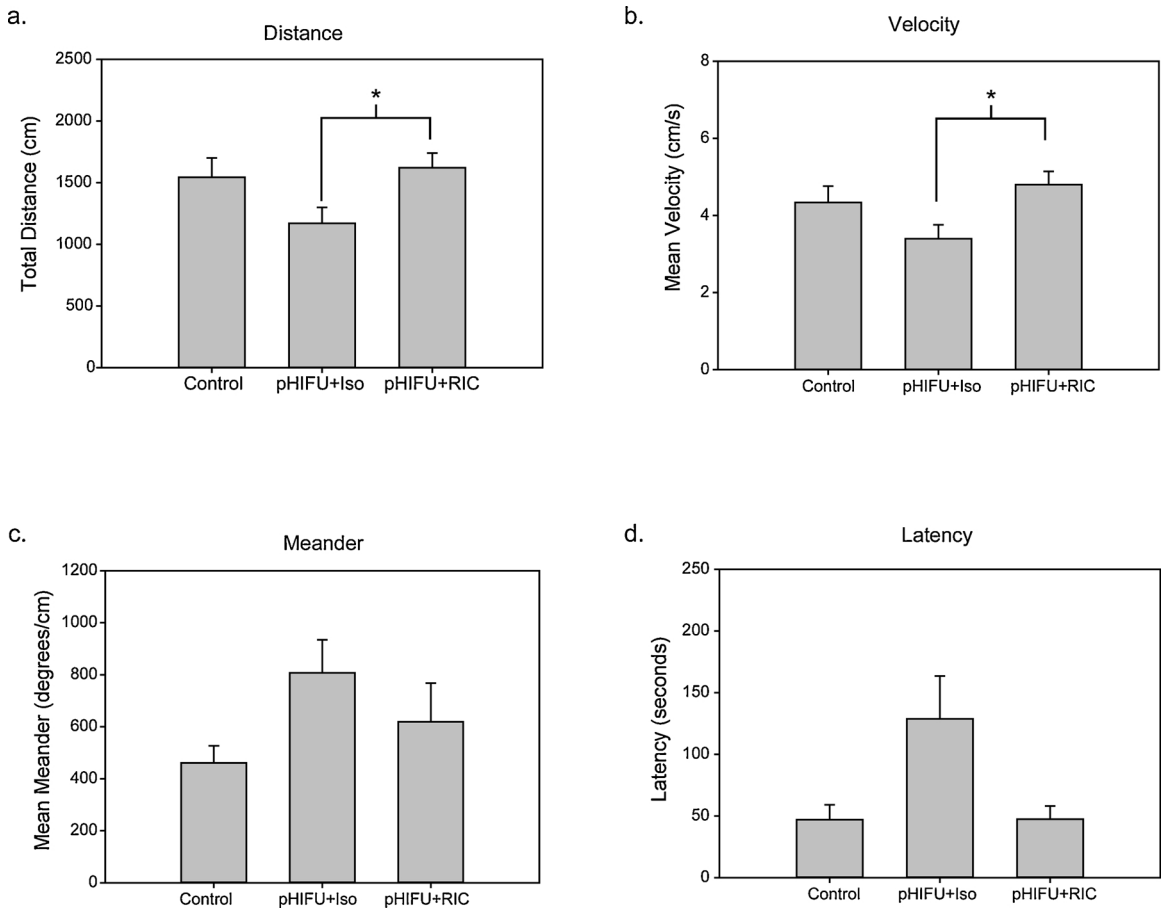

Fig. 4. a) Injured fish administered anesthetic control serum (pHIFU + Iso) had reduced swim distance relative to controls whereas pHIFU + RIC serum treated fish had a significant improvement in total swim distance (*$p < 0.001$). b) Swim velocity was significantly reduced in pHIFU + Iso fish relative to controls and improved in fish treated with RIC serum (*$p < 0.001$). c) The data for meander outcomes suggested an increase in mean meander activity in pHIFU + Iso fish relative to control fish. RIC serum treatment reduced post-traumatic meander activity. d) Similarly, latency values for tank exploration were not significantly different among treatment groups but the data suggested an increase in latency times for exploration in pHIFU + Iso. RIC serum treated fish exhibited a reduction in the latency times relative to isoflurane control treated fish.

unclearly defined (Baker et al., 2011; Sullivan et al., 2018) than more obvious injuries caused by high blast injury levels. Mounting experimental evidence suggests substantial pathophysiological events in the brain that contribute to subtle but significant neurologic impairment after injury (Park et al., 2013, 2011; Pun et al., 2011; Vandevord et al., 2012). Despite the high number of reported mTBI's attributable to blast-related trauma, there remains a paucity in effective clinical therapeutic interventions. In the current study we investigated the potential of a non-pharmacological intervention using remote ischemic preconditioning as an approach to bolster the body's innate reparative and protective systems to provide a biological buffer to low-level primary blast trauma.

The phenomenon of ischemic conditioning was first reported as a cardioprotective mechanism (Murry et al., 1986). This concept was later extended to regional preconditioning whereby alternating ischemic and reperfusion episodes within one vascular bed could protect the myocardium from subsequent arterial occlusion (Przyklenk et al., 1993). Evidence to support remote ischemic preconditioning was demonstrated when ischemic/reperfusion cycles in a the kidney were found experimentally to be protective in myocardial infarction (Kerendi et al., 2005). A host of experimental studies have subsequently demonstrated the effectiveness of RIC on reducing lesion size in myocardial ischemic-reperfusion injury. (Bromage et al., 2017) The concept of preconditioning to protect vital organs has been extended to include the brain in models of global and focal ischemic stroke. (Cheng et al., 2018; Hu et al., 2012; Ren et al., 2018) In this regard we sought in investigate the potential for RIC neuroprotection in a model of primary blast injury.

Although Despite numerous experimental studies demonstrating preclinical neuro and cardio-protection from ischemic-reperfusion injury, the translation to clinical effectiveness has been less clear. (Candilio et al., 2015; Hausenloy et al., 2015; Meybohm et al., 2015) The role and type of anesthetics have been suggested to be potential confounding variables to account for the discrepancy in clinical outcomes (Benstoem et al., 2018, 2017), however a definitive explanation remains elusive. In our studies we observed an effect of RIC on modulation of neurofilament expression patterns that differed from the effect of anesthetic isoflurane controls. It was interesting to note that NF200 levels were increased in response to RIC treatment whereas isoflurane exposure alone resulted in a decrease in NF200 expression. There was a notable increase in NF200 expression beyond normal levels of expression with RIC treatment. With the higher intensity 35 kpA blast injury level, there was a marked reduction in NF200 expression levels. In a previous study (Park et al., 2011) we observed increased NF200 expression levels associated with lower levels of blast injury (11.5 kPa). Whether the difference in NF200 expression between these two levels of injury reflects NF200 accumulation at lower levels versus axonal loss at higher levels of injury remains to be further clarified. However, it is interesting to note that application of RIC in the 35 kPa injury group results in a neurofilament response similar to the NF200 expression levels in the lower intensity 11.5 kPa blast group. This suggests the possibility that RIC acts to buffer the pathophysiological

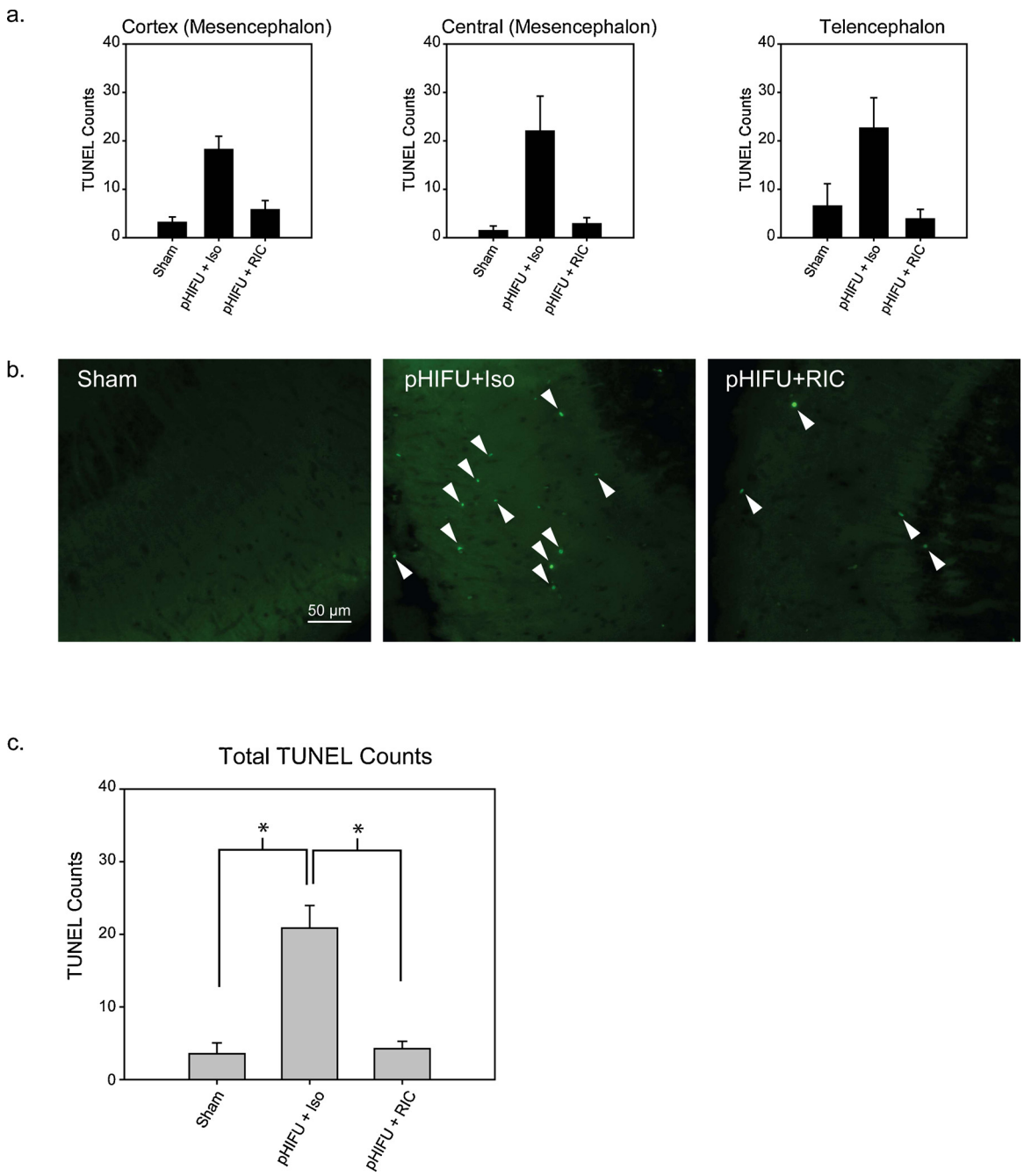

Fig. 5. a) Summarized TUNEL counts from sampling regions of the adult zebrafish brain indicated a increased TUNEL labelling across the sampled regions suggesting that the pHIFU injury was not limited to a single focal area or structure. b) Representative TUNEL brain sections sampled from the cortex of the mesencephalon in sham, pHIFU + Iso and pHIFU + RIC serum treated fish. TUNEL positive cells indicated with arrowheads. c) Summarized total TUNEL count data indicates a significant increase in TUNEL positivity in pHIFU + Iso fish relative to sham and pHIFU + RIC serum treated fish ($p < 0.001$).

neurofilament response in higher blast pressures to that of a lower blast injury response. The implications of altered NF200 expression in Western blots on functional outcome is not entirely clear given that prolonged isoflurane exposure alone also resulted in a drastic reduction in total NF200 expression. Whether, the change in NF200 expression is a downstream pathophysiological response to other events or whether NF200 expression is directly related to isoflurane or RIC remains to be elucidated. To the best of our knowledge this is the first documented report of prolonged isoflurane exposure altering heavy neurofilament expression in rats. There is evidence to indicate that isoflurane exposure can destabilize dendritic spines through actin-dependent mechanisms (Platholi et al., 2014) as well as mounting evidence that anesthetics in general affects synaptic plasticity through numerous signalling pathways. (Jevtovic-Todorovic et al., 2013) Thus it is plausible that isoflurane also acts on pathways that destabilize neurofilament dynamics in reversible manner.

Despite the differing effect of RIC and isoflurane on neurofilament expression, the behavioural outcomes in this study indicated that isoflurane exposure prior to blast injury was sufficiently neuroprotective to provide behavioural improvement in outcome in the absence of RIC intervention. Other report of anesthetics on behavioural outcome have been reported. For example, chloral hydrate and Zoletil have been reported to have neuroprotective effects on ischemic reperfusion injury in the brain but do not exert an additive effect when applied with a RIC intervention. (Silachev et al., 2017) There are numerous lines of evidence that demonstrate neuroprotective effects of both injectable and

volatile anesthetics in models of neurotrauma. (Bell, 2017; Chen et al., 2018; Jiang et al., 2017; Slupe and Kirsch, 2018) Thus careful consideration of anesthetic controls is important for future RIC studies. Based on our behavioural findings with isoflurane alone we did not pursue behavioural tests in rats using the RIC protocol due to a ceiling effect from isoflurane exposure.

In order to isolate the effects of RIC we used serum derived from RIC rats to treat brain injured zebrafish. We had several assumptions in this treatment approach. The first assumption was that the protective factors in the rat serum would share sufficient biological homology to be effective in a xeno-treatment application. The second assumption was that the biological factors would also be protective in a post-injury application. Thirdly effects of isoflurane neuroprotection observed in host rats would not be carried over in the RIC serum collected. The first two assumptions were supported by the data in behavioural and TUNEL assays indicating behavioural and histological improvement. Given the high degree of genetic homology of zebrafish to mammalian vertebrates (Howe et al., 2013), it is plausible that the protective factors in the rat serum were also effective in modulating signalling pathways in the zebrafish. There is also evidence that ischemic preconditioning is effective in both pre-, per- and post- conditioning scenarios. (Basalay et al., 2018; Chen et al., 2018) Although the precise underlying physiological mechanisms of action may differ in these different application paradigms, it proved to be effective in improving outcome in our post-injury TBI model. Furthermore, it has long been assumed that clinical volatile anesthetic clearance occurs rapidly upon removal of exposure. Experimental numbers in mice demonstrate that expired isoflurane drops precipitously within 5 min of anesthetic removal. (Saab et al., 2010) Thus it is unlikely that residual isoflurane exerted neuroprotective effects in our zebrafish injury model. Moreover, isoflurane does not perform as a suitable anesthetic agent in zebrafish with evidence suggesting increased anxiety behaviour with exposure (Collymore et al., 2014) further ruling out any potential effects from isoflurane on zebrafish outcome.

There are notable differences regarding the reparative potential of the zebrafish brain compared to that of mammals.(Ceci et al., 2018; Kizil et al., 2012) Studies in both larvae and adult zebrafish demonstrate a remarkable ability for regeneration of damaged neural tissues following penetrating mechanical insult (Cacialli et al., 2018; Herzog et al., 2019; Shimizu et al., 2018) which is in obvious contrast to the mammalian capacity for neural regeneration of injured tissues. Our model of pHIFU-induced brain injury does not involve obvious penetrating trauma to the brain or overt cell loss as induced in these other studies. The presence of TUNEL expression, however, suggests some degree of cell loss. This in conjunction with post-injury protein expression changes comparable to mammalian TBI responses previously described in the characterization of the adult zebrafish injury model suggest a mild form of TBI (McCutcheon et al., 2017). It is not known to what degree regeneration plays a role in this milder form of brain trauma and requires further investigation. Other studies report that neuroregenerative pathways are activated at 21 days post-injury, (Kishimoto et al., 2012; Maheras et al., 2018) well-beyond the time frame explored in this study but does not rule out the possibility of RIC influencing these pathways at an earlier time point. Signalling pathways responsive to the RIC serum warrants further investigation in order to determine whether the improvements in outcome reported in zebrafish are the same as those associated with modulation of injury response observed in rodents.

Collectively, however, the transferrable protection conferred by xeno-transfer of RIC serum strongly suggests a role for humoral-mediated protection of the brain following injury and RIC treatment. This does not rule out the possibility of RIC modulation on inflammatory pathways as described by others. However, the blast level intensity produced by the Baker lab shock tube device is sufficiently low that signs of neuroinflammation have not been observed through immunohistochemistry for microglia using CD11b/c labeling. There are no appreciable morphological changes in microglial morphology or distribution with the levels of injury described in this study (data not shown). Thus the subclinical blast model is not amenable to test the impact of RIC on neuroinflammation after trauma. Similarly, a neural mechanism of RIC may also play a role in neuroprotection following remote limb ischemia (Basalay et al., 2012; Steensrud et al., 2010), but remains to be further clarified in the context of TBI.

The results of our study demonstrate applicability in both pre- and post- injury paradigms. The feasibility of applying RIC as a prophylactic measure initially seems more applicable as proof-of-concept of neuroprotective potential. However, given the ease of application and safety of the RIC procedure, it could conceivably be applied prior to high risk activities such as contact sports, or prior to engagement in military scenarios to reduce secondary injury severity in the event of brain trauma. Taken together, RIC demonstrates a wide range of application alternatives in the treatment of mild TBI.

Conclusion

The current study demonstrates several novel concepts. First, RIC demonstrates effectiveness in both pre- and post- mild TBI scenarios. Second, prolonged isoflurane exposure demonstrates significant effectiveness in reducing neurobehavioural deficits and should be considered a potential confounder in future behavioural studies evaluating the effects of therapeutics. While the mechanisms and factors responsible for mediating improvement in outcome in both injury models have not been identified in this study, there is sufficient homology in the protective factors resulting in improved outcome in a xeno application system. This easy to apply intervention demonstrates the innate protective potential of the host system to reduce the underlying pathophysiology associated with mild traumatic brain injury.

Conflict of Interest

The authors report no conflicts of interest in this study.

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