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14. ABSTRACT

Purpose: To explore two new neuroprotective strategies in the setting of experimental repetitive mild traumatic brain injury (mTBI): i) hyperbaric oxygen (HBO) preconditioning and ii) HBO treatment combined with intranasal delivery of nicotinamide (NAD). **Scope**: In rat model of a repetitive mild cortical controlled injury, we investigated the neuropathological profile of two mTBI sessions at two interval times (3, 7d) using non-invasive MRI assessments (T2WI, SWI, and DTI), correlated with histology. The effects of i) a prophylactic HBO strategy (pretreatment 3d X 1hr) prior to first mTBI and ii) the comprehensive therapeutic strategy of 3d X 1hr course of HBO combined with intranasal administration of NAD right after the first mTBI impact will be evaluated using the MRI and histological biomarkers. **Major Findings**: Two sessions of mTBI with 3d apart resulted in significant increase in lesion volumes derived from T2WI and hemorrhagic lesion derived from SWI. Ex vivo histology examination confirms the in vivo neuroimaging findings. Pre- or post-treatment HBO significantly reduced the lesion volume and hemorrhagic lesion in rmTBI with either 3d or 7d apart. Similarly, NAD post-treatment also was significantly efficacious in reducing repeated mTBI. HBO+NAD post-treatment was also beneficial, but there were no differences between single or combinatorial treatments.

15. SUBJECT TERMS

Repetitive mild traumatic brain injury, HBO, nicotinamide, intranasal, MRI, rat

Neuroprotective Strategies for Repetitive Mild Traumatic Brain Injury (mTBI)

Table of Contents

Neuroprotective Strategies for Repetitive Mild Traumatic Brain Injury (mTBI)
Principal Investigator: Andre Obenaus, Ph.D., Loma Linda University

INTRODUCTION

Mild Traumatic brain injury (mTBI), or concussion, is an important medical concern for the military in the field. Recurrent mTBI episodes may exacerbate tissue injury and psychosocial outcomes (Huh, 2007; Creeley, 2004). In our current study, we hypothesized that repetitive mTBI (rmTBI) would result in cumulative injuries and that intervention using either HBO alone prophylactically or therapeutically in combination with intranasal delivery of nicotinamide would improve the outcomes in a rodent model subjected to rmTBI. Both intervention paradigms are non-invasive, simple to administer and can be rapidly deployed in the field. We proposed the following three Aims: **Aim 1)** To investigate the neuropathological profile of two mTBI events at two interval times (3, 7d), using non-invasive magnetic resonance imaging (MRI) correlated with histology; **Aim 2)** To investigate the effects of a prophylactic HBO strategy (pretreatment) in the setting of rmTBI. The experimental groups (see Aim 1) underwent a 3d X 1hr course of HBO pretreatment followed by mTBI at two interval times (3, 7d). Outcome assessments were as in Aim 1; **Aim 3)** To investigate the comprehensive therapeutic strategy of 3d X 1hr course of HBO and intranasal administration of nicotinamide for three days after the first mTBI impact.

AIM 1: DEVELOPMENT OF AN rmTBI ANIMAL MODEL

We developed a rat model comprised of a mild cortical deformation (0.5 mm) followed by an identical impact (0.5 mm) either at 3 or 7d after the initial injury but at the same site (Fig. 1). Using MRI we evaluated three key parameters: 1) edema development and formation (T2-weighted imaging; T2WI), 2) localization of extravascular blood (susceptibility weighted imaging, SWI); and 3) at the final time point (14d) we evaluated the integrity of white matter using diffusion tensor imaging (DTI). Histology was used to validate the MRI findings and provide a pathophysiological basis for our results. Detailed methods are provided in the Appendix–Huang, 2010, NNS Poster).

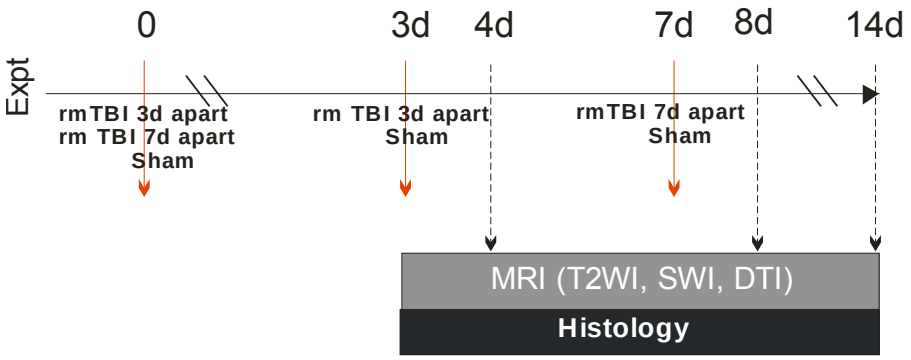

Fig. 1 Experimental design for Aim 1. An initial mTBI was induced at day 0, followed by a second repetitive mTBI at day 3 for the rmTBI 3d apart group and at day 7 for group of rmTBI 7d apart group. Sham animals underwent an identical procedure but without CCI. At 24 hrs after each impact and 14 days after the initial mTBI, multi-modal MRI was acquired. Histology was performed at each time point.

Cortical lesion volume, defined as abnormal T2WI signal intensities (hyperintensity = edema, hypointensity = blood) showed significantly increased lesion size in the 3d compared to the 7d rmTBI group at 14 days after the initial impact (Fig. 2). The interval (i.e. 3d vs 7d) between repetitive injury elicits an altered MRI signature. The rmTBI 3d apart group was associated with a heterogeneous mixture tissue types (hypo- and hyperintensities) at the impact site that was composed of both edema and blood. However, there was little or no edema but only mild bleeding at the injury site when rmTBI is undertaken with a 7d interval (Fig. 3). The evaluation of blood within tissues can be readily visualized using susceptibility weighted imaging (SWI), a new imaging technique. SWI is uniquely sensitive to the iron content of extravascular blood which can be visualized as dark regions on MR. There was a consistently greater SWI derived-hemorrhagic lesion volume in rmTBI with 3d apart (Fig. 4).

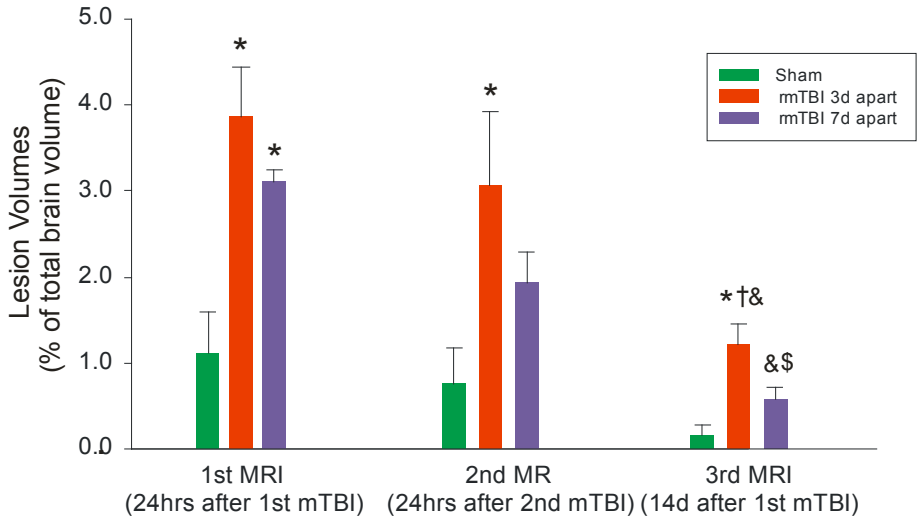

Fig. 2 Lesion volumes derived from T2WI following rmTBI. The first mTBI event resulted in a similar lesion size in both rmTBI 3d and 7d apart groups. When a second mTBI was induced at 3d after the initial impact, there were significant increase in lesion volume compared to Shams and the 7d apart group. *p<0.05 vs Sham; †p<0.05 vs rmTBI 7d apart; $^{\&}$p<0.05 vs 1st MRI; $^{\$}$p<0.05 vs 2nd MRI.

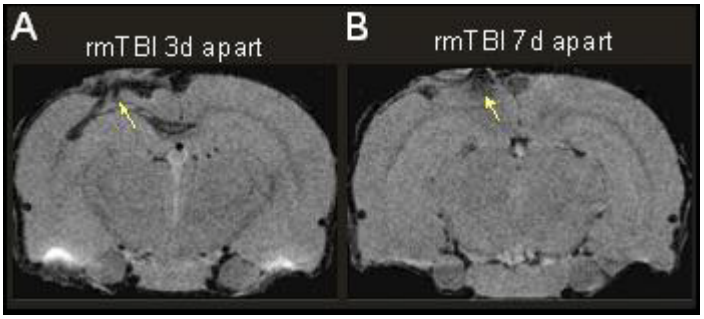

Fig. 3 Representative T2WI showing the signal pattern of the lesion (arrows) in the repetitive mild TBI (rmTBI) groups at 14 days after the initial impact. Hyperintense T2 (bright) is representative of edema formation and hypointensity (dark) indicates blood within the injured brain. A) A heterogeneous signal intensity was evident in animals receiving rmTBI 3 days apart; B) Hypointensities were primarily prominent in the lesion when rmTBI occurred 7 days apart. Also note the smaller lesion area in the rmTBI 7d apart group.

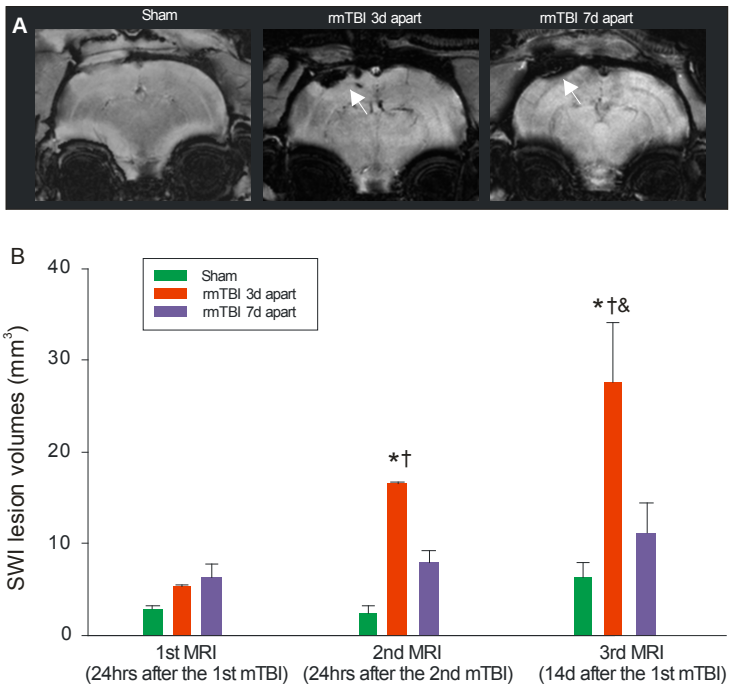

Fig. 4 Hemorrhagic lesion volumes following rmTBI. A) SWI showed the appearance of greater hemorrhage (arrows) in animals who received rmTBI 3d apart compared to 7d apart; B) Quantitative SWI revealed there were significantly increased lesion volumes over 14d when the second mTBI was induced at 3d after the initial impact, which was not observed in the rmTBI 7d apart group. *p<0.05 vs Sham; †p<0.05 vs rmTBI 7d apart group; &p<0.05 vs 1st MRI.

Similar to the T2WI profiles at the final imaging time point, we also observed a significant increase in hemorrhagic lesion volumes at 24hrs after the second injury (Fig. 4), suggesting that shorter intervals (3d) between two mTBI events render the brain more susceptible to bleeding. White matter integrity can be non-invasively assessed using diffusion tensor imaging (DTI), in which water moves preferentially along intact fiber bundles (Obenaus 2007).

A serendipitous finding of this model was an observed mismatch between cerebral blood flow (CBF) and metabolism after mTBI as revealed by SWI (See details in Appendix- Barnes S, 2010 NNS Poster). SWI, as noted above, is uniquely sensitive to deoxygenated blood as well as extravascular blood. Thus, deoxygenated blood in vessels (i.e. veins) can be readily observed. We found a prominent increase in the number and total vessel length of veins between ipsilateral and contralateral hemispheres after the first mTBI in some animals (5 out of 19 rats). This venous asymmetry was not only localized to the area of the impact but was also observed to extend over the entire hemisphere in a rostro-caudal extent (Fig. 5). Longitudinal evaluation (2-3 days later) post injury found that the venous asymmetry was still present (n=2).

The observed venous asymmetry appears to be related to the amount of intracerebral hemorrhage seen on SWI (Fig. 6). Both small and large hemorrhage volumes showed low observed rates of asymmetric veins, whereas moderate hemorrhage showed a very consistent increase in the venous asymmetry. A similar trend was observed with T2 lesion volumes but was not conclusive.

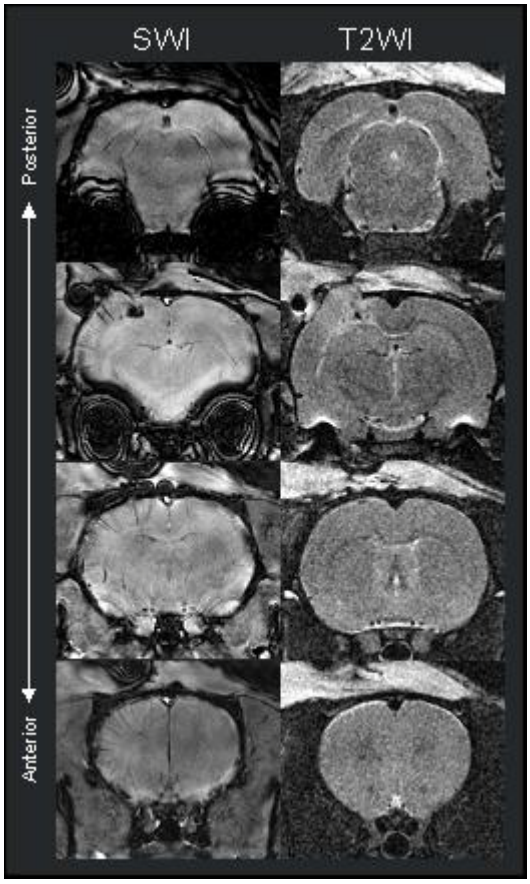

Fig. 5 A unique observation was that using SWI we found a venous asymmetry that extended over the whole hemisphere from anterior to posterior in the injured brain after the 1st impact. Each row represents approximately a 3.3 mm anterior step.

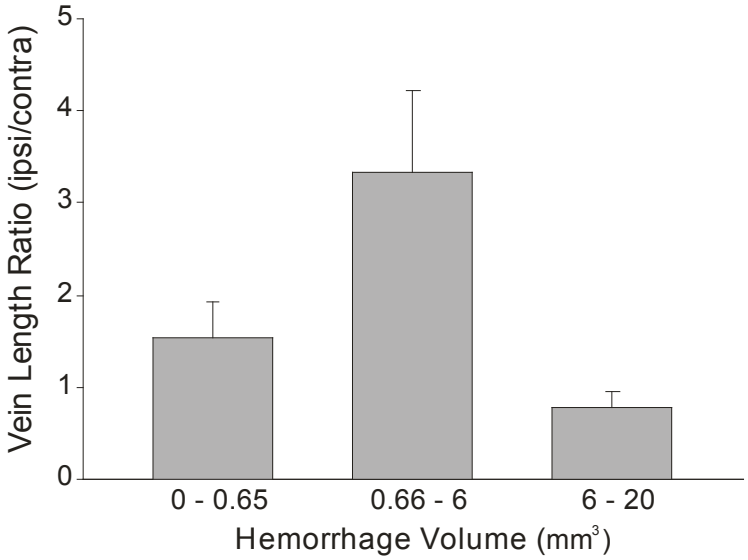

Fig. 6 Hemorrhage volume appeared to influence vein asymmetry. Moderate hemorrhage showed more asymmetry. n=11, 6, and 3, respectively.

These in vivo MRI measures were then validated using histological stains for tissue integrity (cresyl violet, CV) and localization of extravascular blood by staining the presence of iron (Prussian Blue, PB). The lesion size identified by CV staining correlated to the injury severity as defined by in vivo MRI measures, in which the maximum damage was found in brain tissue that received rmTBI at 3d apart (Fig. 7). Similar to the SWI findings, there was more abnormal iron deposition (blood) in animals subjected to rmTBI which was greater in rmTBI with 3d apart compared to Shams and rmTBI 7d apart group (Fig 8). Using a simple quantification method for PB staining revealed a similar severity to the in vivo SWI findings (Fig. 8).

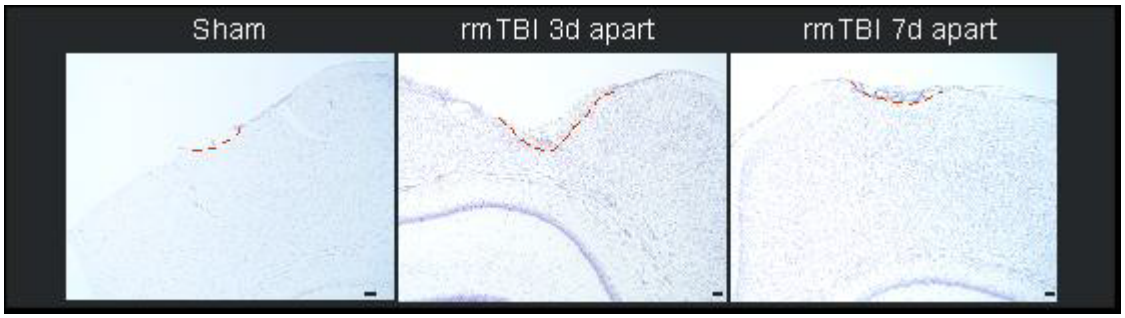

Fig. 7 Cresyl violet (CV) staining at 14 days after the initial mTBI impact. There was an increased lesion area (red dotted line) in 3d apart rmTBI compared to those from the 7d apart group and Shams. These findings were consistent with injury severity assessed by MRI and Prussian blue scoring. Cal bar=100μm

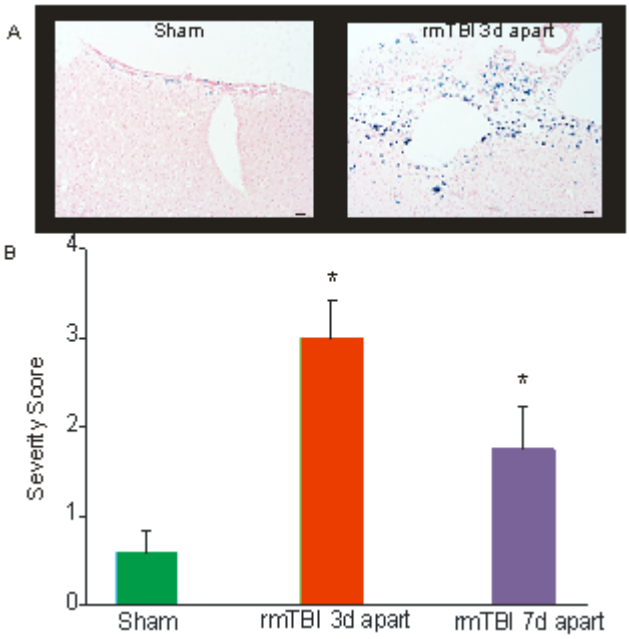

Fig. 8 Prussian blue staining reveled increased iron accumulation at 14 days after the initial mTBI. A) Representative microphotographs showed there was increased Prussian blue staining in brains subjected to rmTBI 3 days apart. Cal bar=100μm; B) Scoring of PB staining confirmed a significant increase in iron deposition within the cortex and corpus callosum of rmTBI groups compared to Shams. Increased iron within tissues was maximum in the rmTBI 3d apart group, suggesting increased hemorrhage and/or bleeding. *p<0.05 vs Sham.

Initial studies evaluating behavioral changes at early times following induction of rmTBI proved to be difficult for a number of reasons including transport to a different building for behavioral testing. In our preliminary studies we found little or no behavioral changes using a variety of measures, including movement initiated escape, turn bias, front and rear paw tape removal tests, open field activity, as well as Morris water maze testing. These preliminary results suggested there were no obvious sensorimotor deficits and cognitive impairments following rmTBI at these early time points.

Several reasons for the lack of behavioral effects could include: i) the very mild injury nature generated in our rat model, ii) the relatively short duration after the mTBI event, as previous studies have shown that behavioral deficits are best observed at later time points, and iii) a relatively small sample size. To test long-term behaviorals change, we extended the testing time point to 3 mo after initial mTBI in a select group of animals from rmTBI 3d apart which showed the largest lesion volumes (Fig. 2 and 4). In this small cohort we observed spatial learning deficits using Morris water maze test (Fig.9) in which rmTBI animals swam longer distances to find a submerged platform.

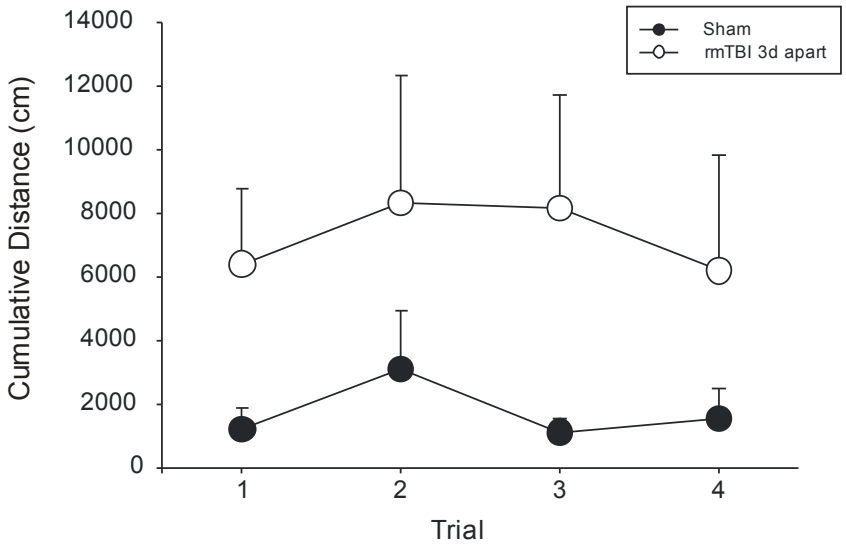

Fig. 9 Morris water maze testing in rmTBI 3d apart animals at 1 mo after the initial impact. rmTBI animals swam for significantly longer distances to find the submerged platform over the 4 trials ($p=0.03$), suggesting the impaired spatial learning memory.

These results have been recently presented (Huang, 2010; Barnes, 2010 NNS Poster).

Summary: The neuroimaging, neurobehavioral and neuropathological profiles of the rmTBI model we have developed provided a strong and valid platform to test our neuroprotective strategies (Aims 2 & 3). Multi-modal MRI is a sensitive monitor for the pathological process in vivo which correlates with histology.

Issues/Limitations: We have not observed any limitations. We originally proposed a battery of early neurological and behavioral tests, but did not observe any significant neurological nor behavioral changes at early post-injury time points. Two outcomes could contribute to this lack of findings: 1) the injury is indeed very mild and our current neurological tests cannot discern any effects of mTBI at the time points we selected in this study. Development of alternate neurological testing paradigms that are more sensitive could uncover neurological deficits. 2) More likely,

however, is that our results suggest that cognitive impairment develops slowly following rmTBI. In future studies, longer time points (months) could be performed for testing the behavioral changes. Indeed, spatial learning deficits were found in a group of rmTBi 3d apart group at 1 mo after the initial impact (Fig. 9).

AIM 2: PROPHALATIC HYPERBARIC OXYGEN PRETREATMENT OF rmTBI

We proposed Hyperbaric Oxygen (HBO) pretreatment to minimize and/or rescue tissues at risk following rmTBI. In our study we treated rats for 1hr/day for 3 consecutive days with 100% oxygen (see methods for details). Previous studies have reported that HBO therapy remains efficacious for up to 12-24hrs after each episode.

Using this experimental paradigm we found several key findings (Figs 10, 11, 12):

1) In animals with HBO pretreatment there were reduced tissue abnormalities at the site of impact,

2) Tissue integrity was improved in all HBO animals both after the first mTBI and after second traumatic event (either 3 or 7d), and

3) At 14d post rmTBI (our final assessment time point) we observed virtually no loss of tissue nor extravascular blood, in contrast to those seen in tissues without HBO pretreatment.

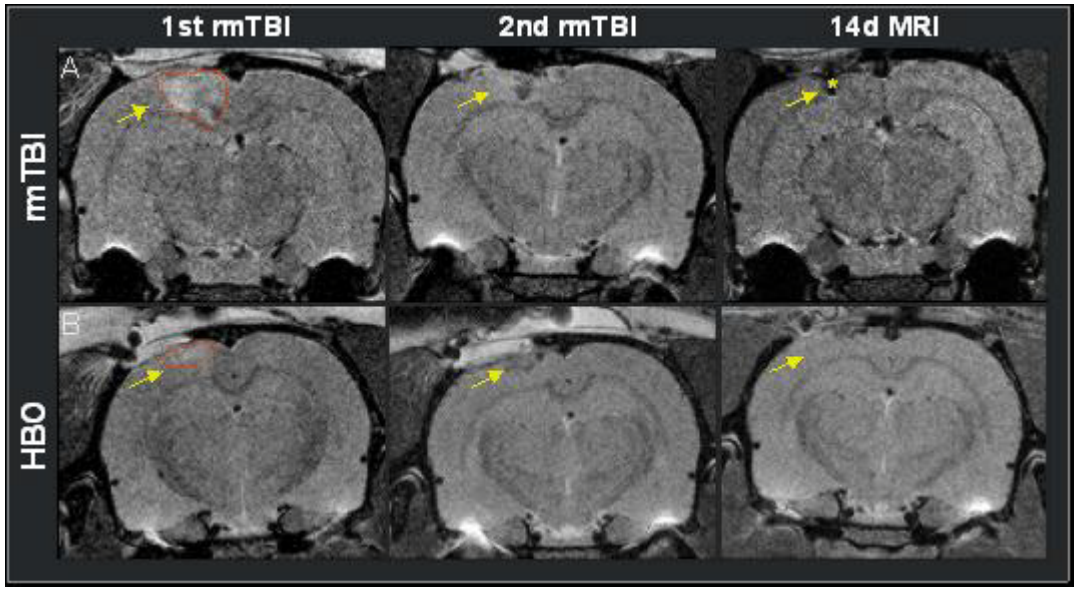

Fig.10 Pretreatment with HBO dramatically rescues tissue at risk following rmTBI. A) rmTBI 3d apart revealed large regions of edema (hyperintensities) with some extravascular blood (hypointensities) on T2WI. Yellow arrows denote the injury site while the red dotted line illustrates the region of tissue abnormality. Over the 14d post-rmTBI there was slow resolution of the edema but significant amounts of extravascular blood remained visible. B) HBO therapy reduced the initial size of the edema (arrow, red dotted line). There were no visible signs of tissue injury after the 2nd rmTBI that was maintained over the 14d time course.

The dramatic ability of HBO to resolve or reduce the amount of extravascular blood within brain tissues is clearly demonstrated in Fig. 11. While there is some blood visible on the SWI images after the 2nd rmTBI, this appears to be primarily on the surface of the brain, compared to blood clearly localized within the brain tissue in rmTBI animals without HBO (Fig 11A).

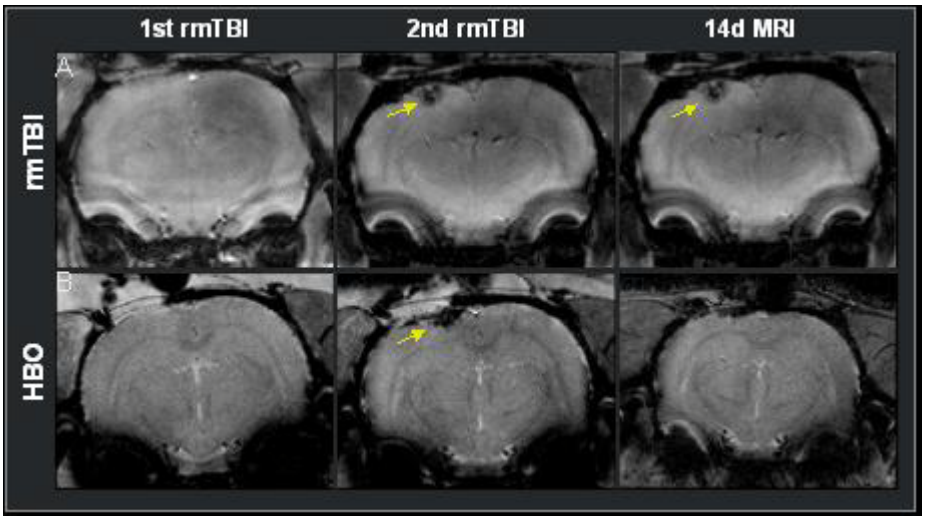

Fig. 11 HBO reduced extravascular blood after rmTBI 3d apart. A) SWI highlighted the location of intra-parenchymal blood after rmTBI which did not resolve over the 14d time course. B) In HBO pretreated animals there was reduced blood visible within the tissues that virtually resolves by 14d.

Quantification of the rmTBI induced lesion volumes at 24hrs after the second hit revealed that there was decreased lesion volume in HBO-treated animals in both the 3d apart and 7d apart groups compared to non-treated animals (Fig. 12A). Similar findings were also found when lesion volume was assessed at 14d (Fig. 12B).

Summary: HBO pretreatment is particularly efficacious in reducing lesion volume, edema associated with rmTBI and extravascular blood.

Issues/Limitations: We have not observed any limitations. HBO pretreatment significantly improved MRI observable outcomes after rmTBI.

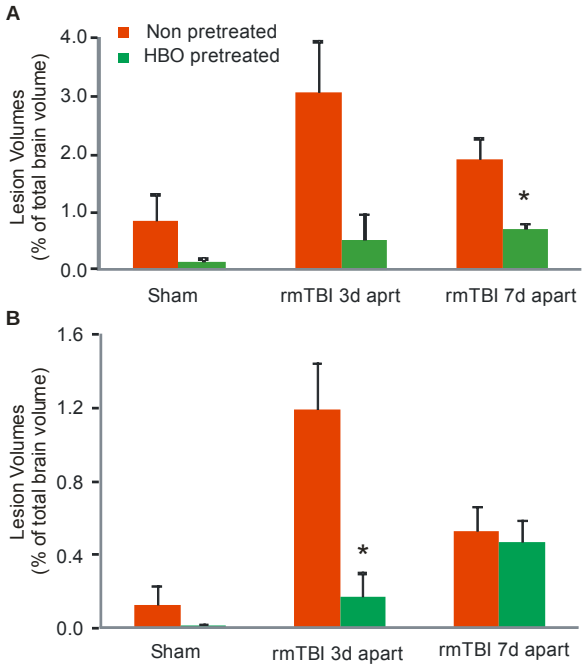

Fig. 12 Lesion volumes derived from T2WI revealed the neuroprotective effects of prophylactic HBO in rmTBI. HBO pretreatment for 3 consecutive days (1h X 3d) prior to rmTBI significantly reduced the lesion volumes at 24hrs after the second mTBI episode (A) and 14d after the initial impact (B). *p<0.05 vs Non-pretreated.

AIM 3: COMBINATORIAL THERAPY (HBO+NICOTINAMIDE) IMPROVES rmTBI OUTCOMES

Based on the efficacious effects of HBO pre-treatment on MR- and histological outcome measures we then undertook a series of experiments to evaluate HBO post-treatment. In addition, use of nicotinamide (NAD) was also proposed to assist tissue recovery by improving tissue metabolic function. Finally, a combinatorial approach of both HBO and nicotinamide together was tested to see if synergistic effects could be obtained by use of both therapeutic approaches.

Using these experimental paradigms we found the following key findings:

1) HBO post-treatment was as effective as pretreatment with HBO. No significant differences were found between the HBO pre- and post-treatment groups. (Figs 13-16)
2) Nicotinamide (NAD), by itself, was also effective in rescuing injured tissues. However, using our outcome measures (lesion volume, etc), NAD did not appear to be as efficacious as HBO. (Figs 17,18)
3) Combined therapy, HBO+NAD was also effective in rescuing rmTBI injured tissues. However, we did not find a statistically significant effect in our outcome measures between single therapy (HBO or NAD) and combinatorial therapy. (Figs 17,18)

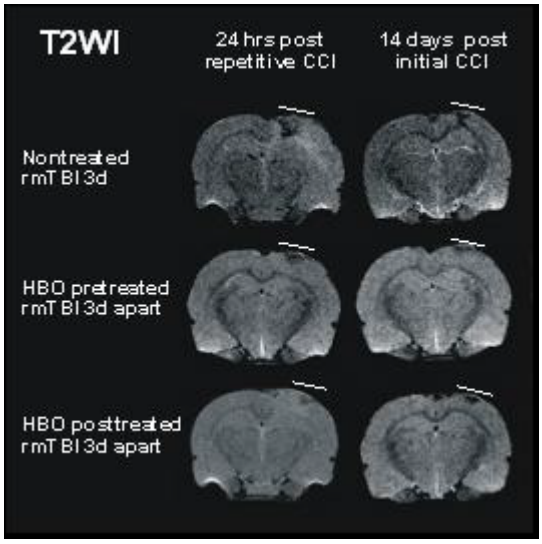

Fig. 13 Representative T2WI images revealed that HBO intervention reduced lesion size following rmTBI 3d apart. HBO pre- and post-treatment protected the brain from cumulative tissue damages including edema (hyperintersity) and hemorrhage (hypointensity) 24 hrs after repetitive injury, respectively. Such neuroprotective effects persisted to 14 days after the initial impact compared to nontreated rmTBI animals. Lines indicate the location of craniotomy.

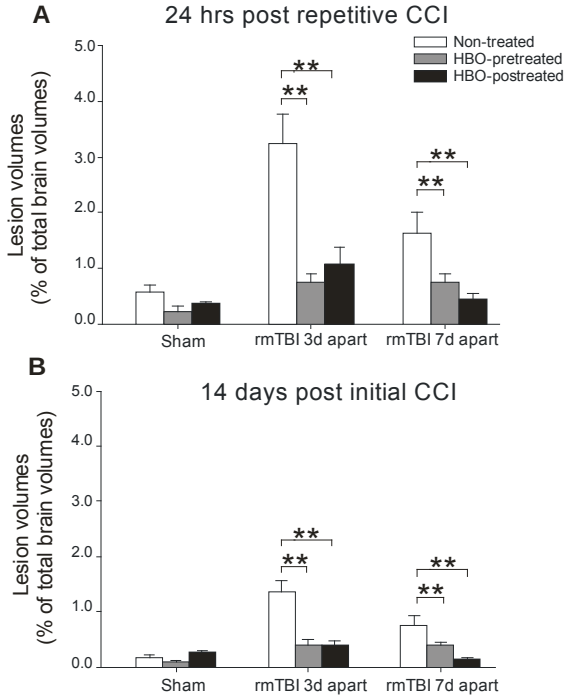

Fig. 14 T2WI-derived lesion volumes after rmTBI. Both HBO pre- or post-treatment significantly decreased cortical lesion volumes 24 hrs in the rmTBI 3d apart group (A), which was still present 14 days after the initial impact (B) compared to non-treated animals. $^{*}p<0.05$, $^{**}p<0.01$ vs non-treated rmTBI 3d apart.

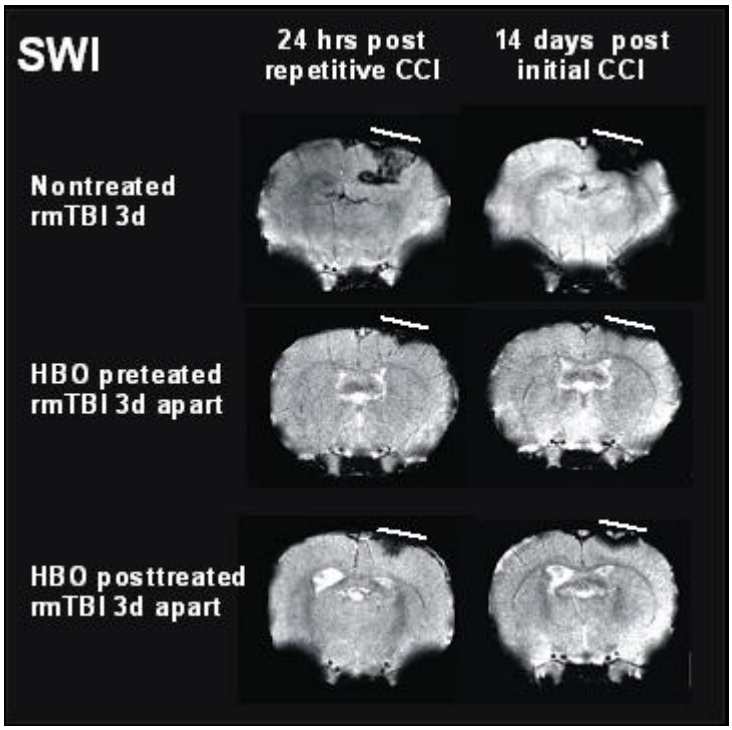

Fig. 15 Representative SWI images revealed that HBO intervention (either pre- or post-treatment) resulted in decreased hemorrhage (hypointensity) within the traumatized brain tissue 24 hrs after repetitive mild CCI in animals subjected to rmTBI 3 days apart. Lines indicate location of craniotomy.

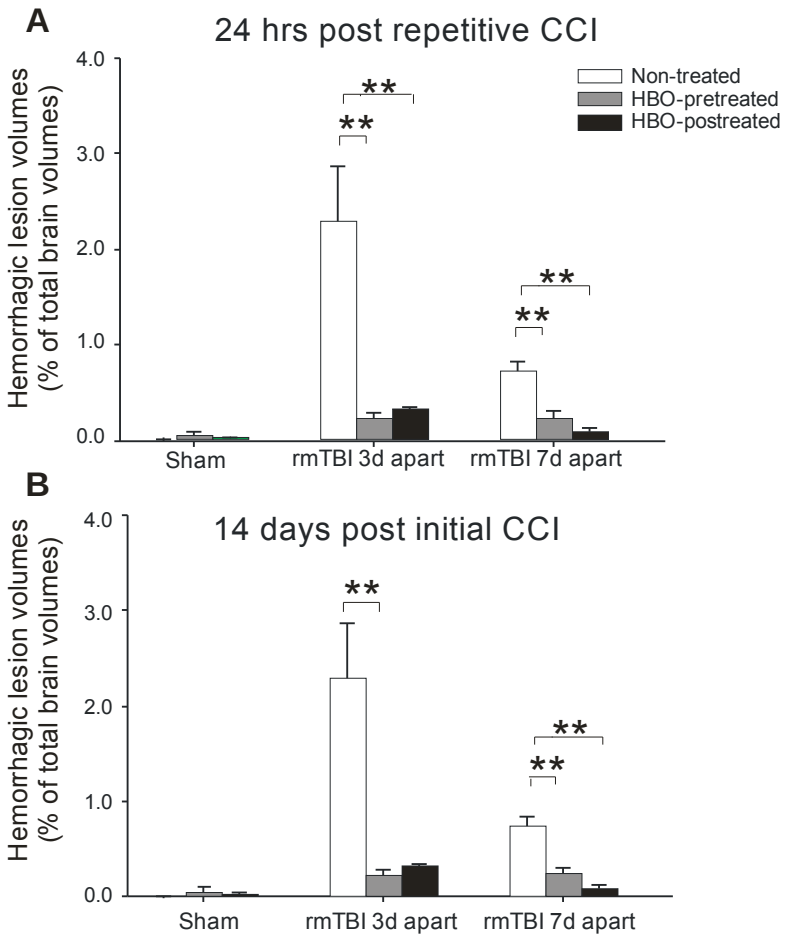

Fig. 16 SWI-derived hemorrhage volumes after rmTBI. Quantifying lesion volumes revealed that both HBO pre- or post-treatment decreased hemorrhagic susceptibility of mild injured brains to repetitive TBI. HBO intervention-induced neuroprotection was most prominent in the rmTBI 3 days apart group, where there was a significantly smaller hemorrhagic lesion volume 24 hrs after rmTBI (A) that was persistent to 14 days after the initial impact (B) compared to non-treated animals. $^{*}p<0.05$, $^{**}p<0.01$ vs non treated rmTBI 3d apart.

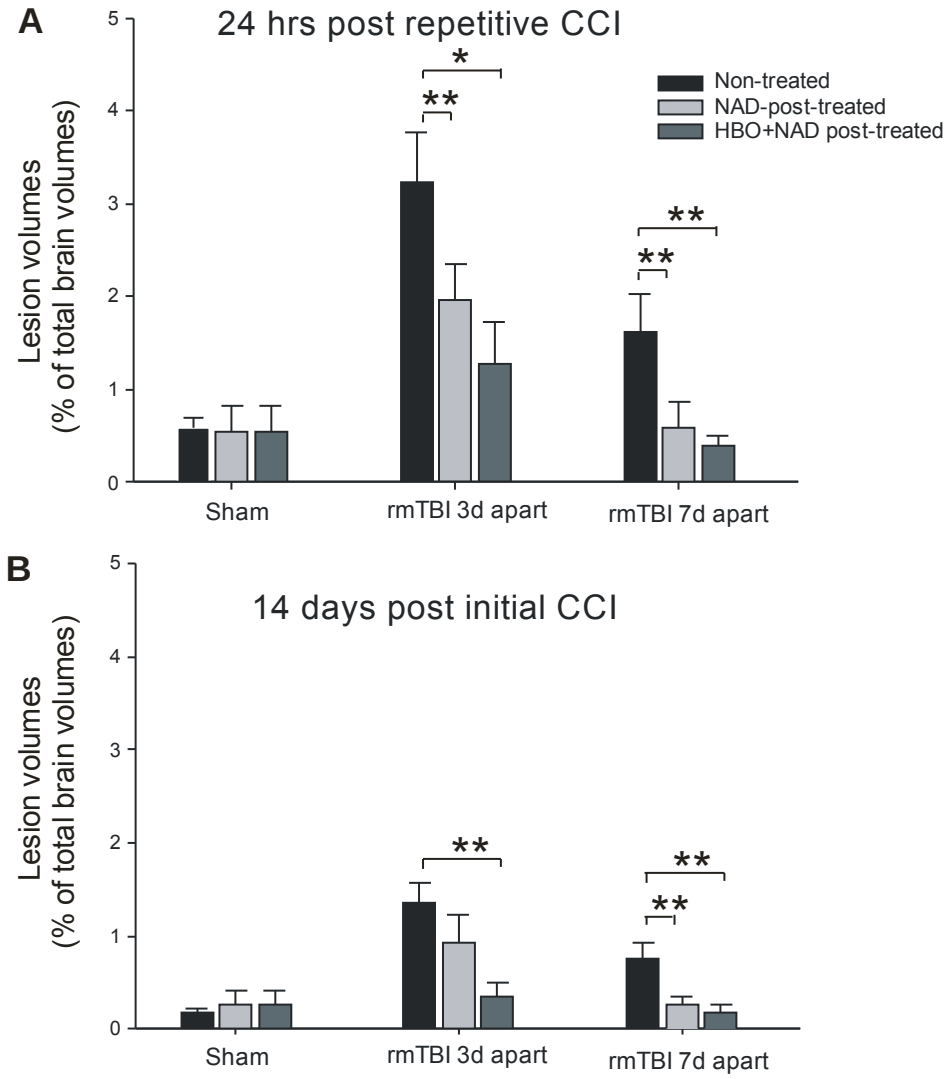

Fig. 17 T2WI-derived lesion volumes after rmTBI. Nicotinamide (NAD) alone or HBO combined with NAD (HBO+NAD) post-treatment significantly decreased cortical lesion volumes 24hrs after a rmTBI (3d apart and 7d apart) compared to non-treated animals (A). These reductions were still present 14 days after the initial impact (B). The HBO combined with NAD was associated with lower lesion volumes compared to NAD alone, but did not reach statistical significance. $^{*}p<0.05$, $^{**}p<0.01$ vs non treated rmTBI 3d apart or rmTBI 7d apart.

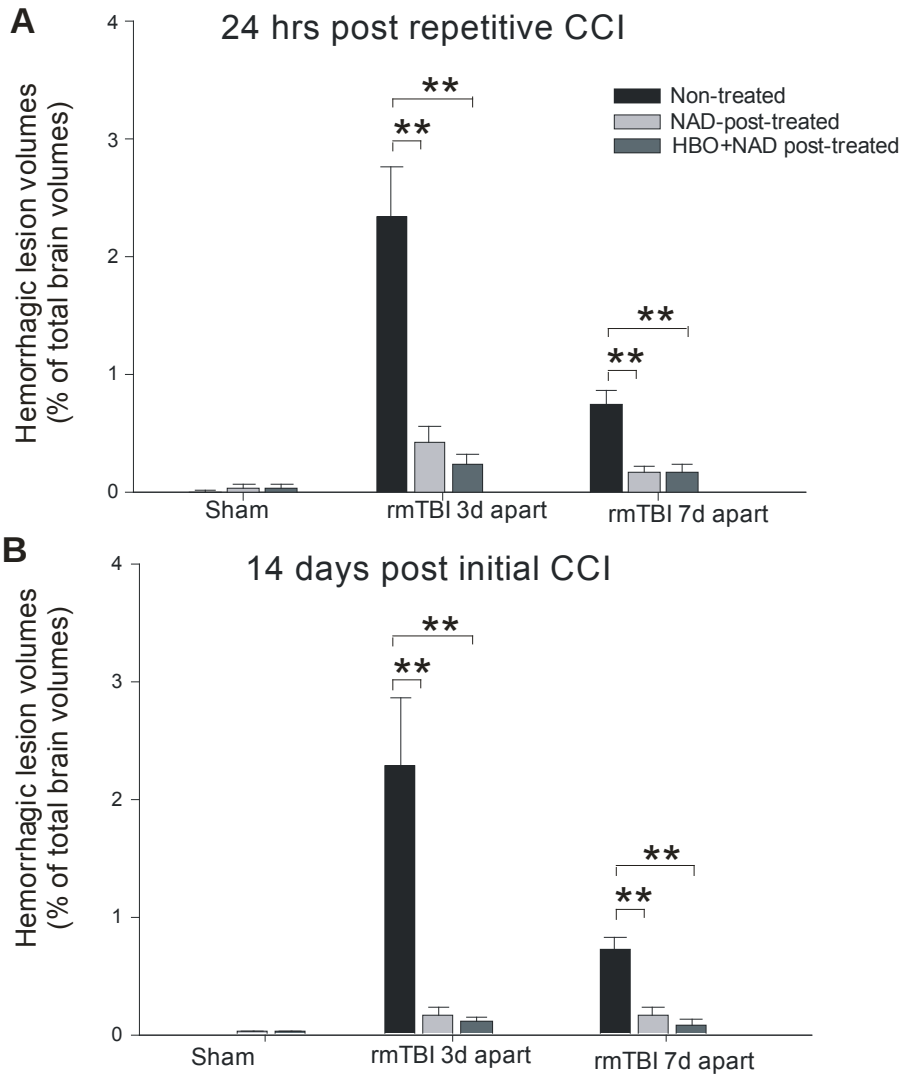

Fig. 18 SWI-derived hemorrhage volumes after rmTBI. Lesion volume assessments revealed that both nicotinamide (NAD) alone or HBO combined with NAD post-treatment decreased hemorrhagic susceptibility in mild injured brains to repetitive CCI at 3 or 7 day apart (A). Neuroprotection was most prominent in rmTBI 3 days apart group, where there was significantly smaller hemorrhagic lesion volume 24 hrs after rmTBI persistent to 14 days after the initial impact (B) compared to non-treated animals. HBO combined with NAD was associated with better neuroprotection than NAD alone, but did not reach statistical significance. $^{*}p<0.05$, $^{**}p<0.01$ vs non treated rmTBI 3d apart or rmTBI 7d apart.

Summary: Single and combinatorial therapies of HBO and NAD were assessed in this Aim. We found that: a) HBO post-treatment was as effective as HBO pretreatment. b) NAD treatment alone was also effective in rescuing injured tissues, but using our outcome measures (lesion volume, etc), NAD was not as efficacious as HBO. c) combined therapy, HBO+NAD, was also effective in rescuing rmTBI injured tissues. However, we did not find a statistically significant effect in our outcome measures between single therapy (HBO or NAD) and combinatorial therapy (HBO+NAD).

Issues/Limitations: No limitations were observed.

KEY RESEARCH ACCOMLISHMENTS

1. Development of model of repeated mild TBI:
    a. After mTBI, the brain is vulnerable to subsequent mTBI events at 3 days post-initial injury in a rat model of controlled cortical impact.
    b. Repeated mTBI 7 days apart did not exhibit as large of a lesion volume
    c. Hemorrhagic lesion volumes were also smaller in the 7day apart group.
2. Demonstration that HBO pretreatment prior to a repeated mTBI:
    a. Prevents the detrimental consequences of either single mTBI or repetitive mTBI events (ie decreased lesion volume).
    b. In animals with HBO pretreatment there were reduced tissue abnormalities at the site of impact,
    c. Tissue integrity was improved in all HBO animals both after the first mTBI and after second traumatic event (either 3 or 7d), and
    d. At 14d post rmTBI (our final assessment time point) we observed virtually no loss of tissue nor extravascular blood, in contrast to those seen in tissues without HBO pretreatment.
3. Single (HBO, NAD) or combinatorial treatment (HBO+NAD):
    a. HBO post-treatment was as effective as pretreatment with HBO. No significant differences were found between the HBO pre- and post-treatment groups.
    b. Nicotinamide (NAD), by itself, was also effective in rescuing injured tissues. However, using our outcome measures (lesion volume, etc), NAD did not appear to be as efficacious as HBO.
    c. Combined therapy, HBO+NAD, was also effective in rescuing rmTBI injured tissues. However, we did not find a statistically significant effect in our outcome measures between single therapy (HBO or NAD) and combinatorial therapy.

REPORTABLE OUTCOMES

Manuscripts: (2 additional in preparation)

Huang L, Obenaus A. Hyperbaric oxygen therapy for traumatic brain injury (review). Medical Gas Research, 2011; 1:21

Huang L, Coats CS, Mohd-Yusof A, Neglerio K, Yin Y, Assad S, Muellner M, Kamper J, Hartman RE, Donovan V, Oyoyo U, Obenaus A. Tissue vulnerability is increased following repetitive mild traumatic brain injury in the rat. Exp Brain Res. 2011; under review

Book Chapters

Obenaus, A., Huang, L., Coats, J., Hartman, R., Badaut, J., Ashwal, S. Animal models of mild pediatric TBI (2011) In: Pediatric Mild Traumatic Brain Injury: From Basic Science to Clinical Management. Editors: Kirkwood, M.W., Yeates, K.O. (in press)

Abstracts:

1. Huang, L., Coats J., Neglerio K., Mohd-Yusof A., Obenaus A. Temporal alterations in lesion volume in a rat model of repetitive mild traumatic brain Injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

2. Coats J., Donovan V., Obenaus A.,Huang L. A contra-lateral model of repeated mild traumatic brain injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

3. Barnes S, Coats J., Huang L., Obenaus A. Mild traumatic brain injury causes unilateral changes in venous blood oxygenation. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

4. L. Huang, J. Coats, A. Mohd-Yusof, A. Obenaus. Hyperbaric oxygen therapy in repetitive mild traumatic brain injury. European Winter Conference on Brain Research, 2011, March 12-19, Les 2 Alpes, France.

5. L. Huang, J. Coats, A. Mohd-Yusof, Y. Yin, A. Obenaus. Hyperbaric oxygen therapy improves neuroimaging outcomes in a rat model of repetitive mild traumatic brain injury. National Neurotrauma Symposium, 2011. July10-13

Personnel Paid by this Research Effort:

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Lei Huang, M.D.

Jacqueline Coats, B.S.

CONCLUSIONS

This completed research proposal was able to demonstrate that a single mild TBI renders the brain vulnerable to subsequent mild TBI event. The period of vulnerability appears to be greater when the mTBI events occur 3 days apart as compared to 7 days apart. This would suggest that the brain tissue has had time to "heal" itself or prepare itself for the next mTBI event. Investigation into the mechanisms underlying this vulnerability were not proposed in the current proposal but should be investigated in the future. A clue to potential mechanisms could be changed vascular and metabolic function, as we found in Aim 1.

HBO has been used for neuroprotection of brain tissues in a variety of injury models, including stroke, ischemia, burns etc. We investigated the effects of HBO pretreatment as a potential therapeutic approach to repeated mild TBI. Treatment of rodents with HBO prior to repeated mTBI (3 or 7 days apart) resulted in significantly reduced lesion volumes and decreased extravascular blood. Thus, in situations where the potential for TBI exisits (sports, military exercises etc) pre-treatment with HBO could be considered as a pretreatment strategy.

We also examined if HBO treatment given shortly after repeated mTBI would be neuroprotective. Interestingly, treatment with HBO after repeated TBI (both 3 and 7 days apart) resulted in significant decrements in lesion volume and extravascular blood. Furthermore, there were statistical significant differences between study HBO groups (pre-vs. post-treatment) that were treated prior or following repeated mTBI. As has been previously reported, the long duration of HBO effects (up to 12-24hrs after a single treatment) are likely potential mechanisms that rescue TBI tissues. The mechanism(s) responsible for these results still require elucidation.

NAD, a compound known to enhance metabolic function, was also tested in repeated mTBI. Our hypothesis that we were testing was that improved metabolic substrates should improve metabolic function in contused tissues. Our findings validated our hypothesis. NAD significantly improved lesion volumes and decreased extravascular blood in both study groups (3 and 7 days apart). While there were no statistically significant differences between HBO and NAD in their ability to reduce lesion volume or the volume of extravascular blood, it was clear that NAD was not as efficacious as HBO in rescuing injured tissues. The mechanism(s) by which NAD rescues injuried tissues has not been investigated and future work should address this question.

Our final intervention was to assess if combined HBO and NAD would be the most effective treatment paradigm. Combined HBO+NAD did result in significant lesion volume and volume of extravascular blood reductions. But of importance is that the reductions in our outcome measures were not significantly different from either HBO or NAD treatments alone. This lack of significance of combined therapy compared to single treatments could be due to timeing of treatments. We believe that this is particularly true for NAD, where initial treatment with HBO followed by a later treatment (ie at 3 days) with NAD may likely be more beneficial in rescuing and improving neuronal function following mTBI.

In summary, we have : a) developed a novel model of repeated mTBI, b) single treatments of either HBO or NAD were effective in reducing MRI- and histological-derived measures of brain injury and c) combined treatments of HBO+NAD were also effective in reducing brain injury. Future work is required to elucidate the mechanisms underlying these changes.

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APPENDICES

Methods:

Detailed methods in support of the final report.

Meeting Abstracts:

Huang, L., Coats J., Neglerio K., Mohd-Yusof A., Obenaus A. Temporal alterations in lesion volume in a rat model of repetitive mild traumatic brain Injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

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Coats J., Donovan V., Obenaus A.,Huang L. A contra-lateral model of repeated mild traumatic brain injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

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Huang L, Obenaus A. Hyperbaric oxygen therapy for traumatic brain injury (review). Medical Gas Research, 2011; 1:21

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Huang L, Coats CS, Mohd-Yusof A, Neglerio K, Yin Y, Assad S, Muellner M, Kamper J, Hartman RE, Donovan V, Oyoyo U, Obenaus A. Tissue vulnerability is increased following repetitive mild traumatic brain injury in the rat. Exp Brain Res. 2011; under review

Methods:

Sprague Dawley adult male rats (2 mo old) were randomized into 10 groups (n=6 each) with two episodes of mTBI: 1) 3 days apart; 2) 7 days apart; 3) sham; 4) 3 days apart pre-treated by HBO; 5) 7 days apart pre-treated by HBO; 6) 3 days apart post-treated by HBO; 7) sham pre-treated by HBO; 8) 3 days apart post-treated by HBO; 9) 7 days apart post-treated by HBO; and 10) Sham post-treated by HBO. Study design is shown in Fig.1 below.

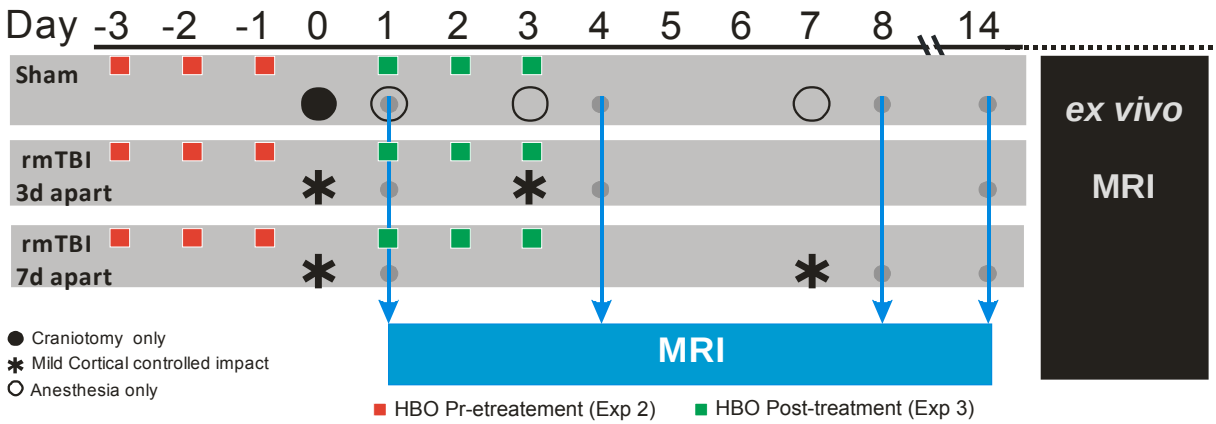

Fig.1 The first mTBI was induced at day 0, followed by a second repetitive mTB at 3 days later for the group of rmTBI 3d apart, at 7 days later for the group of rmTBI 7d apart respectively. Sham animals I underwent the identical procedure except for the delivery of impact injury. For pre-treatment, animals underwent HBO for 3 days in prior to the initial mTBI; HBO post-treatment was administered at 24 hrs after the first mTBI for 3 days in a separate groups of animals. At 24 hrs after each impact and 14 days after the initial mTBI, MRI including T2 weighted imaging (T2WI) and susceptibility weighted imaging (SWI) was acquired. Ex vivo diffusion tensor imaging (DTI) was performed on the fixed brains.

Rat model of repetitive mTBI

Rats were anesthetized with isoflurane (3% induction, 2% maintenance) and the head fixed onto a rat stereotaxic frame. After incision of the skin, a 4.5 mm craniotomy was performed at 4 mm posterior and 3.5 mm lateral to bregma. Care was taken to prevent disturbing the underlying dura and minimizing bleeding at the craniotomy site. A mild controlled cortical impact (CCI) was delivered using an electromagnetic driven piston (0.5 mm depth, 4 mm diameter tip at 6.0 m/s, 200 ms dwelling duration). A second identical impact was delivered at 3 or 7 days after the first CCI event at the same location. Randomized sham animals underwent the same surgical procedure without CCI.

HBO Treatment

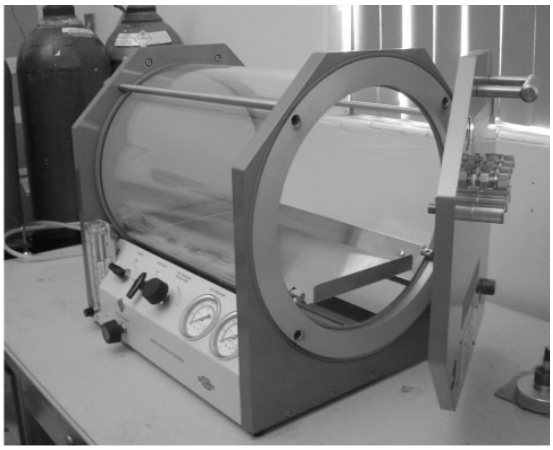

Fig. 2 HBO chamber for small animals. HBO pre-treatment or post-treatment was administered at 2 ATA for 1 hour per day for the total of 3 days.

MRI data collection and analysis

At 24 hr after each CCI and 14 days after the initial impact, in vivo MR data was collected on a Bruker Advance 4.7T MR (Bruker Biospin, Billerica, MA). A T2WI sequence was acquired with TR/TE of 3453 ms/20 ms, 3 cm of field of view (FOV), 25 slices. An SWI sequence was also acquired withTR/TE/Flip angle of 39 ms/20 ms/20, 3 cm of FOV and 48 slices. High resolution ex vivo DTI data were acquired using a Bruker Advance 11.7T (Bruker Biospin, Billerica, MA). A spin echo diffusion sequence withTR/TE of 552.5/15.1 was acquired for a total of seven directions with two b values (43.34 and 2013.46s/ mm2), 2 cm of FOV. All MRI data were collected with matrix of 256X256.

Using Cheshire imaging processing software (Hayden Image/Processing Group, Waltham, MA), the lesion volumes were obtained (including abnormal both hyper- and hypo-intensity) on T2WI images; microstructural integrity of ipsilateral corpus callosum (CC) was analyzed on DTI images at the level of maximal injury (M).

SWI magnitude and phase data were post-processed for high pass filter phase and SWI images using in-house software (Spin). The lesion hypointensity was drawn semi-automatically within cortex and subcortical white matter on each slice and manually checked. Total hemorrhagic volumes were calculated over all slices.

Statistics

One-way ANOVA analysis was performed to statistically examine quantitative measures. Significance was accepted at $p<0.05$. Data were presented as Mean+SEM.

Meeting Abstracts:

Huang, L., Coats J., Neglerio K., Mohd-Yusof A., Obenaus A. Temporal alterations in lesion volume in a rat model of repetitive mild traumatic brain Injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

Abstract: (Your abstract must use Normal style and must fit into the box. Do not enter author details)

Introduction: Repetitive mild traumatic brain injury (mTBI) is an important problem for active military personnel and sport players. Exacerbation of tissue damage and psychosocial outcomes has been documented in repetitive mTBI. In the present study, we characterized the neuropathological profiles of a rat model of repetitive mTBI where the first mild impact was then followed by a second mTBI at intervals of 1, 3, 7 days using non-invasive magnetic resonance imaging (MRI).

Methods: Twenty-four Sprague Dawley adult male rats (2 mo) were randomized into 4 groups with two episodes of mTBI: 1) 1 day apart; 2) 3 day apart; 3) 7 day apart; 4) shams. A mild controlled cortical impact (CCI) was delivered by an electromagnetic driven piston (0.5 mm depth, 4 mm diameter tip at 6.0 m/s, 200 ms duration) at 3 mm posterior and 3 mm lateral to bregma. A second identical impact was induced at 1, 3 or 7 days after the first CCI event. Sham animals underwent the same surgical procedure without CCI. T2 weighted imaging (T2WI) was acquired at 24 hrs after each impact and a final MRI was acquired at 14 day post initial injury. Prussian blue staining was performed on 4%PFA fixed brain tissues.

Results: There were no significant differences in lesion volumes among the three mTBI groups at 24 hrs after 1st CCI. In repetitive mTBI groups of 1 and 3 days but not 7 days apart, a second CCI resulted in increased T2WI lesion volumes at 24 hrs after the 2nd impact that persisted until 14d. The lesion signal intensity patterns also varied among groups with the 1 day apart group being hyper-intense (edema); the 3 day apart exhibiting both hyper- (edema) and hypo-intensities (blood) and the 7day apart group being primarily hypo-intense consistent with increased extravascular blood deposition. Prussian blue staining showed evidence of accumulated iron

Barnes S, Coats J., Huang L., Obenaus A. Mild traumatic brain injury causes unilateral changes in venous blood oxygenation. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

Title:

MILD TRAUMATIC BRAIN INJURY CAUSES UNILATERAL CHANGES IN VENOUS BLOOD OXYGENATION

Abstract: (Your abstract must use Normal style and must fit into the box. Do not enter author details)

Introduction: Susceptibility weighted imaging (SWI) magnetic resonance imaging (MRI) is sensitive to levels of deoxygenated haemoglobin. Increased vascular contrast could indicate less oxygenated venous blood, enlarged veins, or both. We evaluated a novel finding where Sprague Dawley rats that were subjected to a mild traumatic brain injury (mTBI) following a single controlled cortical impact (CCI) were noted to have increased numbers of prominent veins on the ipsilateral brain hemisphere.

Methods: A 5 mm craniotomy was performed, immediately followed by a mild 3mm CCI injury: 6m/s velocity, 200ms dwell time, and 0.5 mm depth. MRI was performed on the animals 24 hours after impact. Nine shams (no CCI) and 12 CCI rats were evaluated. Seven of the animals also underwent 18-FDG positron emission tomography (PET).

Results: A prominent increase in the number of veins was observed between ipsilateral and contralateral hemispheres in 9 of the 12 CCI animals. The volume of the hypointense lesion was measured and the animals were given a haemorrhage score according lesion volume: small (0 – 650nl), moderate (651nl – 6500nl), or large (6501nl - 2000µl). The venous mismatch was observed in 27% of animals with a small haemorrhage (n=11), 86% of animals with a moderate haemorrhage (n=7) and 0% of animals with a large haemorrhage (n=3). Longitudinal evaluation (2-3 days later) post injury found that the venous effect was still present (n=2).

Conclusion: The venous mismatch appeared to be directly related to the amount of intracerebral haemorrhage seen on SWI. Both small and large haemorrhage scores showed low observed rates of prominent veins, moderate scores showed a very

Coats J., Donovan V., Obenaus A.,Huang L. A contra-lateral model of repeated mild traumatic brain injury. Annual meeting of National Neurotrauma Society, June 14-17, 2010, Las Vegas, California (Poster Presentation)

A CONTRA-LATERAL MODEL OF REPEATED MILD-TBI

Abstract: (Your abstract must use Normal style and must fit into the box. Do not enter author details)

Successive mild traumatic brain injury (mTBI) sustained in different brain regions often exacerbates neuropathology and psychological outcomes. An animal model designed to imitate this varied pathophysiology would greatly facilitate translational research. In this study we explored a mTBI model in which cortical impacts were induced days apart on opposite sides of the brain and evaluated the evolution of the lesions with magnetic resonance imaging (MRI).

We randomized Sprague Dawley rats (2-3 mo) into: 1) repeated mTBI groups where two episodes of mTBI were delivered either with a 3 day or a 7 day intervals; 2) mTBI control group with only one impact. We used an electromagnetically driven piston (4mm tip) to deliver a controlled cortical impact (CCI) (0.5 mm depth, 6.0 m/s, 200ms dwell) first to the right cortex 3mm posterior and 3mm lateral to bregma. The second CCI was delivered with the same coordinates and parameters but on the left cortex at 3 or 7 day intervals. MRIs acquired 24hrs after impact included susceptibility weighted imaging (SWI) and T2 weighted imaging (T2WI). A final MRI was acquired immediately before brain fixation 14 days after the first CCI. Cresyl Violet and Prussian blue iron stains were used to evaluate histopathology.

T2WI revealed mild to moderate cortical edema following the first and second impacts in both the 3 and 7day groups. On SWI, mild to moderate cortical hemorrhage was observed after all 1st impacts (right cortex). The second impact (left cortex) delivered at the 3-day interval increased hemorrhage within the initial mTBI lesion in the right cortex.

A repeated mTBI model involving both brain hemispheres may exhibit clinically

L. Huang, J. Coats, A. Mohd-Yusof, A. Obenaus. Hyperbaric oxygen therapy in repetitive mild traumatic brain injury. European Winter Conference on Brain Research, 2011, March 12-19, Les 2 Alpes, France.

Introduction

Repetitive mild traumatic brain injury (rmTBI) is an important public health concern as subsequent injuries can exacerbate existing neuropathology that leads to long-term deficits. In the absence of evident structural damage, the pathophysiological processes of mTBI likely involve cellular, molecular and metabolic perturbations in the traumatic brain, leading to functional disturbances. Thus, a neuroprotective approach should improve the balance between oxygen delivery and demand, in favor of cerebral aerobic metabolism. Hyperbaric oxygen (HBO) fulfills this requirement and has been explored as a novel therapeutic approach for management of TBI. In this study, we investigated the prophylactic HBO strategy (pretreatment) in a rat model of rmTBI.

Methods and Materials

Repetitive mTBI (rmTBI): Sprague Dawley adult male rats (2 mo old) were randomized into Sham, 3d and 7d rmTBI groups. The cortical surface of the brain was exposed via a 5mm craniotomy (3mm posterior and 3mm lateral to bregma). A controlled cortical impact (CCI, 4mm tip diameter, 0.5mm depth at 6.0 m/s with a 200ms dwell time) was performed on the cortical surface with an electromagnetically driven piston. For animals receiving rmTBI, the second CCI was delivered with identical impact parameters at the same location 3 or 7 days later. Sham animals underwent the same surgical procedures without CCI. For preconditioning, HBO (100% oxygen at 2 ATA) was given 1hr daily for 3 consecutive days prior to the initial impact. Neuroimaging and Analysis: T2 weighted imaging (T2WI) and susceptibility weighted imaging (SWI) were acquired on a 4.7T MRI (Bruker Biospin) at 24 hrs after each impact and a final MRI was acquired at day 14 after the initial injury. T2WI derived lesion volumes and SWI-identified hemorrhage volumes were obtained using Cheshire and Spin imaging processing software. Tissue histology: Immuno-histochemistry was performed to assess Glial Fibrillary Acidic Protein (GFAP) staining for astrocytes and Ionized Calcium Binding Adaptor Molecule 1 (IBA1) for microglia.

Results

In animals with HBO pretreatment there were reduced tissue abnormalities at the site of impact. Tissue integrity was improved in all HBO animals both after the first and second mTBI (either 3 or 7d). At 14d post rmTBI (our final assessment time point) we observed virtually no loss of tissue nor extravascular blood, in contrast to those seen in tissues without HBO pretreatment. Quantification of the rmTBI induced lesion volumes at 24hrs after the second hit revealed that there was decreased lesion volume in HBO-pretreated animals in both the 3d and 7d apart groups compared to non-treated animals. Similar findings were also found when lesion volume was assessed at 14d.

Conclusions

HBO pretreatment significantly improved outcomes following rmTBI. It provides a potential neuroprotective strategy that can be applied to victims at high risk for repetitive mTBI. MRI is a sensitive neuroimaging biomarker for monitoring the treatment effects that can be rapidly translated into the military, sports and clinical arenas.

Funding: This work was supported by the Department of Defense, DCMRP#DR080470

L. Huang, J. Coats, A. Mohd-Yusof, Y. Yin, A. Obenaus. Hyperbaric oxygen therapy improves neuroimaging outcomes in a rat model of repetitive mild traumatic brain injury. National Neurotrauma Symposium, 2011. July10-13

Purpose

Repetitive mild traumatic brain injury (rmTBI) is an important public health concern as subsequent injuries are thought to exacerbate existing neuropathology. The pathophysiological processes underlying mTBI likely involve cellular metabolic perturbations in the injured brain and a neuroprotective approach favoring cerebral aerobic metabolism could be beneficial. Hyperbaric oxygen (HBO) fulfills this requirement and has been explored as a novel therapeutic approach for management of TBI. In this study, we investigated both prophylactic and therapeutic HBO strategies in a rat model of rmTBI.

Methods

Sprague Dawley adult male rats were randomized into six groups: Sham, 3d and 7d rmTBI with or without HBO. A mild controlled cortical impact (0.5mm depth) was delivered to the parietal cortex. For animals receiving rmTBI, a second CCI was delivered at the same location 3 or 7 days later. Shams had no CCI. HBO (100% oxygen at 2 ATA) was given 1hr daily for 3 consecutive days either prior to or 24 hrs post the initial TBI. T2 weighted imaging (T2WI) and susceptibility weighted imaging (SWI) were acquired and lesion and hemorrhage volumes were quantified. .

Results

Both HBO pre-treatment and post-treatment improved neuroimaging outcomes following rmTBI in contrast to those seen in tissues without HBO intervention. There were significant reductions in the T2WI-dervided lesion and SWI-identified hemorrhage volumes at 24 hrs post rmTBI. The most dramatic HBO treatment effects were observed in animals receiving rmTBI 3d apart where there was a 3-fold decrease hemorrhage volumes back to Sham levels.

Conclusions

Both prophylactic and therapeutic HBO provides a potential neuroprotective strategy that can be applied to victims at high risk for repetitive mTBI. MRI is a sensitive neuroimaging biomarker for monitoring experimental and clinical treatment effects within the setting of rmTBI.

MEDICAL GAS RESEARCH

REVIEW Open Access

Hyperbaric oxygen therapy for traumatic brain injury

Lei Huang[1] and Andre Obenaus[1,2,3,4,5*]

Abstract

Traumatic brain injury (TBI) is a major public health issue. The complexity of TBI has precluded the use of effective therapies. Hyperbaric oxygen therapy (HBOT) has been shown to be neuroprotective in multiple neurological disorders, but its efficacy in the management of TBI remains controversial. This review focuses on HBOT applications within the context of experimental and clinical TBI. We also discuss its potential neuroprotective mechanisms. Early or delayed multiple sessions of low atmospheric pressure HBOT can reduce intracranial pressure, improve mortality, as well as promote neurobehavioral recovery. The complimentary, synergistic actions of HBOT include improved tissue oxygenation and cellular metabolism, anti-apoptotic, and anti-inflammatory mechanisms. Thus HBOT may serve as a promising neuroprotective strategy that when combined with other therapeutic targets for TBI patients which could improve long-term outcomes.

Keywords: intracranial pressure, metabolism, apoptosis, inflammation, tissue oxygenation, cerebral blood flow

Introduction

Hyperbaric oxygen therapy (HBOT) is a treatment by which 100% oxygen is administered to a patient at a pressure greater than atmospheric pressure at sea level (i.e. one atmosphere absolute, ATA) [1]. The increased partial pressure of oxygen (pO_2) within the blood and subsequent improved mitochondrial metabolism/tissue oxygenation constitutes the net effect of HBOT [2-6]. Given that the dissolved oxygen content in the plasma increases linearly after hemoglobin is 100% saturated [7,8], plasma bound oxygen can be used more readily than that bound to hemoglobin which enables tissue oxygen delivery even in the absence of red blood cells [7,9].

Thus, HBOT induces a much larger oxygen-carrying capacity in the blood that dramatically increases the driving force of oxygen diffusion to tissues. Although HBOT-induced cerebral vasoconstriction appears to be undesirable within the context of ischemic conditions [10,11] this may not be necessarily deleterious due to increased oxygen availability to injured tissues. HBOT may also counter vasodilation of the capillaries within hypoxic tissues, thereby minimizing collection of extravascular fluids (edema) which ultimately reduces brain vasogenic edema and the ensuing decrease in intracranial pressure (ICP) [5,12-14].

Emerging evidence has shown the neuroprotective effects of HBOT in a range of multiple injuries and/or disorders (Additional file 1, Table S1) [15]. The most common clinical applications include decompression sickness, carbon monoxide poisoning, minimization of radiation therapy induced tissue damage and enhancing skin grafts [1,16], which are all covered by insurance/Medicare. There are numerous "unapproved" uses of HBOT that focus on more complex neurological disorders, including autism, multiple sclerosis and stroke, which have shown promising results in experimental settings, but clinical efficacy is still elusive. Recent efforts have applied HBOT to traumatic brain injury [5,14,17]. While significant research on HBOT therapy has been undertaken (> 10,000 citations on PubMed), very little has been reported for HBOT within the setting of TBI (< 30 citations). We now briefly review the experimental and clinical HBO research relevant to TBI.

HBOT in animal models of TBI

Early experimental research focused on the effects of HBO on brain edema, ICP and cerebral blood flow

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(CBF). Dunn and colleagues first demonstrated the neuroprotective effects of hyperoxia in a dog freeze-lesion model of brain injury that simulated a brain contusion. Hyperoxia significantly improved outcomes by reducing mortality [18]. Reduced ICP (30% decrease) and CBF (19%) were also reported in a dog model of brain injury treated by HBOT (2 ATA for 4 hrs) [19]. The absence of changes in cerebrospinal fluid (CSF) lactate, a marker of brain injury, following HBOT further supported the notion that HBO improved tissue oxygen delivery despite the undesirable decrease in CBF subsequent to vasoconstriction [10]. Expanding on the original model, various methods (psyllium seed, extradural balloon) were used to induce brain edema in dogs followed by HBOT [20-23]. Using HBOT at 3 ATA for 45 min [20,21] or at 2 ATA for 4 hrs [22] resulted in a significant decrease (> 50%) in mortality relative to non-treated injured animals. They also reported significantly less brain edema [20] and reduced cisternal CSF pressure [21] in the HBOT groups. Sukoff and colleagues suggested that the improvement seen in their model was due to the effectiveness of HBOT against ischemia, secondary to the induced cerebral edema [21]. Hayakawa et al, however, found that HBOT (3 ATA for 1 hr) did not or barely changed CSF pressure and CBF in most injured dogs [23].

In a rat model of moderate fluid percussion injury, the neuroprotection afforded by HBOT translated into long term cognitive improvements, characterized by a shorter latency to find a hidden platform in Morris water maze (MWM) performance [2]. Within brain tissues, HBOT showed significant protection against hippocampal neuronal loss compared to normobaric oxygen treatment [2]. Importantly, there was no increased free radial peroxide and peroxynitrite production, suggesting the absence of oxygen toxicity after HBOT [2]. Studies in a model of cerebral ischemia concurred that HBOT did not exacerbate lipid peroxidation [24].

The aforementioned neuroprotective efficacy of HBOT was all achieved when intervention was administered during the acute phase (within hours) of post-TBI. The prolonged therapeutic time window of HBOT was further investigated in studies using a focal cortical weigh-drop model of TBI [25-27]. Wang and colleagues have demonstrated that multiple HBOT (3 ATA hourly for 3 or 5 days), delivered at latest 2 days post-injury resulted in significantly reduced overall neurological deficit scores and neuronal apoptosis within brain tissue. But the authors also showed that twelve hours post-TBI is the latest effective window for neuroprotection when a single episode of HBOT was delivered [27]. Moreover, Harch et al [25,26] have tested the effects of low pressure HBOT (90 min twice a day at 1.5 ATA) which started at 50 days after the initial brain injury for a total duration of 40 days. At end of the treatment (100 days post injury), MWM spatial learning performance in the HBOT groups improved significantly and was highly correlated with increased ipsilateral hippocampal blood volume (cerebrovascular density) measured by diaminobenzadine blood stain [25,26]. Given the well-described presence of angiogenesis in HBOT in other brain injury models [28,29], the authors suggested that angiogenesis was the most likely explanation for the HBOT-induced recovery of function. They claimed that coupling of "blood flow and metabolism" and "metabolism and function" were potential mechanisms, as both were increased in animals receiving HBOT. This hypothesis is consistent with the pattern of HBOT-induced increases in blood flow seen on single photon emission computed tomography brain imaging in patients with chronic TBI [30,31]. A caveat is that HBOT failed to improve forelimb placing function, likely due to the reported tissue loss within the sensorimotor cortex following TBI [26].

We recently investigated both prophylactic (pre-treatment) hyperbaric oxygen (HBO) strategy and HBOT (post-treatment) for treatment of repetitive mild traumatic brain injury (rmTBI) (personal communication: Drs. Lei Huang and Andre Obenaus). Repetitive mTBI is an important public health concern for sports athletes and active military personnel as subsequent injuries are thought to exacerbate existing neuropathology. Mild controlled cortical impact (CCI) was used to model rmTBI in adult rats. In rmTBI animals, a second mild CCI was delivered at the same location at 3 or 7 days after the initial impact. HBO pre-treatment or HBOT was given 1 hr daily at 2 ATA for 3 consecutive days either prior to or 24 hrs after the initial TBI, respectively. T2 weighted imaging (T2WI) and susceptibility weighted imaging (SWI) were acquired non-invasively from which lesion and hemorrhage volumes were quantified. Our results clearly demonstrated that both HBO pre-treatment and HBOT improved neuroimaging outcomes following rmTBI, in contrast to those seen in tissues without HBO intervention. There were significant reductions in the T2WI-derived lesion and SWI-identified hemorrhage volumes at 24 hrs after rmTBI (Figures 1, 2). The most dramatic neuroprotective effects were observed in animals receiving rmTBI 3 days apart where a 3-fold reduction in hemorrhage volumes was observed compared to Shams (Figure 2). Given that the pathophysiological processes underlying rmTBI likely involves cellular metabolic perturbations in the injured brain [32], a neuroprotective approach, namely, HBO pre-treatment or HBOT favoring cerebral aerobic metabolism could be beneficial. Similar findings have been reported in HBOT for human severe TBI [14].

Clinical HBOT for human TBI

A variety of human injuries and neurological diseases have applied HBOT to improve outcomes. More overt

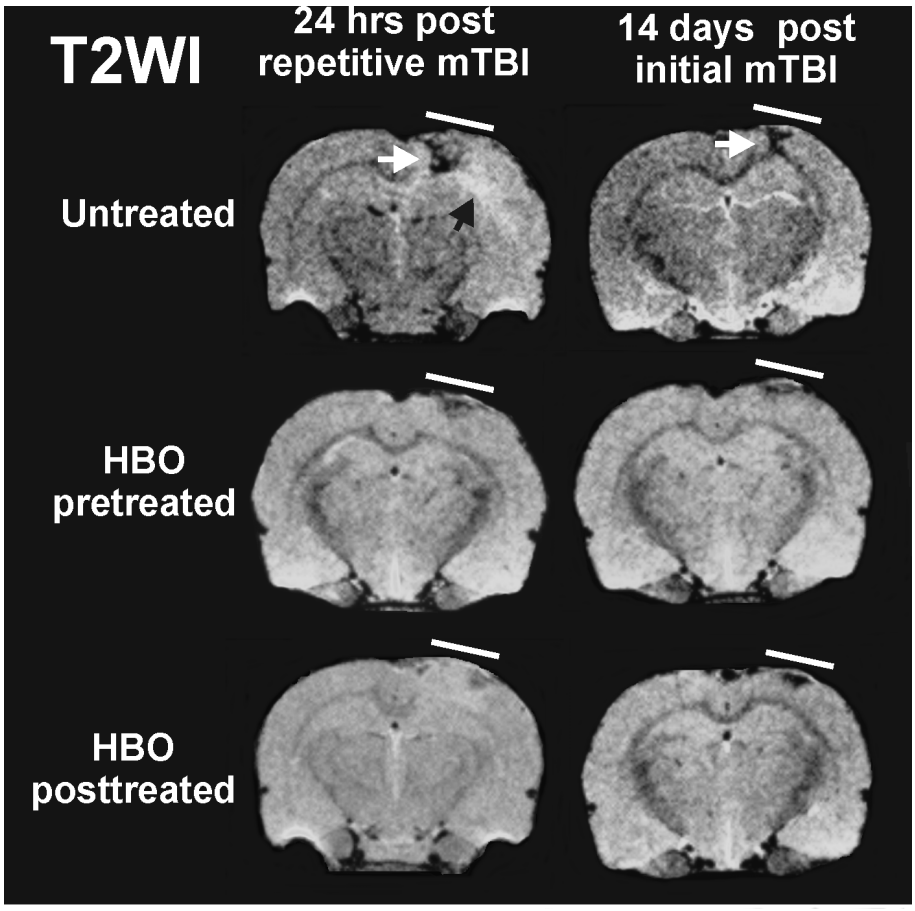

Figure 1 HBO reduces rmTBI lesion volumes. Pre- and post-treatment with HBO reduces lesion volume identified from magnetic resonance imaging (MRI, T2 weighted images). Repetitive mild traumatic brain injury (rmTBI) was induced 3 days apart and resulted in ipsilateral tissue damage. On T2WI, hypointensities (white arrow) are consistent with bleeding while hyperintensities (black arrow) suggest edema formation. At 24 hrs after the rmTBI, HBO pre- or post-treatment significantly reduced the lesion size compared to untreated animals. The neuroprotective effects persisted to 14 days after the initial mTBI.

neurological injuries, such as stroke or TBI have not been aggressively pursued, partly due to apparent or perceived contraindications. While a complete listing of the absolute and relative contraindications for clinical applications are beyond the scope of this review, it should be noted that certain drugs, fever and respiratory ailments limit clinical application of HBOT. However, within the realm of clinically applied HBOT for TBI very little research has been conducted. Enthusiasm for HBOT for brain injured patients was dampened by the findings of a meta-analysis of TBI patients receiving HBOT [33]. Their sobering conclusions were that the risk of death was reduced but there was no apparent change in clinical outcomes. However, as these authors acknowledged, variance in treatment protocols and the limited number of patients in the studies reviewed hampered their analysis. Based on their findings they suggested that HBOT could not be justified for TBI patients.

The poor clinical outcomes of earlier HBOT studies combined with the relative success of normobaric oxygen therapy (NBOT) in TBI have lead some to propose that normobaric oxygen therapy should be used preferentially in brain-injured patients. There are numerous studies that demonstrate an enhanced clinical outcomes by treatment with normobaric oxygen [34]. Much of the enthusiasm for use of NBOT is based on a prospective study of severe TBI patients [4]. Narotam and colleagues [35] evaluated brain tissue oxygen concentrations in patients with severe TBI. Using Licox oxygen probes, 139 patients were studied using a pO_2 protocol that maintained brain oxygen levels to > 20 mm Hg. They elegantly demonstrated that normobaric oxygen therapy significantly reduced mortality, but moreover, they showed improved clinical outcomes at 6 mo post-severe TBI. A similar study found that hyperoxia improved the cerebral metabolic rate of oxygen in severe TBI patients using

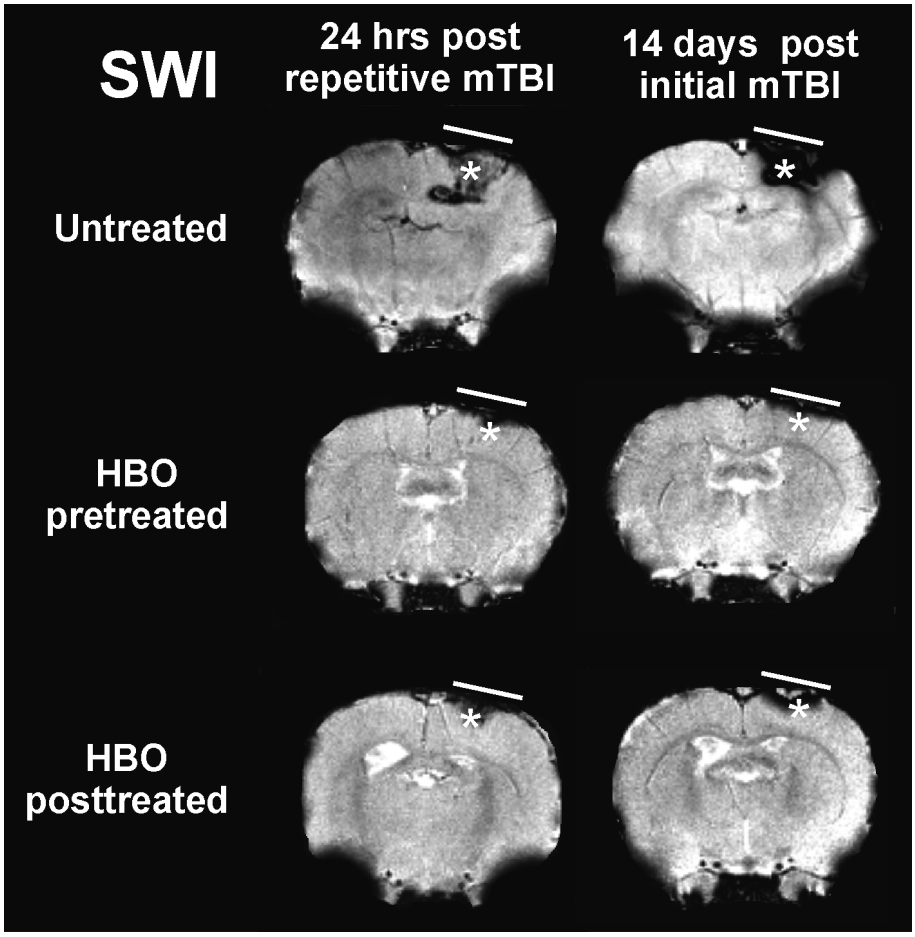

Figure 2 HBOT reduces extravascular blood after rmTBI. HBO pre- and post-treatment improved susceptibility weighted imaging (SWI)-identified intracerebral hemorrhage following repetitive mild traumatic brain injury (rmTBI) 3 days apart. At 24 hrs after rmTBI, HBO pre- or post-treatment significantly ameliorated the hemorrhage (hypointensity, asterisks) compared to untreated animals, which persisted to 14 days after the initial mTBI.

O15-postiron emission tomography, but they did not compare to HBOT treated patients [36]. Thus, at a minimum, NBOT could be beneficial for TBI patients.

The most extensive research into clinical application of HBOT for TBI patients has been conducted by the Rockswolds [5,14,17,37]. Almost 30 years ago they undertook one of the first clinical trials in evaluating the benefits of HBOT for severe head injured patients [17]. In that early study they demonstrated a 50% decrease in mortality but found no changes in the clinical outcome status (i.e. good recovery and moderate disability). As noted above, a meta-analysis of several studies concluded the same findings; reduced mortality but no change in clinical status [33].

While survival was increased, functional recovery was not changed, leading to questions about the timing of the HBOT. In addition, the mechanism(s) underlying HBOT and its effects on cerebral metabolism had not been previously established in severely brain-injured patients. In another prospective clinical trial, Rockswold et al [5], reported increased cerebral metabolic rate of oxygen and decreased lactate measured from cerebrospinal fluid after HBOT (1.5 ATA 1 hr/day every 24 hrs with a maximum of seven sessions). ICP was also reduced but a caveat was noted that these changes did not last till the next session [5]. It also serves to remind the reader that similar results had also been previously reported in NBOT [36].

Based on evidence that NBOT of human TBI patients appeared to have similar outcomes as patients who underwent HBOT, a follow-up study was conducted to compare these two groups after severe TBI to assess the efficacy of therapy [14]. It is important to note, that the standard of care is neither NBOT nor HBOT. Thus, their study design included controls (standard of care), normobaric (3 hrs 100% O_2) and HBOT (1.5 ATA for 60 min) that received their initial treatment within 24 hrs of a severe TBI. Treatments were then conducted daily for 3 consecutive days.

The pO_2 levels within the brain were nearly 3 fold higher in the HBOT compared to the NBOT groups and significantly different from controls [14]. As previously reported, they found increased cerebral metabolic rate of oxygen, decreased lactate and decreased intracranial pressure. They also reported that HBOT increased cerebral blood flow. Perhaps the most important finding was that an indicator of mitochondrial dysfunction, lactate/pyruvate ratios, were significantly decreased only in the HBOT group. They also demonstrated no adverse outcomes or harmful effects in patients receiving HBOT [14]. Thus, HBOT for severe TBI, appears to improve cellular survival which was not observed in NBOT group.

Based on these limited studies, it is clear, that HBOT could be an effective therapy for clinical severe TBI. Compared to NBOT, HBOT assists in improving brain "functions", such as cerebral metabolism and blood flow. However, additional studies are needed not only during the acute phase of the injury, but also long-term studies evaluating outcomes to determine if HBOT is beneficial to TBI patients.

Putative mechanisms underlying the neuroprotection of HBOT following TBI

Over the past several decades, the neuroprotective mechanisms of HBOT have been investigated in a variety of animal models of TBI. The initial work in dogs (see above) have shown the HBOT was able to increase tissue oxygen delivery [10] as well as to protect penumbra tissue from secondary ischemia [21]. Based on the dog model, a similar freeze-induced brain injury was conducted in rats to evaluate local cerebral glucose utilization using the autoradiographic 2-deoxyglucose technique. Compared to animals that underwent NBOT, a four-day HBOT course (2 ATA for 90 minutes daily) significantly reversed the depressed glucose utilization within gray matter ipsilateral to the lesion [38]. Interestingly, HBOT tended to decrease glucose utilization in the sham-operated animals. However, it was still uncertain whether the favorable outcomes were directly attributable to improved glucose metabolism associated with HBOT. HBO-improvements in tissue oxygenation and mitochondrial metabolic function were further investigated in a rat model of fluid percussion injury (FPI) [3]. HBOT (1 hr 1.5 ATA with 3 hrs 100% normobaric oxygen) treatments significantly improved, 1) brain tissue pO_2 (more than 6 fold) near the site of injury; 2) ex vivo brain tissue oxygen consumption (vO_2, more than 1 fold); and 3) recovery of synaptosomal mitochondrial metabolic activity [39,40].

Given that the prognosis of TBI clearly depends on the processes of cell death and survival that occur within the traumatized tissues, neuroprotective therapies need to mitigate and improve survival and function within the remaining viable perilesional brain tissue [41]. The neuroprotective effects of HBOT against secondary brain damage in the penumbra region have been extensively investigated [6,41-43]. Using a model of dynamic cortical deformation (DCD) to produce focal cerebral contusion in rats, HBOT (2 sessions at 2.8 ATA for 45 min/each) were administered at 3 hrs after TBI and compared to the effects of NBOT. There were significantly smaller lesion volumes and decreased numbers of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, a biomarker for apoptosis) positive cells after HBOT. Normobaric oxygen therapy (100%) also improved tissue measures but not to the extent found after HBOT [43]. The anti-apoptotic modulator, B-cell lymphoma (Bcl-2), was increased after HBOT and correlated to reduced tissue apoptosis [41]. Similar changes were found for B-cell lymphoma-extra large (Bcl-xl) expression, while the pro-inflammatory protein, B-cell lymphoma-associated X protein (Bax), was observed primarily in astrocytes instead of neurons. The ratio between pro-apoptotic Bax and anti-apoptotic Bcl-2/Bcl-xl proteins has been shown to act as a "rheostat" that sets the threshold [44] of susceptibility to apoptosis by competitively modulating the opening of the mitochondrial permeability transition pore (mPTP) [45,46]. Enhanced Bcl-2 expression inhibits the mPTP that subsequently preserves mitochondrial homeostasis and therefore the integrity of the electron transport chain [6]. Palzur et al thus hypothesized that HBOT-induced increases in Bcl-2 expression and the resultant increase in intracellular oxygen bio-availability may contribute both to preserve mitochondrial integrity and to reduce the activation of the mitochondrial mediated apoptotic pathway following TBI [6]. In the same animal model, HBOT (2.8 ATA for 45 min at 3 and 24 hrs post-injury) substantially facilitated the recovery of mPTP expression. Subsequently, TBI-induced injury to tissue morphology was reversed with enhanced neuronal survival and preserved axonal architecture within perislesional tissues [6]. Similar findings of improved mitochondrial redox after HBOT in the FPI model of TBI have also been reported [3]. The preservation of mitochondrial integrity by HBOT hindered the activation of mitochondria-associated apoptotic pathways by significantly lowering caspase 3 and 9, but not caspase 8 expression (critical for non-mitochondrial apoptotic pathway) in injured brain tissues [6]. These results underscore the importance of HBOT-induced reductions in delayed cell death within the tissue penumbra after TBI. Such, mechanisms echo the neuroprotection of HBO seen in brain ischemia and subarachnoid hemorrhage [47,48].

Acute inflammation also plays an important role in secondary brain damage after TBI. An influx of inflammatory cells induced by TBI provides the primary source of matrix metalloproteinases (MMPs) activity [49]. MMPs in the injured brain further play a deleterious role and promote cell death, including apoptosis [50]. The effects

of HBOT on inflammatory infiltration and the expression of (MMPs) have been explored in a rat model of DCD. Both HBOT (2.8 ATA for 45 min at 3 hrs after injury and twice a day thereafter for 3 consecutive days) and NBOT significantly decreased myeloperoxidase-positive neutrophils within the traumatic penumbra, but HBOT had a more pronounced effect. HBOT also significantly reduced the elevation of MMP-9 expression associated with neutrophilic infiltration [42]. Thus, HBOT substantially decreases the harmful effects of inflammation by reducing MMP-9 over-expression that then results in a reduction of delayed cell death in penumbral tissues surrounding the site of injury. Interestingly, reduced MMP-9 has also been proposed to be the underlying mechanism associated with HBO pretreatment induced neuroprotection against TBI at high altitude in a rat model [51]. However, what is still unresolved is whether the decreased numbers of apoptotic cells following HBOT, is a direct anti-apoptotic effect or secondary consequence due to HBOT anti-inflammatory effects. Further studies are warranted to disclose the complex mechanisms underlying the neuroprotective effects of HBOT after TBI.

Conclusions

Translational research of HBOT in a variety of TBI models has shown neuroprotective effects in the absence of increased oxygen toxicity when administered at pressures less than 3 ATA. Due to the heterogeneity of human TBI, the efficacy of clinical HBOT and an optimal regimen for HBOT remains elusive. However, all human studies have involved severe TBI patients and it is likely that there may be increased efficacy in mild or moderate TBI patients. Recent clinical trials favor HBOT as promising safe therapeutic strategy for severe TBI patients. Although both NBOT and 1.5 ATA HBOT can be neuroprotective, HBOT exerts more robust and long-lasting effects in the absence of pulmonary or cerebral oxygen toxicity. The improved tissue oxygenation and cellular metabolism, anti-apoptotic as well as anti-inflammatory effects may constitute the multiple and complementary mechanisms underlying HBOT-induced neuroprotection.

Additional material

Additional file 1: Table S1: Current clinical uses for HBOT.

List of abbreviations

ATA: One atmosphere absolute; Bax: B-cell lymphoma-associated X protein; Bcl-2: B-cell lymphoma; Bcl-xl: B-cell lymphoma-extra large; CBF: Cerebral blood flow; CCI: Controlled cortical impact; CSF: Cerebrospinal fluid; DCD: Dynamic cortical deformation; FPI: Fluid percussion injury; HBO: Hyperbaric oxygen; HBOT: Hyperbaric oxygen therapy; ICP: Intracranial pressure; MMPs: Matrix metalloproteinases; mPTP: Mitochondrial permeability transition pore; MWM: Morris water maze; NBOT: Normobaric therapy; pO_2: Partial pressure of oxygen; rmTBI: Repetitive mild traumatic brain injury; SWI: susceptibility weighted imaging; TBI: Traumatic brain injury; TIMP-1: metallopeptidase inhibitor-1; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; T2WI: T2 weighted imaging; vO_2: Oxygen consumption

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Authors' contributions

Both LH and AO contributed intellectually to this review. LH reviewed the HBO studies in animal models of TBI and AO reviewed clinical trials of HBO in severe TBI patients. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Animal Models of Mild Pediatric TBI

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INTRODUCTION

The complex nature of the cause and evolution of traumatic brain injury (TBI) and the wide variety of lesions that occur has made it difficult to develop animal models that accurately reflect the clinical picture. This is particularly true when comparing children with adults. Over the past two decades various experimental approaches have been pursued to develop satisfactory models, initially in larger species, but now more commonly in rodents.

Relevant animal models of TBI must mimic both the injury induction and the short- and long-term neurological deficits seen in human patients. While no single animal model has all of the desired characteristics, a range of models have been developed that are associated with unique neurological impairments and provide insights into particular injury cascades (Fig. 1). As noted by Cernak (2005), experimental models should include the following: (1) the mechanical force used to induce injury must be controlled, reproducible, and quantifiable; (2) the inflicted injury is reproducible, quantifiable, and mimics components of the human condition; (3) the injury outcome, measured by morphological, physiological, biochemical, or behavioral parameters, is related to the mechanical force causing the injury; and (4) the intensity of the mechanical force used to inflict injury should predict outcome severity.

INSERT FIG 1

A significant issue of models and their modes of inducing injury is the definition of the degree of injury severity; however, given the numerous experimental models and their differences (Fig. 1), it is quickly apparent that classification into mild, moderate and severe TBI is difficult. The clinical criteria for mild TBI (mTBI) have been defined by many clinical specialties and organizations (see Chapter 1). What is readily apparent from such criteria is that mTBI is very difficult to model experimentally, in part because of the inability to detect ‘transient amnesia’ or changes in ‘consciousness’ in animals. This is further exacerbated by mTBI criteria that require the virtual absence of any overt abnormalities on neuroimaging (computed tomography, CT; magnetic resonance imaging, MRI). The inability to incorporate lack of

consciousness has plagued virtually every animal model, except the fluid percussion injury model (FPI; (Cernak, 2005; Marklund & Hillered, 2011). Additionally, development of animal models that mimic mTBI based on currently used clinical criteria are necessary but problematic as there often are no overt changes in the animal's physiological function.

We briefly review the major adult and juvenile rodent experimental models of focal and diffuse TBI with a range of injury severities to not only provide the reader with an overview, but also to underscore the virtual dearth of research in pediatric TBI, especially, mTBI. We specifically highlight the ability of MRI to assess injury severity and the tempo-spatial evolution, the role of different MRI modalities, the effects of single vs. repetitive mTBI insults, and finally the behavioral changes seen after pediatric mTBI in rodent and piglet models. We discuss several recent excellent reviews that provide in depth descriptions and analyses of the different TBI models, their biological rationale, advantages and limitations, and scientific findings related to the primary and secondary mechanisms of injury (Cernak, 2005; Morales, Marklund, Lebold, Thompson, Pitkanen, Maxwell et al., 2005; Ucar, Tanriover, Gurer, Onal & Kazan, 2006; Albert-Weissenberger & Siren, 2010; Morganti-Kossmann, Yan & Bye, 2010; Marklund & Hillered, 2011; O'Connor, Smyth & Gilchrist, 2011). In addition, an excellent recently published review on the use of MRI in rodent models of brain disorders is included (Denic, Macura, Mishra, Gamez, Rodriguez & Pirko, 2011).

RODENT ADULT TBI MODELS

The biomechanical forces associated with TBI inflict either dynamic or static injury, depending on the amplitude, duration, velocity and acceleration of the injury (Fig. 1, (Cernak, 2005). Static models are used to focus on morphological and functional processes involved in injury. Dynamic brain trauma includes direct injury where trauma is directly imposed on the brain (e.g., non-accidental trauma, contact sports, falls, etc, where the head is accelerated) and indirect injury where the trauma is imposed on the whole body and imparts its effects indirectly

on the brain (e.g., motor vehicle accidents where the body is rapidly accelerated/decelerated but the head does not directly strike an object).

TBI for modeling purposes can be broken into three distinct types of brain injury: direct, indirect and rotational injuries. “Blast” injuries are not included in this chapter as there is likely little if any relevance to pediatric mTBI. Direct brain injuries can be classified as imparting a more focal injury, whereas at the opposite end of the spectrum rotational types of TBI result in more diffuse injury (Fig. 1). Indirect models of TBI can span this continuum, resulting in a more “mixed” type of injury. These head injury models can be further dichotomized depending on whether the head motion is constrained to a single plane or unconstrained and allowed to move freely (Cernak, 2005).

Other investigators have developed alternative classification schemes for experimental TBI. For example, Marklund and Hillered (2011) stratified animal models into five subgroups (focal, mixed, diffuse, complex, and other; Table 1). This organizational schema attempts to reflect clinical measures and brain injuries that occur in humans. It also offers a more practical approach for researchers to determine the most suitable model that fits the research questions being explored. Relevant points emphasized in this review include: 1) no single animal model can adequately mimic all aspects of human TBI owing to the heterogeneity of the clinical picture; 2) testing in several TBI models and at different injury severities is crucial to successfully develop therapeutic compounds for clinical TBI; and 3) further refinement of animal models and development of functional outcome measures is important.

With regards to experimental features of TBI models, a number of additional criteria need emphasis, including: 1) the importance of being able to precisely grade the severity of injury and that the response must be quantifiable and reproducible between different investigators and laboratories; 2) the model must be able to replicate the type(s) of severity and pathology observed in humans; and 3) the damage produced should represent a component of

an injury continuum, increasing in severity as the mechanical forces applied are increased (Morales et al., 2005).

INSERT TABLE 1

The three most commonly used models of TBI include the weight drop, fluid percussion injury, and controlled cortical impact models and have been extensively reviewed in numerous publications (Morales et al., 2005; Ucar, Tanriover, Gurer, Onal & Kazan, 2006; Albert-Weissenberger & Siren, 2010; Morganti-Kossmann, Yan & Bye, 2010; O'Connor, Smyth & Gilchrist, 2011). Table 2 highlights the most important features of these rodent TBI models and their advantages and disadvantages (Albert-Weissenberger & Siren, 2010). The three primary models are briefly summarized below.

INSERT TABLE 2

Weight drop models

The weight-drop impact model produces a number of characteristics consistent with closed head injury in humans (Cernak, 2005; Morales et al., 2005; Ucar, Tanriover, Gurer, Onal & Kazan, 2006; Albert-Weissenberger & Siren, 2010; Morganti-Kossmann, Yan & Bye, 2010; Marklund & Hillered, 2011; O'Connor, Smyth & Gilchrist, 2011). The advantages of this model include focal and diffuse brain injury, its relative ease of surgical preparation of animals, and the absence of post injury complicating factors such as infection (Table 2; Fig. 1), but a disadvantage is the variability in the degree of injury severity.

Weight-drop models use the gravitational forces of a free falling weight to produce a focal or diffuse (when closed head) injury to the brain (Albert-Weissenberger & Siren, 2010). For focal brain injury, animals are placed on non-flexible platforms to minimize the dissipation of energy whereas for the creation of a more diffuse brain injury, flexible platforms (i.e. foam) allow the head to accelerate. Severity can be graded by using different weights and/or heights.

This model is easy to reproduce but some variability exists, due to: 1) loss of velocity of the weight sliding along a guide tube, and 2) the possibility of a “second hit” induced by the weight rebounding from the skull of the animal resting on the flexible sponge. These shortcomings can be minimized using either an air or an electromagnetic driven impact device to mechanically deform the skull (Table 2; (Cernak, 2005).

Feeney and colleagues (1981) used a weight drop model to impact an intact dura that resulted in a cortical contusion. The pathology included hemorrhage and damage of the blood-brain barrier, subsequent inflammation with microglial and astrocytic activation, neutrophilic and macrophage infiltration, delayed microcirculatory dysfunction and cortical spreading depression. Despite the focal nature of the injury widespread axonal injury has been observed. In the Shohami weight-drop model, the skull is impacted laterally and severity is controlled by weight and height variables (Shapira, Shohami, Sidi, Soffer, Freeman & Cotev, 1988; Flierl, Stahel, Beauchamp, Morgan, Smith & Shohami, 2009). This model induces an ipsilateral cortical contusion with blood-brain barrier disruption, edema formation, activation of the complement system, progressive cell death and a post-injury inflammatory response.

A third commonly used weight-drop model is the ‘impact acceleration’ model developed by Marmarou and colleagues (Foda & Marmarou, 1994; Marmarou, Foda, van den Brink, Campbell, Kita & Demetriadou, 1994). The head is placed on a foam bed that allows freedom of movement and acceleration and produces diffuse injury including hemorrhage, neuronal cell death, astrogliosis, diffuse axonal injury, and cytotoxic brain edema. Others have extensively modified this model by attaching a stainless steel disc to the surface of the skull to allow a more diffuse injury and to prevent potential skull fractures, particularly in mice. In adult rats, a mild diffuse brain injury without local lesion or contusion results in no mortality or skull fractures, stable hemodynamic activity, a brief (5- to 10-second) apneic period and mild microscopic neuronal, axonal, astrocytic and small vessel changes (Foda & Marmarou, 1994; Marmarou et al., 1994).

Tang and colleagues (Tang, Noda, Hasegawa & Nabeshima, 1997b; Tang, Noda, Hasegawa & Nabeshima, 1997a) used acrylic weights combined with a silicon rubber platform to create a mild injury in mice. It generated concussive-like brain injury without skull fractures eliciting transient behavioral and long-lasting learning and memory impairments in the absence of motor deficits, similar to the reversible loss of consciousness and persistent cognitive deficits observed in human mTBI. A similar approach in adult rats to model cerebral concussion injury also exhibited: 1) loss of muscle tone, righting and corneal reflexes, and whisker responses, that recovered in <10 minutes; 2) global hemodynamic depression with a short transient decrease of arterial pO_2, an increase in mean arterial blood pressure, and a reduced heart rate; 3) no visible abnormalities, similar to what has been reported in human mTBI; 4) quantitative MRI analysis identified subtle structural and functional alterations despite normal conventional MRI; 5) learning and memory deficits on Morris water maze tests, and 6) cellular neuropathology with cortical and hippocampal neuronal loss (Henninger, Dutzmann, Sicard, Kollmar, Bardutzky & Schwab, 2005; Henninger, Sicard, Li, Kulkarni, Dutzmann, Urbanek et al., 2007).

Fluid percussion injury models

Fluid percussion injury (FPI) models produce a brain injury by a rapid pressure pulse direct onto the dural surface through a craniotomy either centrally/medially (CFPI/ MFPI) over the sagittal suture midway between bregma and lambda or laterally (LFPI) over the parietal cortex. Graded injury severity can be achieved to produce focal (usually with LFPI) or diffuse (usually with CFPI) injury (Tables 2, 3; Fig. 1). FPI can result in cortical contusion, hemorrhage, and cytotoxic or vasogenic edema formation in moderate and severe injuries, but little has been reported on mild FPI injures. Immediate physiological responses include changes in blood pressure (transient hypertension), brief respiratory arrest, elevated intracranial pressure, reduced cerebral blood flow, decreased cerebral perfusion pressure, and increased cerebral vascular resistance (Cernak, 2005). FPI models result in cellular alterations in ion homeostasis

(e.g., increased intracellular calcium and sodium, decreased intracellular magnesium, reduced potassium homeostasis), reduced cerebral metabolic activity, and depression of electroencephalographic activity. A delayed progression of brain damage seen with FPI is accompanied by astrogliosis, diffuse axonal injury, inflammation, cortical spreading depression, and neurodegeneration (Albert-Weissenberger & Siren, 2010). Moderate LFPI is associated with observable and quantifiable MR imaging changes that are in stark contrast to those seen in CCI models (Obenaus, Robbins, Blanco, Galloway, Snissarenko, Gillard et al., 2007).

FPI results in testable behavioral abnormalities in rodents and have been used as a model of post-traumatic epilepsy. An important caveat is that even a small shift in the craniotomy site is often associated with marked differences in neurological outcomes and lesion size, indicating that the FPI model requires extensive methodological modification to obtain standardized outcome measures (Albert-Weissenberger & Siren, 2010). Although FPI still remains one of the most commonly used TBI models, one of its primary limitations is related to increased brainstem injury, which often results in apnea and increased morbidity as well as the development of neurogenic pulmonary edema.

Controlled cortical impact models

Controlled cortical impact (CCI) models utilize a pneumatic or an electromagnetic piston to deform an exposed dura and the underlying cortex, providing a precise and controlled impact (Cernak, 2005; Morales et al., 2005; Albert-Weissenberger & Siren, 2010; Marklund & Hillered, 2011). Animals are placed in a stereotactic device allowing accurate cortical deformation and the craniotomy site is often varied laterally and in the rostral caudal direction, allowing injury to the frontal cortex or at sites adjacent to the hippocampus. Dependent on the severity of injury, CCI results in an ipsilateral injury with cortical contusion, hemorrhage, and blood-brain barrier disruption. Extensive cortical tissue loss, and hippocampal and thalamic damage can occur (Marklund & Hillered, 2011), particularly in severe CCI (Mac Donald, Dikranian, Song, Bayly,

Holtzman & Brody, 2007). Typical neuropathological features include neuronal degeneration, astrogliosis, microglial activation, inflammation and axonal injury. Cytotoxic and vasogenic edema formation are commonly seen after CCI (Obenaus et al., 2007), which makes this an excellent model to study edema formation and resolution that is well known to occur clinically. Seizures also occur frequently after severe CCI, so it has also been used as a model of posttraumatic epilepsy. We have recently developed a single and repeated mTBI model using an electromagnetic impactor which results in transient observable changes on neuroimaging with histological and behavioral changes (Huang, Coats, Mohd-Yusof, Neglerio, Yin, Assad et al., 2011). Closed head models can use a similar impactor to induce CCI injury without craniotomy, particularly in mice (Obenaus, unpublished results; (Dikranian, Cohen, Mac Donald, Pan, Brakefield, Bayly et al., 2008).

Advantages of the CCI model include its ease of use, reproducibility, and the ability to adjust the degree of injury severity but drawbacks include that the injury may be extensive, destroying large cortical regions, and may not be comparable with the extent of brain injury observed in survivors of human TBI. Since a large craniectomy is often performed and if the bone flap is not replaced after injury, the effects of secondary brain swelling may be attenuated thus mimicking a decompressive craniotomy used in alleviating raised intracranial pressure (ICP) in humans.

PEDIATRIC TRAUMATIC BRAIN INJURY MODELS

The pediatric brain is not simply a “smaller” adult brain, but represents a complex process of development and maturation. The physiological consequences of pediatric TBI are therefore expected to be equally complex and dependent upon the time and location of injury. The appropriate age in the rodent (i.e., new born vs. juvenile vs. adolescent) is particularly important in modeling TBI in children. Watson et al. (2006) summarized many of the postnatal characteristics that are critical when comparing rodent to human development. For example, in

the human brain, aerobic metabolism is mature by 1 year of age while in the rat the same level of maturity is reached by post-natal day 21 (PND21). Similarly, onset of myelination in the rat starts at PND14-17 and within 2-3 months in the human and is complete by PND90 and 20-30 years of age in the rodent and human, respectively (Watson, Desesso, Hurtt & Cappon, 2006; Clancy, Finlay, Darlington & Anand, 2007; Danzer, 2008). While considerable debate continues, some consensus exists that in the rat PND10 is equivalent to the time of birth in humans while others have utilized PND7 (Ikonomidou & Turski, 1996); both PND7 and 10 are combined in our review. The PND35 in the rat is considered equivalent to puberty in the human (Watson, Desesso, Hurtt & Cappon, 2006). A developmental model in the rat by Prins and colleagues (1996) demonstrated significant differences in the evolution of TBI in PND17 and 28 rats compared to adults (Prins, Lee, Cheng, Becker & Hovda, 1996). Thus, many researchers use a broad range of ages to model pediatric TBI, and we review these studies based on age and the type of TBI model.

Controlled cortical impact in rodent models

PND7-10. Accidental brain injury is the predominant cause of TBI in the human infant, and neonatal mTBI models have been established in the PND7 rat or mouse using closed head controlled cortical impact (CCI; see above for detailed model description, (Ikonomidou, Qin, Labruyere, Kirby & Olney, 1996; Bittigau, Sifringer, Pohl, Stadthaus, Ishimaru, Shimizu et al., 1999). In these CCI models of mTBI, little evidence of cavitation, distortion or hemorrhage to the cortical mantle at the impact site or in the contralateral hemisphere was found. Mild TBI in infant mice resulted in very early and rapidly progressing axonal degeneration in white matter structures, including the cingulum and external capsule. At later time points, substantial apoptotic cell death was observed in the injured cortex and in functionally and anatomically connected neuronal populations between the injury site and the thalamus (Dikranian et al.,

2008). Similar injury patterns have been reported in mTBI in the developing rat brain (Bayly, Dikranian, Black, Young, Qin, Labruyere et al., 2006).

Although substantial damage to the hippocampal region in these models is not evident, the extrahippocampal circuitry ipsilateral to the injury is selectively and irreversibly damaged, particularly when the injury is delivered to the skull overlying the parietal cortex. Importantly, orientation and spatial learning functions rely on an intact hippocampal circuit relaying information between the hippocampus, the retrosplenial cortex, the subicular complex, the anterior thalamic nuclei, and the mammillary nuclei; thus, damage to these connections results in behavioral and neurological deficits (Aggleton, Neave, Nagle & Sahgal, 1995; Aggleton, Hunt, Nagle & Neave, 1996; Aggleton & Brown, 1999; Mitchell, Dalrymple-Alford & Christie, 2002). These closed head CCI models of mTBI are useful for studying the long-term consequences of TBI in the developing brain, but additional studies are needed to elucidate the underlying mechanisms.

PND35. Although the incidence of TBI varies with age, particularly in sports-related injury, children 5–14 years of age can account for up to 40% of all brain injuries (Kraus, Rock & Hemyari, 1990). Using the PND35 rat as a model of the preadolescent human, Prins and colleagues have developed a closed head injury model of mTBI where the head is placed obliquely in a molded wooden block and the skull is impacted using an electronically controlled pneumatic piston allowing the head to freely move in the direction of the injury (Prins, Hales, Reger, Giza & Hovda, 2010). Histological assessment showed no axonal death in either the cortex or the hippocampus. However, subtle axonal damage using β-amyloid precursor protein immuno-histochemistry was observed 24 hours after a single impact with no change in the open field behavioral tests. When mTBI animals were challenged at 24-hours post injury by inclusion of a novel object, both the single (and repeated) mTBI animals exhibited a significant decrease in the percent time spent with the novel object relative to sham animals. These results

demonstrate that in the PND35 rodent, measurable cognitive deficits can be observed early after mTBI in the absence of gross pathology.

PND21 and PND35. More recently we completed a series of preliminary studies evaluating the effects of a single or repetitive mTBI in PND21 and 35 rodents using a model of closed head CCI (Obenaus, unpublished data). In both groups, neuroimaging for edema (T2WI), alterations in water mobility (DWI), and determination of the presence of extravascular blood (susceptibility weighted imaging; SWI) did not demonstrate any overt changes within the parenchyma one day post injury (Fig. 2). Consistent with previous reports and clinical criteria, our mTBI model of closed head injury did not reveal any changes on structural MRI. Even a repetitive injury to the contralateral hemisphere, 2-4 days after the initial mTBI, did not cause observable changes on neuroimaging. These findings suggest that this model, at least from an imaging point of view, does not result in a direct injury to the underlying cortical or subcortical tissues. This is in contrast to the kind of injury seen in CCI models where brain tissues are directly impacted.

INSERT FIGURE 2

While structural imaging appeared to be negative in our model, functional imaging, specifically perfusion weighted imaging (PWI), demonstrated decreased cerebral blood flow and volume (Fig. 3). When contralateral cortex was impacted 3d later, we observed further reductions in cerebral blood flow and volume at the site of the second impact. Notably, since structural imaging is often negative in mTBI, emerging imaging modalities may provide more sensitive indicators of subtle brain injury.

INSERT FIGURE 3

Previous work by Holshouser and colleagues has demonstrated the utility of magnetic resonance spectroscopy (MRS) to evaluate pediatric patients with varying degrees of injury (Ashwal, Holshouser & Tong, 2006; Ashwal, Wycliffe & Holshouser, 2010). MRS is technically difficult to do in small animal models, but as shown by Signoretti and colleagues, MRS has the potential to provide significant additional information about an injury that may not be apparent

on structural MRI (Signoretti, Marmarou, Tavazzi, Lazzarino, Beaumont & Vagnozzi, 2001; Vagnozzi, Signoretti, Tavazzi, Cimatti, Amorini, Donzelli et al., 2005; Belli, Sen, Petzold, Russo, Kitchen, Smith et al., 2006; Vagnozzi, Tavazzi, Signoretti, Amorini, Belli, Cimatti et al., 2007).

In a PND21 rat model of closed head injury, using MRS to identify metabolite changes one day after the first injury we found no changes in key brain metabolites, including N-acetyl-aspartate (NAA, a neuronal marker), choline (Cho, a marker of cell membrane integrity) and creatine (Cre, a marker for cell energy utilization; Fig. 4). However, after a second contralateral impact (4 days after the initial mTBI), we observed a decreased Cre peak (Fig. 4). This finding is consistent with reduced energy metabolism/utilization associated with neuronal injury, resulting in an uncoupling between cerebral perfusion and metabolism (Fig. 3). This cause-effect relation may increase edema or result in other cerebro-vascular alterations that reduce cerebral blood flow and results in the spectroscopic changes that we observed. In moderate TBI, clinical studies have shown that patients with the appearance of a lactate peak have worse clinical outcomes (Tong, Ashwal, Holshouser, Nickerson, Wall, Shutter et al., 2004; Holshouser, Tong & Ashwal, 2005; Babikian, Freier, Ashwal, Riggs, Burley & Holshouser, 2006). Overall, these preliminary findings suggest that methods such as PWI and MRS may be more sensitive in detecting brain injury after mTBI than conventional (structural) imaging techniques.

INSERT FIGURE 4

Piglet model of nonimpact closed head injury

A critical link for successful translational research is the development of large animal models of brain injury that provide a platform to correlate meaningful functional outcome measures with well-characterized histological and molecular substrates. Piglets have been used to model acute asphyxial damage to the developing brain resulting from circulatory arrest (Brambrink, Ichord, Martin, Koehler & Traystman, 1999), cardiopulmonary bypass (Kurth, Priestley, Golden, McCann & Raghupathi, 1999; Schultz, Creed, Schears, Zaitseva, Greeley,

Wilson et al., 2004), and TBI (Armstead, 2002; Raghupathi & Margulies, 2002; Raghupathi, Mehr, Helfaer & Margulies, 2004). Moreover, the histopathology and acute cerebral physiologic responses seen in piglets in these injury paradigms more closely resemble those seen in human infants (Hagberg, Peebles & Mallard, 2002). The piglet brain also more closely resembles human cortical gyral and white matter distribution than the pachygyric rodent brain.

Mild TBI in piglets is induced by rapid axial head rotations using a pneumatic actuator (Raghupathi & Margulies, 2002; Raghupathi, Mehr, Helfaer & Margulies, 2004), similar to that described for adult pigs (Smith, Nonaka, Miller, Leoni, Chen, Alsop et al., 2000). Behavioral testing in these mTBI piglets found a delayed return of the pinch reflex by ~2.4 minutes (Raghupathi & Margulies, 2002; Raghupathi, Mehr, Helfaer & Margulies, 2004), but serial behavioral testing up to 11 days post-injury using an open field paradigm, found no differences between the mTBI and sham groups. Macroscopic and microscopic histological brain examination found no evidence of subarachnoid hemorrhage or ischemia (Friess, Ichord, Owens, Ralston, Rizol, Overall et al., 2007). These studies have now been extended to repeated TBI (see below).

Weight drop model

Although the weight drop model has been used in adult models of TBI, it has not yet been reported in pediatric models. Such a model clearly has the potential to be useful, even if comparisons will need to be made to the more commonly used pediatric CCI models.

Repeated mild injury models

At the present time, only three published reports exist of repeated experimental pediatric mTBI (Friess et al., 2007; Huh, Widing & Raghupathi, 2007; Prins, Hales, Reger, Giza & Hovda, 2010), each employing different species. Margulies and colleagues using rotational injury in neonatal piglets (PND3-5) evaluated the effects of two rotational injuries given in quick

succession (15 min apart). No differences were apparent in cellular density (axonal) measures of DAI on histopathology between single or double rotational injuries, but the double rotational injury group showed a wider distribution of axonal swelling (Raghupathi, Mehr, Helfaer & Margulies, 2004). No measureable neurobehavioral changes were found (Friess et al., 2007).

A follow-up study investigated repeated rotational injuries, either 1 day or 1 week apart (Friess, Ichord, Ralston, Ryall, Helfaer, Smith et al., 2009). The 1 day-apart rotational group experienced significantly higher mortality, raising the question whether this model was a mild injury. Visual based problem solving was impaired in the 1-day compared to the 1-week apart group and shams. Also, white matter injury was significantly increased in the 1-week apart group compared to other groups and correlated with the degree of injury severity.

The effects of recurrent TBI in young rats (PND11) with 1, 2 and 3 successive impacts (5 min apart) have been reported (Huh, Widing & Raghupathi, 2007). Although the authors did not specify injury severity, based on their previous studies (Huh, Widing & Raghupathi, 2008; Huh, Widing & Raghupathi, 2011b), we surmise that injury induction in this study was mild due to the lack of skull fractures. Seven days after mTBI, demonstrated graded tissue injuries were found where the two-repeated mTBI resulted in enlarged ventricles and white matter atrophy, while the three-repeat mTBI animals exhibited the same neuropathology but were visible earlier (i.e., 3 days post injury). In addition, the three-repeat mTBI group showed evidence of distant injury, within the thalamus. The glial response to repeated mTBI was also increased in a graded fashion. Despite these neuropathological changes, all three groups performed similarly on behavioral testing.

In a closed head injury model the effects of single and double mTBIs 1 day apart in young rats (PND35, (Prins, Hales, Reger, Giza & Hovda, 2010) found a 10% mortality, which was lower than that described in the rotational piglet studies (Friess et al., 2009). Behavioral measures from the novel object recognition task revealed increased memory impairment in the repeated mTBI group. Acute axonal injury in the posterior portion of the brain was significantly

higher in the repeated mTBI group compared to single mTBI animals along with a consistent bilateral increase in astrocytic reactivity compared to the single mTBI group (Prins, Hales, Reger, Giza & Hovda, 2010).

Taken together, these studies suggest that repeated mTBI results in an increase in axonal injury and in proliferation of glial responses (Raghupathi, Mehr, Helfaer & Margulies, 2004; Huh, Widing & Raghupathi, 2007; Friess et al., 2009; Prins, Hales, Reger, Giza & Hovda, 2010). Although comparison of these findings across species and models is difficult, few differences in behavioral outcomes were found between sham, single and repeated mTBI groups. Long-term studies during the chronic phase of mTBI have not yet been reported, but likely would provide meaningful and hopefully clinically relevant data.

Direct controlled cortical impact

Currently no published studies have focused on direct mild mechanical CCI in pediatric-aged animals. One potential reason is the difficulty in obtaining reproducible injuries in the pediatric rodent, particularly for mTBI. A large number of tests (see below), from imaging to behavioral indices, are required to reveal the existence of mild deficits. In unpublished experiments, we modeled a single mild CCI to the right frontal cortex (Fig. 5) in PND17 rats with a 0.5 mm cortical deformation without rupture of the underlying dura. Assessments 7d later revealed a small cortical deformation with loss of tissue parenchyma that was very mild compared to a moderate (i.e., 1.5mm depth) injury (Ajao, Pop, Kamper, Adami, Rudobeck, Huang et al., 2011).

INSERT FIGURE 5

Neuroimaging of this model revealed early edema formation at 1 day post injury. Similarly, DWI showed reduced water mobility at the impact site, consistent with swelling of the cortical tissue in the vicinity of the impact (Fig. 6). At 3 days post-injury, T2 imaging normalized, but increased water mobility was seen on DWI at the impact site, suggesting mild cell death.

The injury at 7 days appeared to have some degree of tissue herniation with a secondary edema phase that was visualized on neuroimaging and is consistent with the onset of delayed vasogenic edema formation.

INSERT FIGURE 6

We also evaluated the potential for extravascular blood deposition in this model using susceptibility weighted imaging (SWI), which is sensitive to the detection of blood products after TBI (Ashwal, Holshouser & Tong, 2006). We observed no evidence of hemorrhage early after injury but did observe the presence of subtle petechial hemorrhages at 7 days post-injury (Fig. 7). Visualization of suspected blood in these tissues combined with a secondary phase of edema formation is consistent with neuroinflammation in response to the initial injury. Future studies are required to evaluate these outcomes more rigorously.

INSERT FIGURE 7

These very preliminary studies suggest that cortical deformation using the CCI model of mTBI in pediatric rodents could be useful for studying mechanisms associated with secondary injury in children and that these effects of mTBI can be visualized non-invasively using MRI.

Neurobehavioral correlates in pediatric models

In humans, TBI sustained from birth through adolescence can lead to behavioral deficits (motor, cognitive, affective) that worsen with age and can also result in long-term deficits that are more severe than a similar TBI acquired later in life (Anderson, 2005; Donders & Warschausky, 2007). The behavioral effects of mild or moderate brain injury in animal models are often difficult to detect or interpret, but behavioral observations ultimately offer the most clinically relevant paradigms for testing therapeutic targets. One of the first studies to assess behavioral dysfunction in juvenile PND17 rats used a closed-head weight drop model (Adelson, Robichaud, Hamilton & Kochanek, 1996; Adelson, Dixon, Robichaud & Kochanek, 1997). Testing demonstrated a prolonged recovery time of reflexes as impact severity increased, such

that moderately injured rat pups took three times longer than shams to recover, and severely injured pups took five times as long. In more extensive behavioral testing of mild and moderate TBI animals, no changes in grip strength or water maze swim speed were found, suggesting no differences in motor performance up to 11 days after injury. The severe group had marked motor deficits 1 day post-injury that persisted for 10 days, as well as water maze performance deficits that lasted for at least 22 days, suggesting possible hippocampal involvement (Adelson, Dixon, Robichaud & Kochanek, 1997). These studies did not have an explicit mTBI group and if so, behavioral deficits would have been difficult to ascertain.

Long-term behavioral testing (i.e., 3 months post-injury) was reported in a weight drop model in PND17 rat pups (Adelson, Dixon & Kochanek, 2000). The weight used (150 gm) was considered a severe injury in their previous studies (Adelson, Robichaud, Hamilton & Kochanek, 1996). Similar to their earlier findings (Adelson, Dixon, Robichaud & Kochanek, 1997), these injured rats exhibited severe motor deficits over the course of the 10 days of testing, although performance improved with learning deficits being present up to 3 months post injury. The presence of performance deficits on the cued water maze task (in which the animal can see the escape platform) suggests that the deficits observed in the spatial water maze task (in which the escape platform rests just below the surface of the water) may primarily be due to sensorimotor rather than spatial learning deficits.

Behavioral assessment in a weight drop model in PND7 rat pups assessed the protective effects of resveratrol, a polyphenolic compound found in grapes and other fruits (Sönmez, Sönmez, Erbil, Tekmen & Baykara, 2007). Of note, the authors did not state whether this was a mild or moderate injury. TBI rats were hypoactive and performed poorly on the object recognition task that was reversed by a single injection of resveratrol at 100 mg/kg immediately following the injury. Resveratrol also ameliorated (by ~50%) the detected memory deficits.

In contrast to the findings reported in the weight drop studies by Adelson (2000), studies by Prins and colleagues using a FPI model in PND17 rats revealed that in moderately injured rat

pups no behavioral deficits in Morris water maze learning could be found (Prins & Hovda, 1998). Although the PND17 rat pups did not exhibit behavioral deficits, PND28 injured animals had transient performance deficits that resolved after the first week of testing, whereas animals injured as adults had subtle performance deficits throughout the 14 days of testing post-injury. These results suggest that a moderate injury produced using the FPI model at PND17 was insufficient to induce long-term learning deficits but that a brain injury of similar physical magnitude in older juveniles and adult animals found deficits.

The effects of an enriched environment (which more closely approximates wild-type living conditions) in juvenile TBI were assessed using behavioral measures in PND19 rat pups (Giza, Griesbach & Hovda, 2005). A moderate FPI injury to the left parietal cortex found no deficits in water maze performance at 30 days post-injury, whereas raising the pups in an enriched environment immediately after injury improved the performance of shams, but not the TBI group. When the enrichment paradigm was delayed until 2 weeks post-injury, spatial learning performance of shams and injured rats improved, but spatial memory, as assessed by the water maze probe trial, only improved for sham rats. These results suggest that moderate TBI in juvenile rats can induce behavioral deficits, but that standard laboratory housing conditions could mask underlying deficits.

The same investigators published an extension of these studies evaluating three different degrees of fluid percussion injury (2.65, 2.8, and 3.2 ATM) in PND19 male rats (Gurkoff, Giza & Hovda, 2006). In spite of the lack of neuronal loss in the ipsilateral cortex or CA3 field of the hippocampus, subtle deficits in water maze performance were found 1-10 days later. However, in contrast to the findings of Adelson (1997), no correlation was found between injury severity and the degree of behavioral deficits. In a related study, the effects of using the ketogenic diet (i.e., high fat, low protein/carbohydrate) in a closed head CCI model (PND17, 35, 45 and 60) showed deficits in beam balance testing but only in the PND35 group (Appelberg, Hovda & Prins, 2009). These beam balance deficits were transient at 3-4 days post-injury and

recovered by 5 days post-injury. The behavioral deficits were ameliorated by a ketogenic diet only in younger animals.

What is missing from many of these pediatric TBI studies is the long-term assessment of behavioral outcomes. The longest behavioral assessment in a pediatric study is by Adelson et al (1997), who measured outcomes at 3 months post-injury (Adelson, Dixon, Robichaud & Kochanek, 1997). Recently, we have examined the emergence of behavioral deficits as early as 30 days that persist until 90 days (Ajao et al., 2011). Perhaps more interesting is that we observed the development of anxiety-like behaviors similar to that reported in humans after TBI (Whelan-Goodinson, Ponsford, Johnston & Grant, 2009). We are now extending these studies to 6 months post-injury. Given the lack of long-term outcome data, we suggest that other future studies also make a concerted effort to evaluate persistent changes in behavior.

The only non-rodent study to assess behavior in an animal model of juvenile TBI used PND3-5 female piglets (see above; (Friess et al., 2009) subjected to moderate rotational TBI with either a single TBI or double TBI (1 day or 1 week apart). As noted above, piglets subjected to 1 day-apart double TBI had a higher mortality (43%) and more severe visual-based problem solving deficits compared to piglets with single or 1 week-apart double injury.

In reviewing studies of pediatric mTBI, a number of limitations are evident, with the most obvious being a dearth of reports using either PND17 or PND35 rodents. In addition, most studies assessed behaviors only acutely or less than 1 month post-injury with the longest being 3 months. No studies have recorded behavior in older adult animals that were injured as juveniles with the exception of our recent study (Ajao et al., 2011). Also missing from the literature are studies in mice. Although porcine models provide a much more appropriate model of white matter involvement than rodents, transgenic / knockout mice can provide valuable tools for therapeutic and mechanistic studies. A broad and comprehensive test battery for behavioral testing in pediatric rodents is also lacking. To more fully characterize the spectrum of behavioral disorders observed in children following TBI, testing of a wide range of behavioral domains,

including affective responses, fine and gross motor skills, and learning and memory, by multiple paradigms will likely produce a number of behavioral targets for prognostic and therapeutic studies.

Overall, the above findings demonstrate that rodent closed-head impact models have dominated the field of experimental behavioral testing in pediatric mTBI. Closed-head impact consistently has resulted in acute and relatively long-term (up to 3 months post-injury) behavioral deficits affecting motor and cognitive functions. Open-skull FPI has shown only very subtle, if any, deficits in water maze testing, whereas open-skull CCI has produced clear-cut water maze deficits (Appelberg, Hovda & Prins, 2009). Increasing the severity in FPI does not result in increased behavioral deficits (Gurkoff, Giza & Hovda, 2006) but increased severity in closed-head impacts has been associated with worse behavioral deficits (Adelson, Dixon, Robichaud & Kochanek, 1997; Huh, Widing & Raghupathi, 2011a). In one study of repeated closed-head TBI (PND17), no behavioral differences were observed between animals hit once or twice (Huh, Widing & Raghupathi, 2011a), but in another report (PND35), two hits led to more severe deficits (Prins, Hales, Reger, Giza & Hovda, 2010). The only study of TBI using repeated rotational injury likewise showed that double injury led to more severe deficits than single injury when the second injury was generated within 1 day (Friess et al., 2009). This was the only study to find a relation between neuropathological correlates of injury severity and the degree of behavioral deficits.

Finally, of the studies that assessed TBI at different ages (Prins & Hovda, 1998; Huh & Raghupathi, 2007; Raghupathi & Huh, 2007; Appelberg, Hovda & Prins, 2009), only the FPI model induced worse deficits in older (PND35) animals (Prins & Hovda, 1998). The closed-head impact at age of PND11 (Huh & Raghupathi, 2007; Raghupathi & Huh, 2007) and open-skull CCI (Appelberg, Hovda & Prins, 2009) models (PND35 and PND75) each corroborated human clinical data suggesting that earlier injury is associated with more severe behavioral outcomes (Anderson, 2005; Donders & Warschausky, 2007). Only the closed-head impact models

exhibited that increasing injury severity produced worse behavioral outcomes (Adelson, Dixon, Robichaud & Kochanek, 1997; Huh, Widing & Raghupathi, 2011a).

SUMMARY

The literature on neonatal, juvenile, and adolescent mTBI animal studies is extremely limited, resulting in very little scientific knowledge about the progression and long-term outcomes of brain injury. Unlike adult models of TBI, virtually no pediatric studies have used neuroimaging as a biomarker to assess disease progression. Behavioral paradigms that reflect functional injury in the adult clearly are not very useful in pediatric models, and more sensitive or perhaps an entirely different battery of neurological and behavioral testing needs to be devised to observe the effects of mTBI. Finally, only a handful of studies have evaluated potential therapeutic compounds in the pediatric population and additional studies are needed to define the appropriate pediatric mTBI model to test potential therapeutics. Together, significant research needs to be undertaken to fill these knowledge gaps and to ameliorate the clinical consequences of mTBI in human patients.

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Figure Legends

Fig. 1. Traumatic brain injury model schematic

This simplified schema outlines the primary types of injury (direct, indirect, and rotational) and the predominant type of neuropathology that often results. Direct TBI can also be classified as penetrating injuries, while indirect TBI can relate to non-penetrating injuries. The types of models that can induce these injuries are detailed below each type of injury. For more detailed schema see Cernak (2005) and O'Connor et al. (2010). Abbreviations: CCI – cortical contusion injury, WD – weight drop, FPI – fluid percussion injury, CHI – closed head injury.

* - typically injuries are delivered to the midline

+ - may include a rotational component

Fig. 2. Neuroimaging of mild juvenile closed head injury (CHI) at two developmentally sensitive ages

The initial mild CHI (●) was performed in rat pups at PND21 and PND35 followed by the second contralateral mild injury (●●) 2 days after the initial CHI. Multimodal MR imaging was acquired at 24 hrs after each impact. In both age groups there were no overt signal intensity changes on any imaging modality (T2, DWI or SWI). Thus, in a mild CHI conventional MR imaging may not reveal tissue level changes. (PND21 CHI: tip diameter=4mm, displacement=1.5mm, location=2mm lateral, 3mm posterior from bregma. PND35 CHI: tip diameter=4mm, displacement=3mm, location=3mm lateral, 3mm posterior from bregma)

Fig 3: Perfusion weighted imaging (PWI) after closed head injury in a PND21 juvenile rat.

PWI was performed using an injection of a contrast agent (gadolinium, Gd) to assess perfusion deficits. A reduction (left panel, arrow) in cortical blood flow (CBF) was observed 24 hrs post single mild CHI (tip diameter=4mm, displacement=1.5mm, location=2mm lateral, 3mm posterior

from bregma). At this time point there was a modest decrement in cerebral blood flow (CBF) seen on the contralateral side of the brain, consistent with diffuse bilateral changes. A second CHI was induced on the contralateral side 3 days after the first impact. At 14 days after the first single impact (right panel), these initial deficits in perfusion were still observed at the site of single injury (arrow on the right hemisphere). PWI also revealed reduced CBF in the contralateral cortex where the double impact occurred (arrow on the left hemisphere). Additional regions of reduced CBF appeared in subcortical areas. These results suggest that there are long-term deficits in CBF (possibly reflecting metabolic changes) after a mild CHI.

● - site of impact

Fig 4. Magnetic resonance spectroscopy (MRS) after closed head injury in a PND 21 juvenile rat.

MRS was acquired at site of 1st CHI and 2nd CHI (a 3.5 X 3.5 cm^2 voxel), respectively, at variable time points using a PRESS sequence on a 4.7T Bruker MRI. Closed head injury at 24 hrs after a single injury (1d) revealed no overt changes in brain tissue spectral metabolite peaks on the side of the 1^{st} CHI. The second contralateral CHI (2^{nd} CHI) was induced 3d after the 1^{st} CHI MRS was performed 1 day later (4d). At 4 days (arrow) after the 1^{st} CHI (but 1d after the 2^{nd} CHI) there was a decrease in the tissue creatine (Cre) peak on the side of 2^{nd} CHI. The reduction in Cre is consistent with the decrements in CBF in our PWI studies (see Fig 3). By 17 days after the initial injury there were no observable metabolite changes within brain tissues at the initial injured site.

Fig 5. Induction of juvenile mTBI using the CCI model.

The CCI model requires a craniotomy to directly impact the cortex for mTBI induction. A) Craniotomy site in a juvenile rat pup (PND17) immobilized in a stereotactic device following a right frontal 2.7 mm craniotomy procedure. Care was taken not disrupt the underlying dura. B)

Higher magnification of the craniotomy site prior to CCI induction, illustrating that no injury to the dura occurred. C) The same craniotomy site immediately after impact with a 2 mm stainless steel tip at a depth of 0.5 mm depth (duration: 250 ms, speed: 6 m/s) demonstrating a localized contusion site. D) At seven days after mild CCI, the injury site is barely visible after perfusion fixation. A small amount of blood is still present within the impact site (arrows) but only minor disruptions are seen in the overlying cortex. (Cal bar = 0.5 cm)

Fig 6. Temporal neuroimaging of mild juvenile CCI.

T2-weighted (T2WI) and diffusion-weighted imaging (DWI) studies in a juvenile rat pup (PND17) after CCI (● - injury site) showed the temporal evolution of this mild lesion (arrows). On T2WI, 1 day post-injury, there was development of edema (hyperintensity, arrow) that slowly resolved by 3 days. At 3 days (also on T2WI) extravascular bleeding (hypointensity, arrow) could be observed. By 7 days edema was present again (hyperintensity, arrow) accompanied by tissue deformation within the ipsilateral cortex. DWI, a biomarker for brain water mobility, revealed reduced water mobility (hyperintensity, arrow) corresponding to cytotoxic edema formation and gliosis at 1 and 7 days. Also, at 3 days there was an increase in water mobility (hypointensity, arrow) that could be due to inflammatory processes associated with the extravascular blood that was observed on the T2WI at 3 days post injury.

Fig 7: Susceptibility weighted imaging (SWI) of mild juvenile CCI.

SWI in a juvenile rat pup after mild CCI at PND17 revealed no extravascular bleeding within the cortex at 1 day post-injury (● - injury site) on either the magnitude image (Mag; raw MRI) or the SWI (phase corrected) image. The minimum intensity projection (MIP; 2 slices) images also did not show any extravascular blood at 1 day post-injury. However, at 7 day after mTBI, all three SWI image types (Mag, SWI, MIP) consistently revealed a heterogeneous signal change

(hyperintensity and hypointensity, arrows) within the ipsilateral cortical and hippocampal regions, consistent with blood deposition at the injury site.

Table 1: Experimental TBI model classification scheme[1]

Focal
Controlled cortical impact
Weight drop injury
Bi-frontal contusion
Acute subdural hematoma
Epidural hematoma
Mixed
Lateral fluid percussion injury (LFPI)
Diffuse
The impact/acceleration ("Marmarou") model
Midline (central) fluid percussion injury (CFPI)
Diffuse TBI models -"CCI-based" models
Complex
CCI and impact/acceleration models with hypoxia and/or hypotension
LFPI model with hypoxia and/or hypotension
Other
Repetitive models
Blast injury models
Penetrating injury models

1-Modified from Markland and Hillered 2010.

Table 2: Adult Rodent Models of Head Injury1

Model	Species	Injury	Strengths	Weaknesses
Weight-drop				
Feeney's weight-drop	Rat	Predominantly focal	Injury mechanism and inflicted injury is close to human TBI	High mortality rate due to apnea and skull fractures
Shohami's weight-drop	Rat, Mouse	Predominantly focal	Injury severity can be adjusted	Not highly reproducible
Marmarou's weight-drop	Rat, Mouse	Predominantly diffuse	Well characterized neuro-scores immediately after injury allows randomization	Reproducibility
Fluid Percussion Injury				
Medial FPI	Rat	Mixed	Injury severity can be adjusted	Requires craniotomy that may compensate for ICP increases
Lateral FPI	Rat, Mouse	Mixed	Inflicted injury is highly reproducible within the same laboratory but variable across laboratories	No immediate post-injury neuroscoring used and injury is variable between laboratories. There is also a relatively high

				mortality rate due to apnea
Controlled Cortical Impact				
	Rat Mouse	Predominantly focal	Severity of injury can be adjusted and is highly reproducible	Requires craniotomy and no immediate post-injury neuroscoring usually used

1 - Adapted from Albert-Weissenberger and Sirén (2010)

mTBI Book Chapter Figs

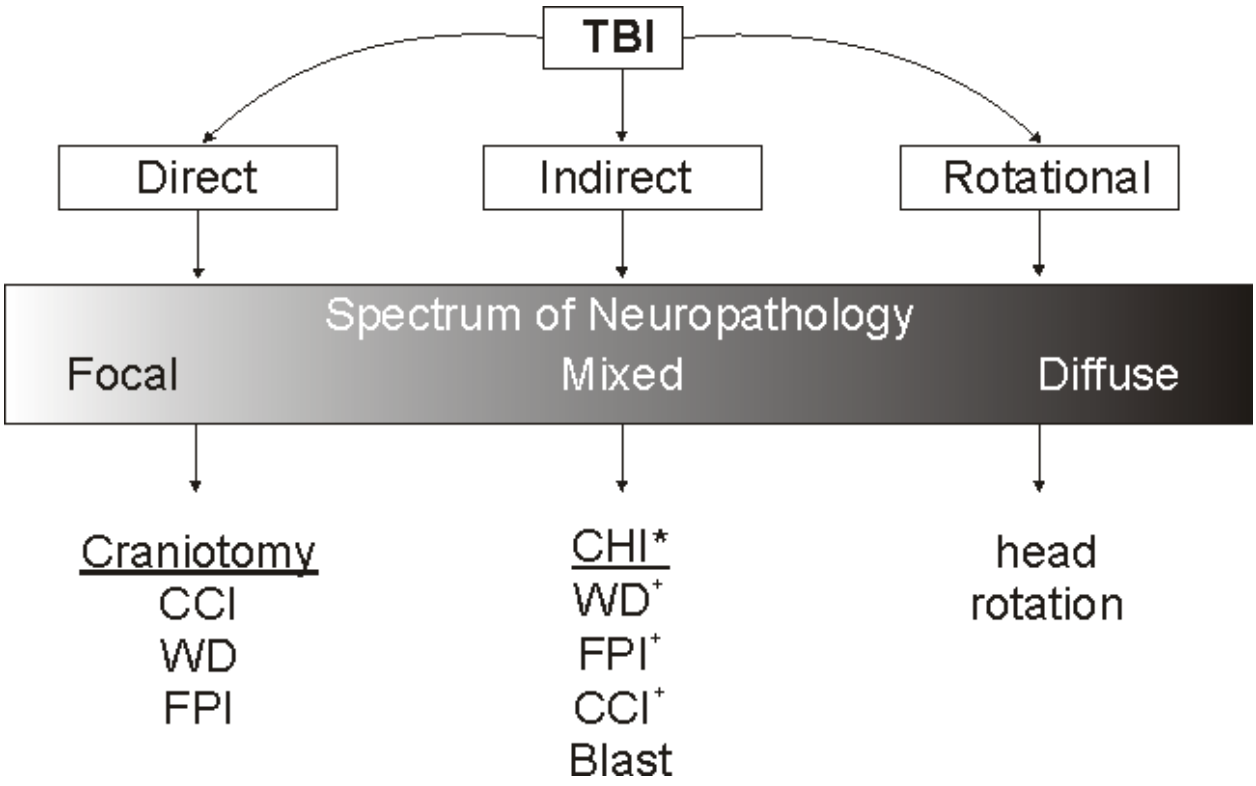

Fig. 1. Traumatic brain injury model schematic

This simplified schema outlines the primary types of injury (direct, indirect and rotational) and the predominant type of neuropathology that often results. Direct TBI can also be classified as penetrating injuries, while indirect TBI can relate to non-penetrating injuries. The types of models that can induce these injuries are detailed below each type of injury. For more detailed schema see Cernak (2005) and O'Connor et al. (2010). Abbreviations: CCI – cortical contusion injury, WD – weight drop, FPI – fluid percussion injury, CHI – closed head injury.

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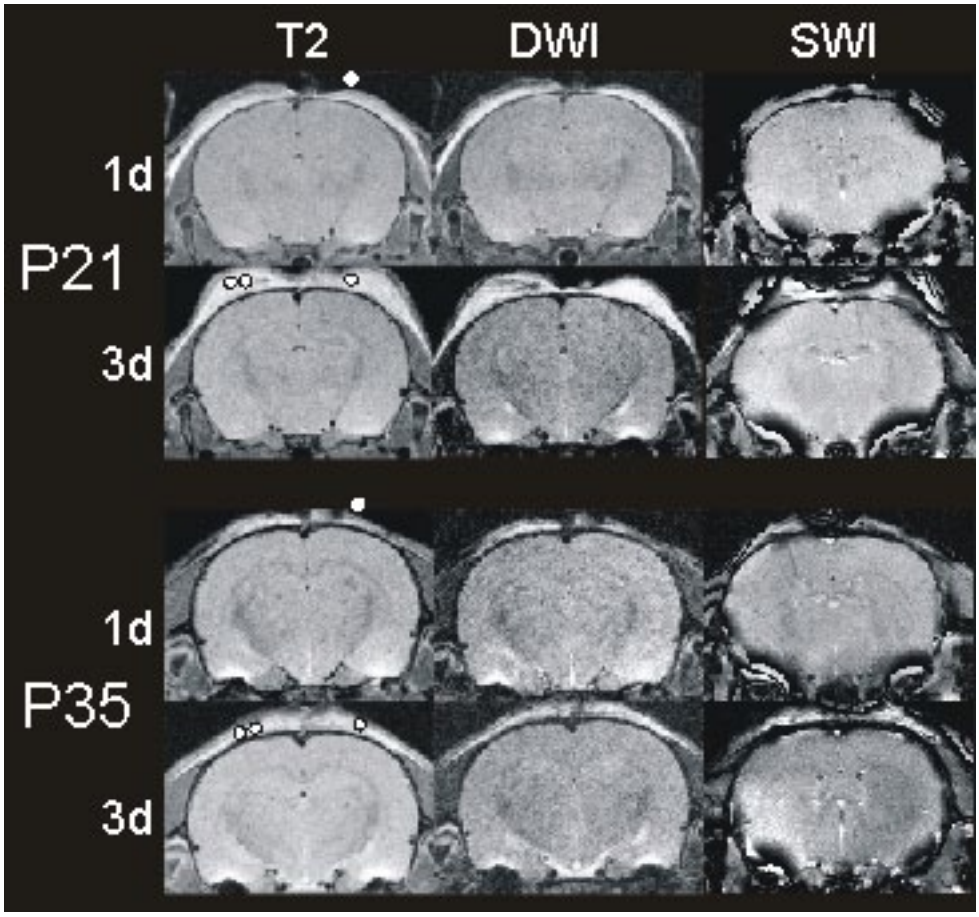

Fig. 2. Neuroimaging of mild juvenile closed head injury (CHI) at two developmentally sensitive ages

The initial mild CHI (●) was performed in rat pups at PND21 and PND35 followed by the second contralateral mild injury (●●) 2 days after the initial CHI. Multimodal MR imaging was acquired at 24 hrs after each impact. In both age groups there were no overt signal intensity changes on any imaging modality (T2, DWI or SWI). Thus, in a mild CHI conventional MR imaging may not reveal tissue level changes. (PND21 CHI: tip diameter=4mm, displacement=1.5mm, location=2mm lateral, 3mm posterior from bregma. PND35 CHI: tip diameter=4mm, displacement=3mm, location=3mm lateral, 3mm posterior from bregma)

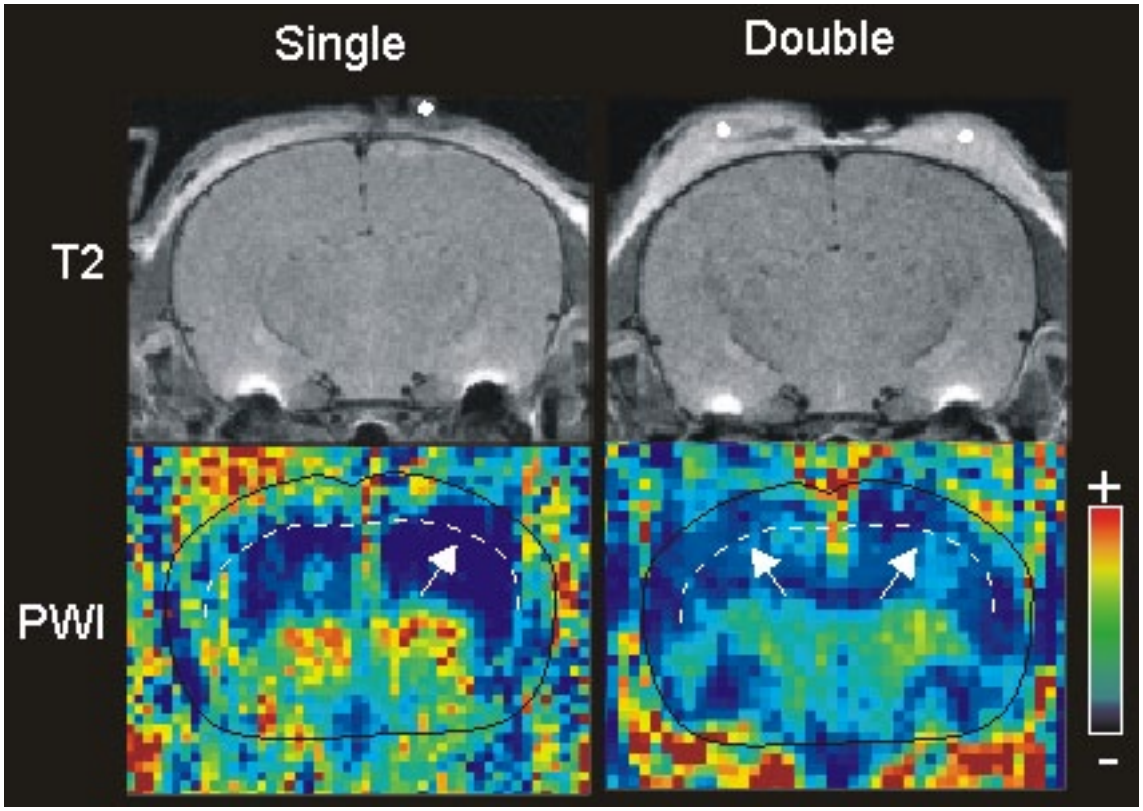

Fig 3: Perfusion weighted imaging (PWI) after closed head injury in a PND21 juvenile rat.

PWI was performed using an injection of a contrast agent (gadolinium, Gd) to assess perfusion deficits. A reduction (left panel, arrow) in cortical blood flow (CBF) was observed 24 hrs post single mild CHI (tip diameter=4mm, displacement=1.5mm, location=2mm lateral, 3mm posterior from bregma). At this time point there was a modest decrement in cerebral blood flow (CBF) seen on the contralateral side of the brain, consistent with diffuse bilateral changes. A second CHI was induced on the contralateral side 3 days after the first impact. At 14 days after the first single impact (right panel), these initial deficits in perfusion were still observed at the site of single injury (arrow on the right hemisphere). PWI also revealed reduced CBF in the contralateral cortex where the double impact occurred (arrow on the left hemisphere). Additional regions of reduced CBF appeared in subcortical areas. These results suggest that there are long-term deficits in CBF (possibly reflecting metabolic changes) after a mild CHI.

● - site of impact

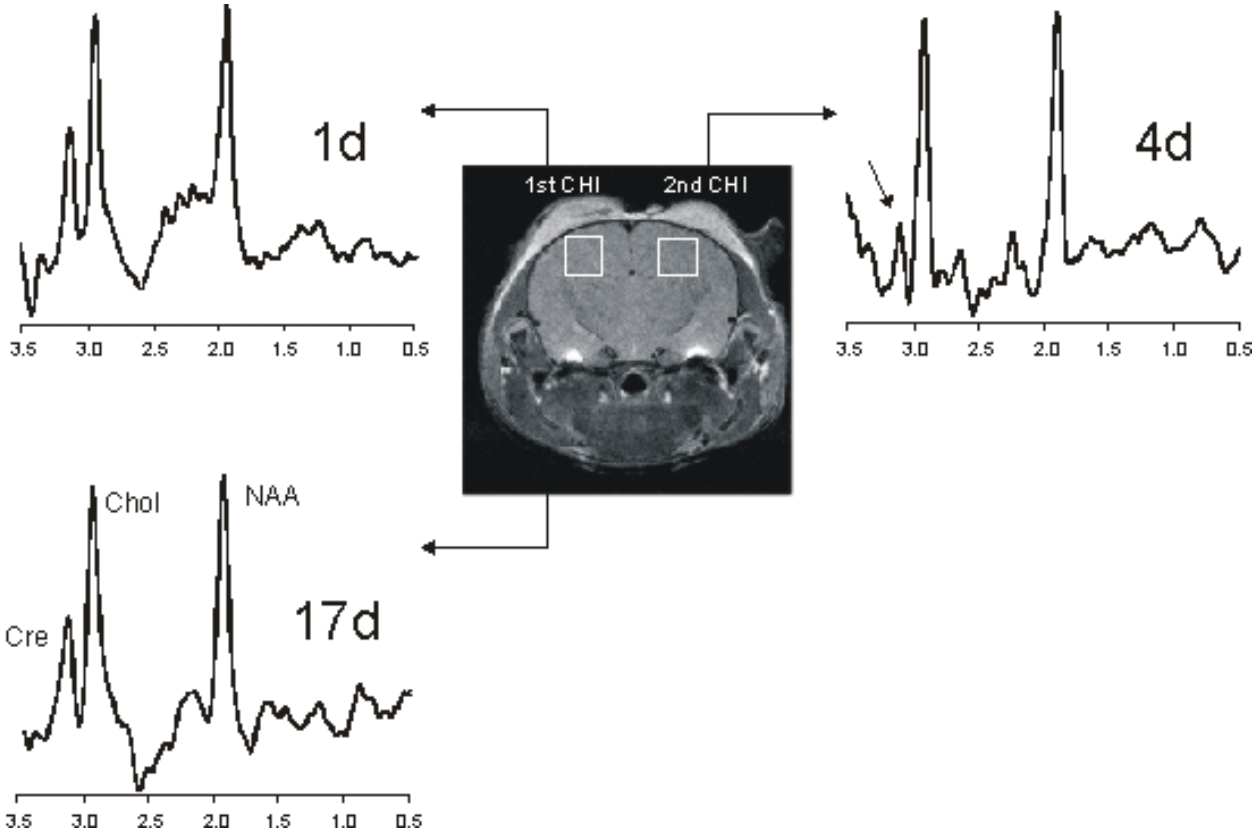

Fig 4. Magnetic resonance spectroscopy (MRS) after closed head injury in a PND 21 juvenile rat.

MRS was acquired at site of 1st CHI and 2nd CHI (a 3.5 X 3.5 cm^2 voxel), respectively, at variable time points using a PRESS sequence on a 4.7T Bruker MRI. Closed head injury at 24 hrs after a single injury (1d) revealed no overt changes in brain tissue spectral metabolite peaks on the side of the 1st CHI. The second contralateral CHI (2nd CHI) was induced 3d after the 1st CHI MRS was performed 1 day later (4d). At 4 days (arrow) after the 1st CHI (but 1d after the 2nd CHI) there was a decrease in the tissue creatine (Cre) peak on the side of 2nd CHI. The reduction in Cre is consistent with the decrements in CBF in our PWI studies (see Fig 3). By 17 days after the initial injury there were no observable metabolite changes within brain tissues at the initial injured site.

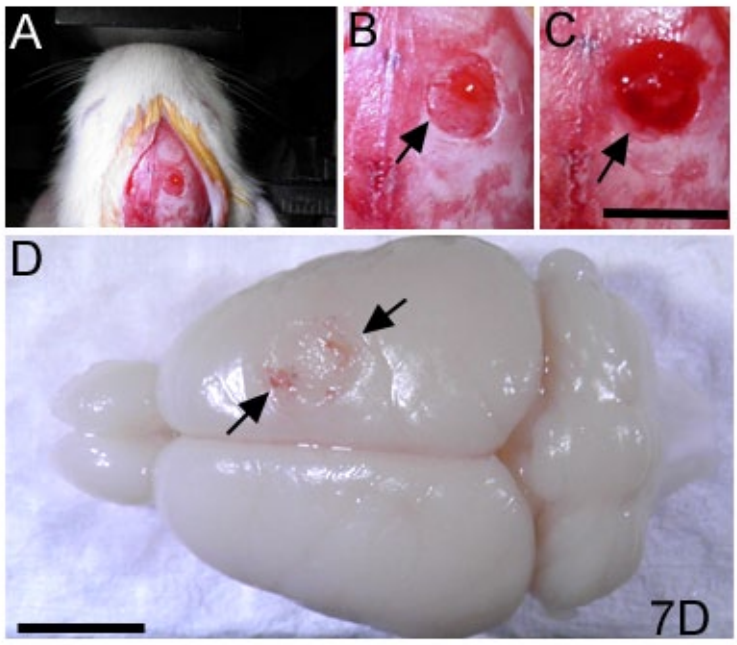

Fig 5. Induction of juvenile mTBI using the CCI model.

The CCI model requires a craniotomy to directly impact the cortex for mTBI induction. A) Craniotomy site in a juvenile rat pup (PND17) immobilized in a stereotactic device following a right frontal 2.7 mm craniotomy procedure. Care was taken not disrupt the underlying dura. B) Higher magnification of the craniotomy site prior to CCI induction, illustrating that no injury to the dura occurred. C) The same craniotomy site immediately after impact with a 2 mm stainless steel tip at a depth of 0.5 mm depth (duration: 250 ms, speed: 6 m/s) demonstrating a localized contusion site. D) At seven days after mild CCI, the injury site is barely visible after perfusion fixation. A small amount of blood is still present within the impact site (arrows) but only minor disruptions are seen in the overlying cortex. (Cal bar = 0.5 cm)

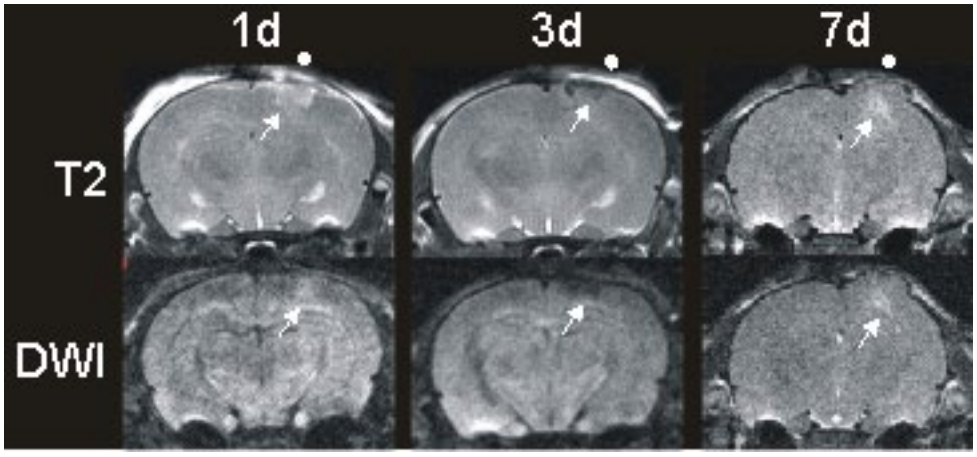

Fig 6. Temporal neuroimaging of mild juvenile CCI.

T2-weighted (T2WI) and diffusion-weighted imaging (DWI) studies in a juvenile rat pup (PND17) after CCI (● - injury site) showed the temporal evolution of this mild lesion (arrows). On T2WI, 1 day post-injury, there was development of edema (hyperintensity, arrow) that slowly resolved by 3 days. At 3 days (also on T2WI) extravascular bleeding (hypointensity, arrow) could be observed. By 7 days edema was present again (hyperintensity, arrow) accompanied by tissue deformation within the ipsilateral cortex. DWI, a biomarker for brain water mobility, revealed reduced water mobility (hyperintensity, arrow) corresponding to cytotoxic edema formation and gliosis at 1 and 7 days. Also, at 3 days there was an increase in water mobility (hypointensity, arrow) that could be due to inflammatory processes associated with the extravascular blood that was observed on the T2WI at 3 days post injury.

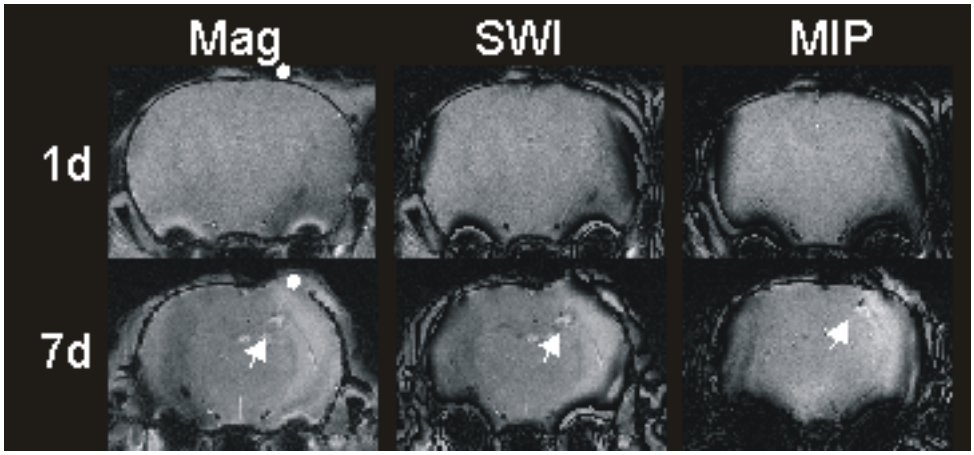

Fig 7: Susceptibility weighted imaging (SWI) of mild juvenile CCI.

SWI in a juvenile rat pup after mild CCI at PND17 revealed no extravascular bleeding within the cortex at 1 day post-injury (● - injury site) on either the magnitude image (Mag; raw MRI) or the SWI (phase corrected) image. The minimum intensity projection (MIP; 2 slices) images also did not show any extravascular blood at 1 day post-injury. However, at 7 day after mTBI, all three SWI image types (Mag, SWI, MIP) consistently revealed a heterogeneous signal change (hyperintensity and hypointensity, arrows) within the ipsilateral cortical and hippocampal regions, consistent with blood deposition at the injury site.

Tissue vulnerability is increased following repetitive mild traumatic brain injury in the rat

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Keywords:	magnetic resonance imaging, diffusion tensor imaging, cumulative injury, white matter, rat, gliosis

Title: Tissue vulnerability is increased following repetitive mild traumatic brain injury in the rat

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Abstract

Repetitive mild traumatic brain injury (rmTBI) is an important medical concern for active sports and military personnel. Multiple mild injuries may exacerbate tissue damage resulting in cumulative brain injury and poor functional recovery. In the present study, we investigated the time course of vulnerability in the brain to rmTBI in a rat model of mild cortical controlled impact. An initial mild impact was followed by a second injury at an interval of 1, 3, or 7 days. Animals with rmTBI were compared to animals with a single mTBI and shams. Neuropathology was assessed using multi-modal magnetic resonance imaging (MRI), neurobehavioral testing followed by tissue immunohistochemistry. Animals with rmTBI 1 or 3 days apart had significantly greater lesions on MRI compared to single mTBI and shams. A second mTBI 3 days after the first resulted in a significant increase in local blood deposition. Animals with rmTBI 3 days apart were also associated with long-term reduced exploratory behaviors and spatial learning memory deficits. Diffusion tensor imaging of the corpus callosum revealed axonal injury following single mTBI that was exacerbated by an rmTBI (1, 3 or 7 days apart). Increased cortical damage and astroglosis assessed by histology/immunohistochemistry correlated with *in vivo* MRI findings where shorter intervals (1 or 3 day apart) resulted in increased neuropathology. Collectively, our findings suggest that the brain is more vulnerable to tissue damage when rmTBI is delivered 3 days apart. Axonal measures of white matter were vulnerability in all rmTBI groups.

Keywords: magnetic resonance imaging; diffusion tensor imaging; cumulative injury; white matter; rat; gliosis

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Introduction

Mild traumatic brain injury (mTBI; or concussion), is an important global public health issue that accounts for upwards of 75% of all TBI patients each year. There is a particularly high incidence of mTBI in individuals engaged in contact sports (McCrory 2011) with ~300,000 sport-related concussions occurring annually in the United States (Thurman et al. 1998; Theye and Mueller 2004). Similarly, active military personnel have high rates of mTBI with the majority of veterans of Iraq and Afghanistan conflicts having mTBI diagnoses (Hoge et al. 2008; Terrio et al. 2009). Many of these individuals with mTBI/concussion often report multiple events. The Brain Injury Association (http://www.biausa.org/) has estimated that an individual with a previous brain injury is three times more likely to experience a second brain injury (Annegers et al. 1980; DeFord et al. 2002).

After an initial mTBI, transient neurological and cognitive impairments reflect a cascade of cerebral pathophysiology (Barkhoudarian et al. 2011). Disturbed cellular metabolism following the initial post-injury period may render the brain vulnerable to future mTBI events. Repetitive mTBI has been shown to significantly exacerbate the impairment of cellular oxidative metabolism within brain tissue similar to that found in severe TBI (Vagnozzi et al. 2005). A mild *in vitro* model of stretch-induced mechanical injury produced no cellular damage but repeated mild stretching at 2-min intervals induced cellular damage (Weber 2007). *In vivo* animal studies have consistently demonstrated that repeated mild injury increases hippocampal pathology and accelerates β-amlyoid deposition in white matter resulting in cognitive deficits (Laurer et al. 2001; DeFord et al. 2002; Slemmer et al. 2002; Uryu et al. 2002; Creeley et al. 2004; Raghupathi et al. 2004; Longhi et al. 2005). Repetitive mTBI patients present with persistent and often latent long-term cognitive impairments (Guskiewicz et al. 2005; McCrory 2011). An emerging hypothesis is that pathologies induced by repeated mTBI events are cumulative resulting in the long-term consequences including cognitive impairments. However, there is a lack of experimental evidence about the brain's period of vulnerability after a concussion.

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Magnetic Resonance Imaging (MRI) is widely used clinically to evaluate moderate to severe as well as mTBI patients and provides a tool to monitor the neuropathological evolution as well as potential treatments non-invasively (Lee and Newberg 2005; Ashwal et al. 2006). Conventional neuroimaging, such as T2 weighted imaging (T2WI) is sensitive to edema and large hemorrhagic contusions (Kent et al. 1994; Lee and Newberg 2005). Taking advantage of blood-induced phase shifts in the local magnetic fields (Haacke et al. 2004), susceptibility weighted imaging (SWI) is superior in the detection of TBI-induced microhemorrhages (Ashwal et al. 2006; Tong et al. 2008). In clinical studies on concussion patients, there was a 30% prevalence of abnormal findings on MRI in patients with a normal computed tomography (CT) scans (Bazarian et al. 2006). Cortical contusions can be visualized as multifocal hyperintensities on T2WI indicating shearing white matter injury or hypointensities due to microhemorrhages (Ashwal et al. 2006; Tong et al. 2008). Diffusion tensor imaging (DTI) is an advanced MRI technique that can assess the disruption of white matter microstructure (Cubon et al. 2011) that often occurs following TBI (Blumbergs et al. 1994). Quantitative DTI measures, such as fractional anisotropy (FA) or relative anisotropy (RA) are believed to reflect many factors including the degree of myelination, axonal density and/or white matter integrity (Arfanakis et al. 2002; Song et al. 2003; Wilde et al. 2008). Axial diffusivity represents water mobility parallel to axonal fibers while radial diffusivity is a measure of water mobility perpendicular to axonal fibers. While changes in axial diffusivity reflect the pathology status of the axon (Niogi and Mukherjee 2010) an increase in radial diffusivity appears to be more strongly correlated with myelin abnormalities, either dysmyelination or demyelination (Song et al. 2003; Niogi and Mukherjee 2010). DTI can provide robust early detection of white matter damage following mTBI, which may serve as a potential prognostic clinical indicator (Niogi and Mukherjee 2010). In both experimental and human studies analysis of axial and radial diffusivity has been shown to provide detailed insight into mTBI-induced pathophysiology (Niogi and Mukherjee 2010; Li et al. 2011).

In the present study, we evaluated the effects of cumulative brain injury following two repeated episodes of mTBI using multi-modal MRI, neurobehavioral assessments and immunohistochemistry. We hypothesized that

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there would be a period of vulnerability in the mildly injured brain to repetitive mTBI. This hypothesis was tested by evaluating three time intervals between the initial and subsequent mTBI events.

Material and Methods

All protocols were approved by the Animal Health and Safety Committees of Loma Linda University (LLU) and were in compliance with Federal regulations. A schematic of the experimental time line is shown in Fig. 1B.

Animals

A total of 55 male Sprague-Dawley rats (65-75 days old) were randomized into five experimental groups: 1) sham (n=18), 2) single mTBI (Single, n=6), 3) repetitive mild TBIs (rmTBI) induced 1 day interval (n=6), 4) 3 days interval (n=19) or E) 7 day interval (n=6). Six animals /group underwent temporal MR imaging followed by histopathology. An additional cohort of shams (n=12) and rmTBI 3d apart (n=13) animals were used for neurological and behavioral testing. All rats were housed in a vivarium for a minimum of five days prior to TBI induction with a 12-hour light-dark cycle and free access to food and water.

TBI Induction

Rats were anesthetized with isoflurane (3% induction, 1-2% maintenance) and the head was secured in a stereotaxic frame. Lidocaine HCl 2% and Epinephrine (1:100,000) was administered by intradermal injection to the underlying skin followed by a midline incision and the skin was then retracted to expose the skull. A 5 mm craniotomy was performed 3 mm posterior and 3 mm lateral to bregma (Fig. 1A) to expose the right cortical surface. Care was taken to prevent disturbing the underlying dura and minimize bleeding at the craniotomy site. Animals were excluded if the dura was ruptured. A mild controlled cortical impact (CCI) was delivered using an electromagnetic driven piston with 4 mm diameter tip at a depth of 0.5 mm, speed of 6.0 m/s, and 200 ms contact duration (dwell) (Leica Biosystems Inc., Richmond, IL). For animals in the rmTBI groups, a second identical impact was delivered either 1, 3 or 7 days after the first CCI at the same craniotomy site. After each

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impact, a sterile nylon flap (~1 cm^2) was put on the top of impact site to prevent adhesive tissue reactions. The wound was closed with 5-0 nylon sutures and Buprenex (0.01mg/kg, IM) was administered to minimize pain and discomfort after TBI induction. Randomized sham animals underwent the same surgical procedures, but without CCI. Body temperature was maintained at 37±1°C with a heating pad during the surgery. Animals were placed in a warmed recovery chamber and monitored till they recovered from anesthesia.

MRI data collection and analysis

MRI assessments were performed on six animals in each group. *In vivo* MRI data were collected on a Bruker Advance 4.7T 30 cm horizontal bore instrument equipped with a 116 mm (internal diameter, ID) quadrature receiver coil (Bruker Biospin, Billerica, MA). Sequences were designed to capture multiple contrast levels including a T2WI (TR/TE/FA = 3453 ms/20 ms/20°, 25×1 mm slices) and a SWI (TR/TE/FA = 1248.8 ms/20 ms/20°, 32×0.8 mm slices), both which were acquired with a 256×256 matrix, a 3 cm field of view (FOV), and a contiguous interslice distance of 1 mm. MRIs were acquired 24 hrs after each CCI and 14 days after the initial CCI (Fig. 1B). For the MRI acquisition rats were anesthetized using isoflurane (3% induction, 1% maintenance) and body temperature was maintained at 37±1 ºC using a thermostat controlled heated water cushion.

High resolution e*x vivo* DTI data were acquired from 4% paraformaldehyde (PFA) fixed brains (Fig. 1B, see *Histopathology* below) using an 11.7T Bruker Advance 8.9 cm horizontal bore instrument equipped with a 89 mm (ID) receiver coil (Bruker Biospin, Billerica, MA). A T2WI (TR/TE/FA=1769.9 ms/16.2 ms/0°, 25×1 mm slices) and a diffusion sequence (TR/TE = 552.5 ms/15.1 ms) was acquired in seven directions with two *b*-values (43.34 and 2013.46 s/mm^2), a matrix of 256×256, and a 2 cm FOV.

All analysis was conducted blinded to the groups. *Cheshire* image processing software (Hayden Image/Processing Group, Waltham, MA), was used to manually outline whole brain and lesion volumes on T2WI images. Lesions were identified as hyper/hypo- signal intensities within the cortex (Fig. 1C). In a small

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subset of animals, edema was observed within ipsilateral corpus callosum (CC) 24 hrs after the initial mild impact and the edematous portion of CC was not included in our lesion calculation. Whole brain and lesion volumes (mm^3) were extracted and the percentage of T2WI-derived cortical lesion volume (%) was calculated by dividing the lesion volume by the brain volume. *Spin* image processing software (The MRI Institute for Biomedical Research, Detroit, MI) was used to generate SWI images by post-processing SWI magnitude and phase data using a 48×48 filter (Haacke et al. 2004). Regions of interest (ROIs) of hemorrhagic lesions were identified as hypointense signals on each slice using an operator guided threshold technique and volumes were extracted. Some SWI data were excluded from the analysis (n=3) due to phase artifacts that could not be adequately removed by filtering. The percentage of SWI-derived cortical lesion volumes (%) were calculated as SWI lesion volumes divided by T2WI defined brain volumes.

DTI images were analyzed using *Cheshire* to assess the microstructural integrity of the CC. Ipsilateral and contralateral ROIs were drawn on three slices under the lesion including the slice with maximal injury (M) and one slice anterior (A) and posterior (P) to the lesion (Fig. 1C). Data means were extracted for each ROI for relative anisotropy, axial diffusivity (mm^2/sec), radial diffusivity (mm^2/sec) and trace (equivalent to mean diffusivity, mm^2/sec).

Neurological and behavioral testing

Functional outcomes were assessed in an independent cohort of rmTBI 3d apart (n=13) and sham animals (n=12). Beam balance and foot fault scores were used to evaluate sensorimotor function 24 hrs after each mTBI and 14 days after the initial impact. In the beam balance test, animals were placed in the middle of a 1.3 cm wide Plexiglas sheet (61 cm long, 48 cm high) and the following data were collected and summed over two 60 sec trials: A) fall latency (time it took the rat to fall off the beam), B) total distance travelled (5 cm intervals), C) number of left and D) right turns (both shoulders changed direction). For the foot fault test, rats were placed atop an elevated (1m) wire ladder (30 cm wide, 145 cm long, with 2 mm diameter rungs at 2.5 cm apart) where

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the total number of times a limb fell or slipped through the grid was recorded and summed over two 60 sec trials (Baskin et al. 2003).

We hypothesized that 14 days after the initial injury might be too early to see neurobehavioral changes associated with rmTBI. Thirty days after the initial mTBI activity levels were assessed using the open field test and learning and memory was assessed using a water maze test (Recker et al. 2009) in the same cohort of rmTBI 3d apart and sham animals. Briefly, activity levels were assessed by recording the animals for 30 minutes in an open field and analyzing their movements with a computerized tracking system (Noldus Ethovision 3.1, Wageningen, Netherlands). The water maze consisted of a pool of water containing a small platform that the animal could find and climb onto it. Cued learning, in which the escape platform's surface was visible 2 cm over the surface of the water, was performed to determine whether the animals could see, swim, and were motivated to find the platform. Ten trials (60 sec maximum) were administered with a 30 sec inter-trial interval. The platform's location was changed after every 2nd trial. Spatial learning, in which the escape platform's surface was submerged 2 cm below the water's surface, was tested over the next 3 days. The water was made opaque with non-toxic black tempera paint to obscure the platform's location. The animals were given 10 trials (60 sec maximum), with a 30 sec inter-trial interval per day. The platform's location remained in the same location for all 10 trials, but changed on subsequent days. A probe trial, in which the platform was removed and the animal allowed to swim freely for 60 sec, was administered at the end of each day. To determine whether a more difficult task might highlight potentially subtle spatial learning differences, a separate subset of animals (n=3 per group) underwent an additional accelerated water maze paradigm, in which they were required to learn different 3 locations (4 trials each) in one day.

Histopathology

After the final *in vivo* MRI (Day 14, Fig 1B), animals were anesthetized with a mixture of ketamine hydrochloride (90mg/kg, Vedco, Inc.) and xylaxine (10mg/kg, Vedco, Inc.). When a surgical level of anesthesia

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was reached the animals were perfused intracardially with a chilled (~8°C) fixative solution of 4% PFA in phosphate buffered saline (PBS) with a pH of 7.4. Following perfusion, brains were removed from the skull and postfixed overnight in 4% PFA at 4°C. The fixed brains underwent two 30 min rinses in PBS and were then stored in PBS at 4°C until *ex vivo* MR imaging was performed and they were prepared for cutting. Prior to sectioning the brains were soaked in 30% sucrose at 4°C for 24 hrs followed by embedding in optimal cutting temperature compound (O.T.C., Tissue Tek; Sakura Fine Tek, Torrance, CA) at -80°C. Frozen sections were sliced beginning at the center of lesion and progressed caudally through the lesion using a Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar Germany) at a thickness of 30 μm. Histology sections were mounted directly on gelatin-chrome-alum-coated slides and slices for immunohistochemistry were stored as free-floating sections in cryoprotectant at 4°C.

Cresyl violet acetate staining (CV) was used to determine the presence of tissue damage. To validate MRI observed hemorrhage Prussian blue (PB) staining was used to identify the presence of iron. A simplified PB scoring system was used to assess the amount of blood on tissue slices where the lesion was maximal. A point was given for the presence of a cortical lesion and a point was given for positive PB staining in each of the following regions: ipsilateral cortex, contralateral cortex, ipsilateral CC and contralateral CC. A total score of five points was possible with higher scores indicating more extravascular blood within the injured tissues.

Immunohistochemistry was performed on free floating sections at the level of the maximal cortical lesion. Sections were first incubated in PBS with bovine serum albumin (BSA, 1%) three times to block endogenous peroxidase activity and then incubated in PBS containing 0.25% BSA with 0.25% Triton X-100 for 2 hrs at room temperature on a shaker to block nonspecific staining. Sections were incubated in the primary antibodies of 1) mouse anti-glial fibrillary acidic protein (GFAP, Millipore, Temecula, CA, 1:400) ; 2) rabbit anti-ionized calcium binding adaptor molecule 1 (IBA1, Dako, Carpinteria, CA, 1:400); 3) rabbit anti- neurofilimamment 200 (NF 200, Sigma-Aldrich, St Louis, MO, 1:1000) and 4) mouse anti-myelin basic protein (MBP, Millipore,

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Temecula, CA, 1:1000) overnight at 4°C, respectively. Sections were washed three times in PBS prior to incubation with the secondary antibody conjugated to either goat anti-mouse Alexafluor AF488 (Invitrogen, Carlsbad, CA, 1:400) or goat anti-rabbit rhodamine (Millipore, Temecula, CA, 1:200) for 2 hrs at room temperature and protected from light. Sections were then washed in PBS. After allowing tissue to air dry at room temperature, slides were mounted with Vectashield Hardmount (Vector Laboratories, Inc. Burlingame, CA) for epifluroescence imaging (Olympus IX2-UCB, Olympus America Inc., Center Valley, PA). Randomly selected tissue samples from each group were processed concurrently. For negative controls the primary antibody was omitted during the staining procedure.

Statistics

All statistical analyses were performed with SAS v9.1.3 (SAS, Cary. NC, USA). The distribution of quantitative MRI measures was tested for normality with a Kolmogorov-Smirnov test. Distributions failing to meet the requirements of normality were analyzed using nonparametric measures. Appropriate nonparametric post hoc comparisons based on median ranks were performed for groups of unequal size. The Kruskal Wallis test was used to determine whether there was a significant difference between the study groups at each time point post injury.

For DTI, there were no statistical differences in mean values between hemispheres; therefore, we combined the data from the ipsi- and contralateral CC for each animal. Body weight and T2WI lesion volumes and area of the CC were considered as covariates for statistical modeling of group differences in DTI measures. Neurological and behavioral testing data were analyzed using repeated-measures ANOVA. All tests of hypotheses were two-sided and conducted at an alpha level of 0.05. Data were presented as Means ± SEM.

Results

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There was no mortality and no significant differences in body weights were between mTBI (332±22 g) groups and shams prior to first surgery (326±14 g). Over the 14 days post-initial mTBI comparable increases in body weights were observed in all injured animals (16±3.8 g) and shams (17±4.1 g).

Lesion volumes and tissue characteristics derived from T2WI

At 24 hrs after the initial impact, mTBI induction resulted in a 2.5% T2WI-derived cortical lesion volume of whole brain volume. The observed hyperintensities on T2WI suggested mild edema at the injury site (Fig. 2, 3). There were no significant differences in lesion volumes between any of the mTBI experimental groups after the first injury. At 24 hrs after a second mTBI, a significantly larger cortical lesion volume was found in the 1d and 3d apart rmTBI groups relative to single mTBI and sham animals (Figs. 2, 3A). While rmTBI 1d apart was primarily associated with edema (hyperintensities) on T2WI, edema and hemorrhage (hypointensities) were evident in the rmTBI 3d apart animals (Fig. 2). The increased extravascular blood in the rmTBI 3d apart group persisted for the 14 days experimental duration (Fig. 3B). When a second mTBI was delivered 7d apart, it also resulted in an increase in lesion volume within the ipsilateral cortex, but was not statistically different from shams or single mTBI (Figs. 2, 3).

Hemorrhage volume identified by SWI

At 24 hrs after the initial mTBI, SWI-derived hemorrhagic (hypointensity) lesion volumes (0.53±0.08%) were not statistically different from shams (0.02±0.01%). RmTBI 1d or 3d apart resulted in a significant increase in SWI-observable hemorrhage at 24 hrs after the second impact which persisted to the 14 day end point (Fig. 4). Consistent with the T2WI-derived total lesion volume, the time window of brain vulnerability to the second mild impact was most evident in the 3d apart rmTBI group, where the hemorrhagic volumes were increased 3 fold compared to a single mTBI. Cerebral hemorrhage was also increased when a second mTBI was delivered 7d later, but was not significantly different from single mTBI or shams.

Microstructural damage within corpus callosum identified by DTI

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There was no statistical significance in laterality (ipsilateral vs contralateral) for all DTI metrics at 14 days after the initial mild injury. Group differences were only found in DTI indices of axial diffusivity and trace, where posthoc pairwise comparisons revealed that repetitive mTBI (1, 3 or 7d) animals exhibited significantly decreased axial diffusivity and trace compared to single or sham injured animals (Table 1). Single mTBI animals exhibited significantly increased axial diffusivity and trace compared to shams (Table 1). Although rmTBIs animals were also associated with decreased relative anisotropy, this did not reach statistical significance compared to single and shams (Table 1).

Neurological and behavioral testing

No neurological deficits were observed in the beam balance and foot fault tests in the animals receiving rmTBI 3d apart either at 24 hrs after the second injury or at 14 days after the initial impact (data not shown).

At 30 days post the initial mTBI, the distance that the rmTBI 3d apart animals moved was significantly less compared to shams (Fig. 5A, B) in the open field test. Our standard water maze testing protocol (5 day testing regime) did not show differences in swim speed or turn bias (a measure of hemiparesis) between the rmTBI animals and shams (Fig. 5C). However, when 3d apart rmTBI animals were cognitively challenged using an accelerated water maze test (1 day regime), they displayed significant learning deficits ($p<0.05$) at the 3rd spatial location (Fig. 5D).

Histopathology

Confirming our macroscopic structural MRI findings, tissue sections stained with Cresyl violet showed that rmTBI 3d apart had the largest cortical lesion compared to single rmTBI and sham animals while rmTBI 7d apart animals did not have a significant increase in tissue damage relative to single mTBI (data not shown). Prussian blue staining confirmed significant greater iron deposition in brain tissue subjected to rmTBI that was most prominent in the 3d apart rmTBI animals (Fig. 6A, B). SWI-derived hemorrhagic lesion volumes *in vivo* correlated significantly ($p<0.01$, $r=0.85$) with histological evaluation of iron deposition (Fig. 6C).

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Post-injury gliosis was observed in all TBI groups (single and rmTBI) at 14 days after the initial impact. Increased GFAP immunostaining was localized around the impact site at the ipsilateral cortex (Fig. 7). However, increased astrogliosis was evident in the 1 or 3d apart rmTBI group relative to the 7d apart and single mTBI animals. We also observed increased microglial activation was also more evident in rmTBI 1d or 3d, but not 7d group compared to shams and single mTBI animals using IBA1 immunostaining (data not shown).

Axonal microstructure was assessed by antisera against heavy neurofilament protein (NF200). At 14 days post initial mTBI, evidence of traumatic axonal injury was associated with either a single mild injury or rmTBI. Axonal bulb retractions and swelling was observed within the corpus callosum (Fig. 8) with increased punctate NF200 staining evident in animals subjected to rmTBI. Contiguous axonal swelling along with reduced immuorreactivity was observed in single mTBI animals. Staining intensity for MBP was comparable across all four experimental groups and shams, consistent with the relative preservation of myelin microstructure at 14 days posts the first mTBI (data not shown).

Discussion

Repetitive mTBI exacerbated clinically observable outcomes, confirming our hypothesis that there is a period of increased vulnerability to a second traumatic event. Based on our MR derived observations, we demonstrate that 1-7 days between two episodes of mild injury resulted in cumulative harm using a number of measures. Multimodal MRI found dynamic signatures of tissue damage following rmTBI at variable intervals, which were associated with chronic behavioral impairments. Our novel findings are: 1) significantly increased lesion and hemorrhagic volumes in the 1d or 3d apart rmTBI groups, but not in the 7d apart cohort. These differences persisted to the end time point of our study; 2) neurobehavioral impairments were observed at 30 days after the initial mTBI in the 3d apart rmTBI group in the absence of sensorimotor deficits, 3) microstructural changes within the corpus callosum were associated with rmTBI, with significantly decreased axial diffusivity, suggestive of ongoing diffuse axonal injury; and 4) histopathology correlated with MRI findings.

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Macroscopic structural changes following rmTBI

In the absence of frank structural damage, human mTBI is typically associated with post-traumatic edema (Tokutomi et al. 1997), reduced cerebral blood flow (Golding et al. 1999) and altered cellular metabolism (Masdeu et al. 1994; Vagnozzi et al. 2010). The mild nature of our novel rat model of mTBI was validated by the subtle observation of edema and bleeding.

Post-concussive pathophysiological processes involve a complicated cascade of cellular metabolic disturbances may exist in the absence of overt clinical symptoms (Barkhoudarian et al. 2011). Many of these events (i.e. calcium influx, glutamate excitotoxicity) may enhance brain vulnerability to a secondary insult (Jenkins et al. 1989; Vagnozzi et al. 2005). Second-impact syndrome has been associated with athletes who suffer repeated concussions when playing contact sports. In these individuals, a second concussion can result in massive brain swelling, subdural hematoma, increased intracranial pressure, and occasionally death before symptoms from an earlier concussive event have subsided (Bailes and Cantu 2001). Swelling and edema are often seen in rmTBI patients (Kelly et al. 1991). Clinical imaging studies of professional boxers with a history of multiple concussions also have shown a higher prevalence of pathological lesions (Ross et al. 1987).

In our rat model of rmTBI, we observed exacerbated MRI measures in animals receiving rmTBI, especially 1 or 3 days apart. There was significantly increased tissue damage at 24 hrs after a second mTBI that persisted up to 14 days later compared to single mTBI and sham-injured animals. The size of lesion was less than 5% total brain volume affirming the mild nature of our model in terms of the level of macroscopic structural damage. Given that the tissue and cellular pathological processes are distinct at variable time points post mTBI, the vulnerability of the brain to a second mTBI is critically sensitive to the interval between subsequent TBI events. While rmTBI 1d apart was primarily associated with brain edema, the dramatic appearance of intra-cerebral hemorrhage in the 3d apart rmTBI animals suggested enhanced brain vulnerability. Our neuroimaging results echo previous histopathologal studies of rmTBI. Similar to our study, in a mouse model of repetitive brain

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injury 24-hours apart, there was a significant increase in cerebral edema (Laurer et al. 2001), but when the interconcussion interval was 3 days (Longhi et al. 2005). When the interval was increased between injuries (i.e. rmTBI 7d apart) we did not observe significant exacerbation of tissue damage compared to single mTBI animals. Our results suggest that enhanced brain vulnerability to a second injury occurs within the first 3 days after a mild impact, namely macroscopic tissue damage. Using metabolic measures a similar time window of vulnerability to repetitive experimental concussive brain injury has also been reported by Vagnozzi and colleagues (Laurer et al. 2001; Vagnozzi et al. 2005). The extent of the biochemical modifications was associated with the temporal interval between consecutive concussive injuries and influence overall injury severity (Vagnozzi et al. 2005). When a second TBI was delivered 3 days apart, N-acetylaspartate (NAA) and adenosine 5'-triphosphate (ATP) concentrations were comparable to rats receiving a single severe TBI (Vagnozzi et al. 2005). Thus, a shorter temporal interval between two episodes of mTBI resulted in a more significant cellular metabolic perturbation that may lead to more severe macroscopic tissue damage found in our 1d or 3d apart rmTBI animals.

Microstructural changes in the corpus callosum

Disruption of white matter integrity (axonal injury) leading to cerebral functional disconnection has been implicated in cognitive impairments that are related to TBI severity (Spain et al. 2010). Diffusion tensor imaging (DTI) is a sophisticated MRI technique that measures white matter axonal and myelin integrity by sampling the directionality of water diffusion (Beaulieu 2002). In our study we chose the corpus callosum as it is the largest commissural white matter bundle in the rat brain and is sensitive to traumatic axonal injury (Parizel et al. 1998).

To the best of our knowledge we are the first to utilize DTI to assess white matter integrity following multiple concussions in an animal model of rmTBI. Two episodes of mTBI with variable intervals (1, 3 or 7d apart) revealed significantly reduced axial diffusivity relative to single mTBI and shams, suggesting that cumulative

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axonal injury occurs. Unlike edema or the appearance of hemorrhage, axonal vulnerability to rmTBI was also observed in the 7d apart group. Decreased axial diffusivity has been correlated yo histological detection of axonal injuries in rat model of moderate to severe traumatic brain injury (Mac Donald et al. 2007). Such axonal injuries were characterized by an accumulation of β amyloid precursor protein (APP) in axonal retraction bulbs. The mean diffusivity reflects the overall cellular composition and tissue structure (Basser and Pierpaoli 1996), and our decrements in mean diffusivity are consistent with a disruption of axonal structural integrity (decreased axial diffusivity).

It is notable that we also observed a significant increase in corpus callosum axial diffusivity in single impacted animals at 14 days post-injury. Axonal degeneration over time has been shown to underlie the increased white matter axial diffusivity in chronic mTBI patients (Kraus et al. 2007). Such elevations in axial diffusivity in our single mTBI group were likely due to chronic evolution of injury rather than recovering axonal pathology. Axonal atrophy increases the interstitial space and the overall water mobility (Buki and Povlishock 2006). Our significantly increased mean diffusivity further supports the conclusion of chronic axonal injury. In mTBI patients with chronic postconcussive symptoms, axonal injury in white matter tracts also resulted in increased mean diffusivity (Salmond et al. 2006; Kumar et al. 2009).

Increased radial diffusivity has been associated with disrupted myelin sheaths (Song et al. 2003). We observed no change in this DTI metric in any of the groups studied, suggesting that at the 14d time point post injury there exists a relatively intact myelin microstructure. Irreversible demyelination is usually a chronic pathological process that occurs secondary to axonal injury (Kraus et al. 2007; Mac Donald et al. 2007). Myelin damage is typically either spared or reversible after a single mTBI (Kraus et al. 2007; Kumar et al. 2009).

Unlike most reports in the TBI literature (Kumar et al. 2009; Niogi and Mukherjee 2010), the relative anisotropy in our repetitive impacted animals did not differ significantly from single mTBI and sham animals. Relative anisotropy reflects the degree of alignment of cellular structures within the fiber tracts and their

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structural integrity (Basser and Pierpaoli 1996), which takes into account the directional composition and extent of changes in the axial and radial diffusivity (Beaulieu 2002). The complexity of the variable stages of pathology in axons and myelin following rmTBI may compromise the sensitivity of relative anisotropy as a TBI index in our mild TBI model. The exact biological basis underlying these DTI detectable biophysical alterations within white matter need to be further explored.

Functional changes

Animal studies of mTBI have shown selective behavioral deficits in the absence of neurological impairments (Hogg et al. 1998; Zohar et al. 2003). In the present study, although MRI identified significantly increased macrostructual damage following rmTBI, the relatively small lesion size resulted in undetectable sensorimotor dysfunction at 14 days post injury. However, long-term behavioral changes were observed when we evaluated a cohort of rmTBI animals 30 days post-initial injury. The open field data revealed reduced spontaneous exploratory activity, which others have reported to be associated with depressive-like behavior (Sousa et al. 2004). Depressive-like states have been demonstrated in animals following experimental mTBI or rmTBI (Milman et al. 2005; Shultz et al. 2011). Our results are similar to clinical observations of increased risk of depression as a potential neurological consequence of recurrent sport-related concussions (Guskiewicz et al. 2007). Future experiments using the Porsolt forced swim test (Shultz et al. 2011) could be helpful to assess more accurately those affective disorders associated with rmTBI.

Multiple episodes of mTBI have been shown to result in impaired cognitive performance in mice (DeFord et al. 2002; Creeley et al. 2004; Shultz et al. 2011) and in a transgenic mouse model of Alzheimer amyloidosis (Uryu et al. 2002). A number of clinical studies performed over the past decade suggest that cognitive performance is significantly poorer in patients who have one or more concussions than those without a history of concussion (Guskiewicz et al. 2005; McCrory 2011). Chronic cognitive impairments, as seen in boxers, hockey and football players are associated with accelerated and/or increased neurodegeneration (Bailes and Cantu 2001; Guskiewicz

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et al. 2005; McCrory 2011). In our study, the spatial learning and memory impairments were only observable at 30 days after the initial impact in the rmTBI 3d apart group, corresponding to increased tissue lesion volumes, blood deposition and astrogliosis seen at 14 days post-injury. It is interesting that the mild cognitive deficits were only uncovered by a more challenging cognitive paradigm, implicating subtle cognitive alterations. In human studies, early stage behavioral deficits also go undetected in standard neurological and/or neuropsychological tests. However, such altered cognition may evolve over time as neuronal and/or white matter degeneration progresses, leading to chronic cognitive deficits. Similarly, others also reported a slow progression of mild cognitive deficits following multiple episodes of mTBI, which were attributed to brain β APP accumulation and oxidative stress (Laurer et al. 2001; Uryu et al. 2002). Long-term studies are warranted for monitor the time course of developing behavioral impairment following rmTBI.

Histopathology correlated to MRI findings

Histopathological assessment of brain tissue confirmed increased tissue damage in animals subjected to rmTBIs 1d or 3d apart that was not seen in the rmTBI 7d apart animals. The degradation products of the red blood cells containing iron were detected by Prussian blue staining, which strongly correlated with SWI-derived hemorrhagic volumes *in vivo*. Similar to our own findings, increased long-term iron accumulation was also reported at 16 weeks after rmTBI in a mouse model of Alzheimer's disease (Uryu et al. 2002).

Pathological TBI insults trigger glial cell responses and we found an increase astrogliosis primarily in brains subjected to second mild impact either 1d or 3d apart. The increased staining intensity can be attributed to either greater glial cell numbers or a hypertrophic glial morphology. It is well accepted that astrocytic activation contributes to the secondary injury process, as well as neuronal repair mechanisms (Laird et al. 2008). Activated astrocytes may release proinflammatory mediators and chemokines to attract inflammatory cells to the site of injury, resulting in further tissue damage (Csuka et al. 2000). Hamberger et al also demonstrated increased reactive astrocytosis in the cerebral cortex in an animal model of concussion ((Hamberger et al. 2009). In our

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study, rmTBI resulted in exacerbated astrocytic responses 14 days after the initial insult, accompanied by microglia accumulation. Such gliosis was greatest in animals with intervals of 1d or 3d between two mTBI events but not 7d. Abundant astrocytic scarring around the lesion may impede recovery by preventing axonal repair (McGraw et al. 2001).

Consistent with our DTI findings, axonal, but not myelin injuries were identified within corpus callosum tissues following mTBI at 14 days after the initial injury. Intact neuronal cytoskeleton, formed by microtubules and neurofilaments (NFs), are essential for axonal transport (Hamberger et al. 2003), where trauma can lead to axonal swelling and cytoskeletal damage, resulting in focal accumulations of NFs and impaired axonal transport (Smith et al. 1999). Repeated mild injury results in diffuse axonal injury in the corpus callosum and subcortical white matter where NF200 accumulates in the neuronal perikaya 10 days after impact (Hamberger et al. 2009). Following mild closed head rotational acceleration injury, there is also evidence of accumulation of NF200 in damaged axons in both single and multiple impacted animals (Raghupathi et al. 2004). We observed the increased punctuate NF200 in axons suggesting the existence of axonal bulb retractions within the corpus callosum following rmTBI. This may result in our observed decreased axial diffusivity. In the single impacted animals, NF200 accumulation appeared in a contiguous pattern consistent with axonal swelling. The reduced NF200 immunoreactivity may also be representative of axonal degeneration over time. Consistent with our findings of relative normal radial diffusivity, intact myelin microstructures were observed by normal MBP staining.

Conclusions

Our novel rat model of rmTBI can serve as a platform for investigation into the temporal evolution of neuropathology following rmTBI. The outcomes obtained in the study provide the basis for further testing of putative therapeutic strategies. In summary, our findings suggest that rmTBI, induced cumulative injuries that are clearly dependent upon the interval between traumatic events. While animals exhibited heightened

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vulnerability to macrostructural tissue damage to a second mild traumatic insult up to 3 days after an initial mTBI, exacerbation of white matter damage appeared to be detectable by DTI even in the 7d apart rmTBI group. The underlying tissue histopathology correlated with MRI results. MRI is a sensitive neuroimaging biomarker for monitoring the pathological evolution after repetitive mTBI that can be rapidly translated into the clinic for military and sport induced injury assessments.

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Table 1: DTI metrics within corpus callosum (CC)

Group	Axial Diffusivity	Radial Diffusivity	Relative Anisotropy	Trace
Sham	0.483±0.009	0.193±0.004	0.505±0.010	0.860±0.013
Single mTBI	0.592±0.013*	0.226±0.005	0.528±0.018	1.059±0.019**
rmTBI 1d apart	0.424±0.009**††	0.187±0.005	0.481±0.014	0.798±0.016**††
rmTBI 3d apart	0.435±0.011††	0.216±0.013	0.466±0.010	0.836±0.019††
rmTBI 7d apart	0.395±0.027**††	0.189±0.011	0.434±0.029	0.772±0.043*††

*p<0.05, **p<0.01 vs Sham; ††p<0.01 vs Single mTBI

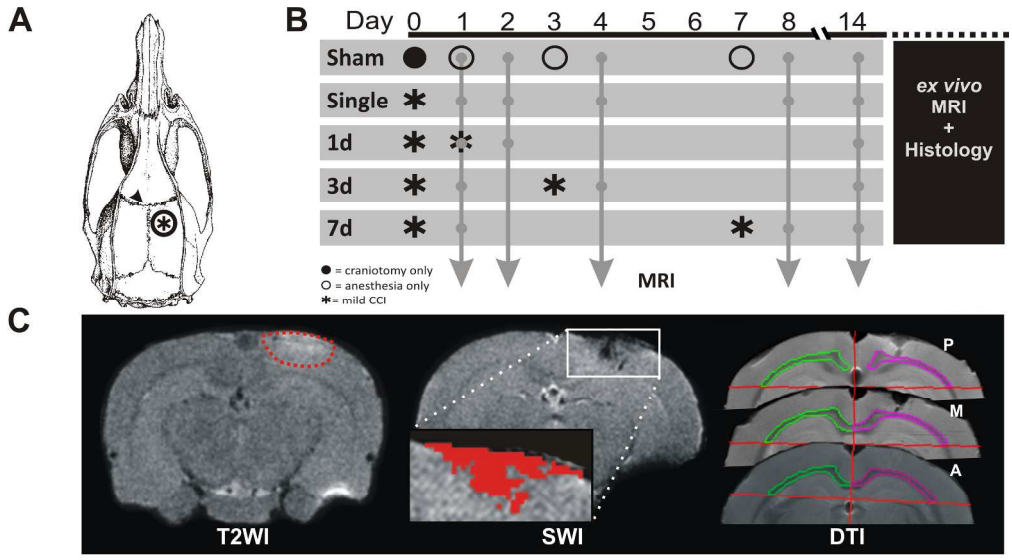

Fig. 1

Fig. 1 Experimental design and methods. A) Injury location: the brain was exposed via a 5 mm diameter craniotomy (circle) 3 mm lateral and 3 mm posterior to bregma (arrow head) on the right hemisphere; B) Time line: craniotomies were performed on all animals on day 0. This was followed by the first mild controlled cortical impact (CCI, asterisk) with 4 mm tip and 0.5 mm depth for TBI animals. A second mild TBI was performed 1, 3, or 7 days after the first TBI in the rmTBI groups. *In vivo* MRI's (gray dots) were acquired 24 hrs after each surgery and at the final 14 day time point. *Ex vivo* MRI's were acquired brain prior to histological examination; C) Regions of interest on multi-modal MRI: Cortical signal abnormalities (hyper- and hypo-intensities) were drawn manually (red dotted line) on T2WI's. An operator-guided thresholding method was used to segment hemorrhages (inset) on SWI's. For DTI analysis, the right (magenta) and left (green) corpus callosum was outlined on three slices at the level of the maximal lesion (M) with an additional slice anterior (A) posterior (P).
254x149mm (300 x 300 DPI)

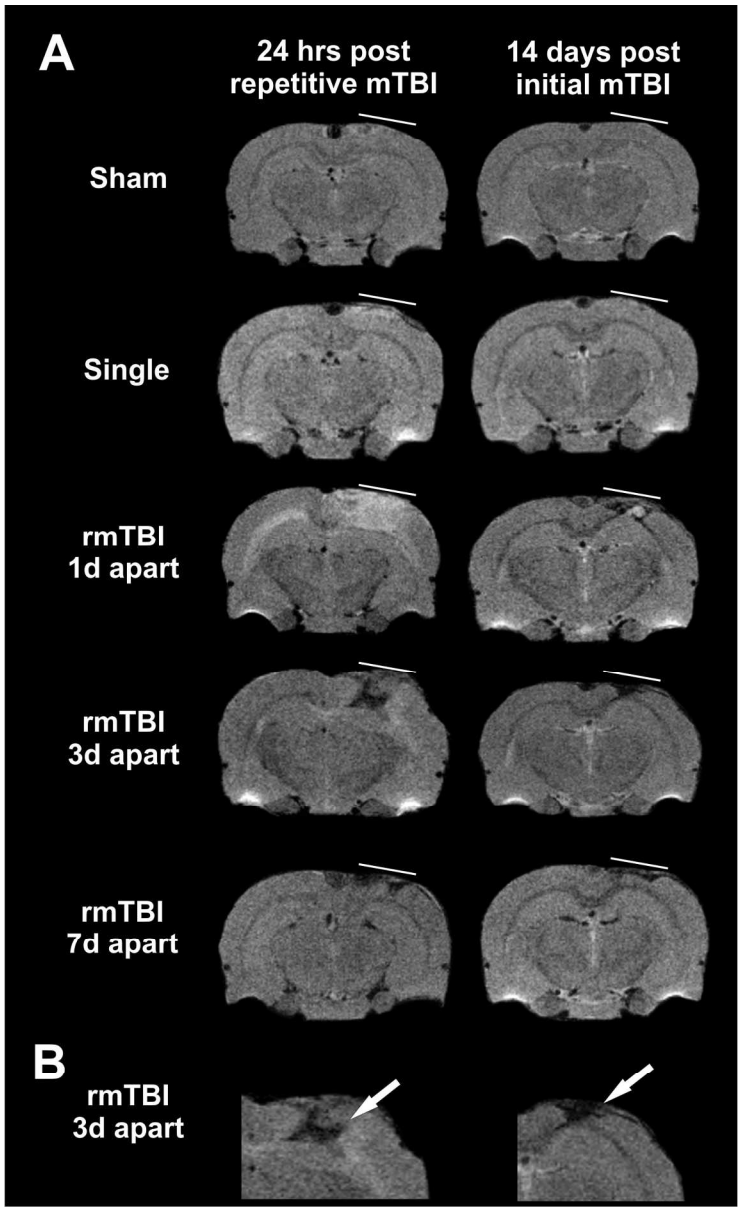

Fig. 2 Representative T2WI images revealed cumulative injury following rmTBI. A) rmTBI 1d or 3d apart resulted in increased tissue damage including edema (hyperintersity) and hemorrhage (hypointensity) 24 hrs after injury induction, which persisted to 14 days after the initial impact compared to sham and single mTBI animals. These changes were not as evident in rmTBI 7d apart injured animals; B) There was an increased hemorrhagic component (arrows) that was most prominent in animals receiving rmTBI 3d apart. Lines indicate the location of craniotomy and CCI.
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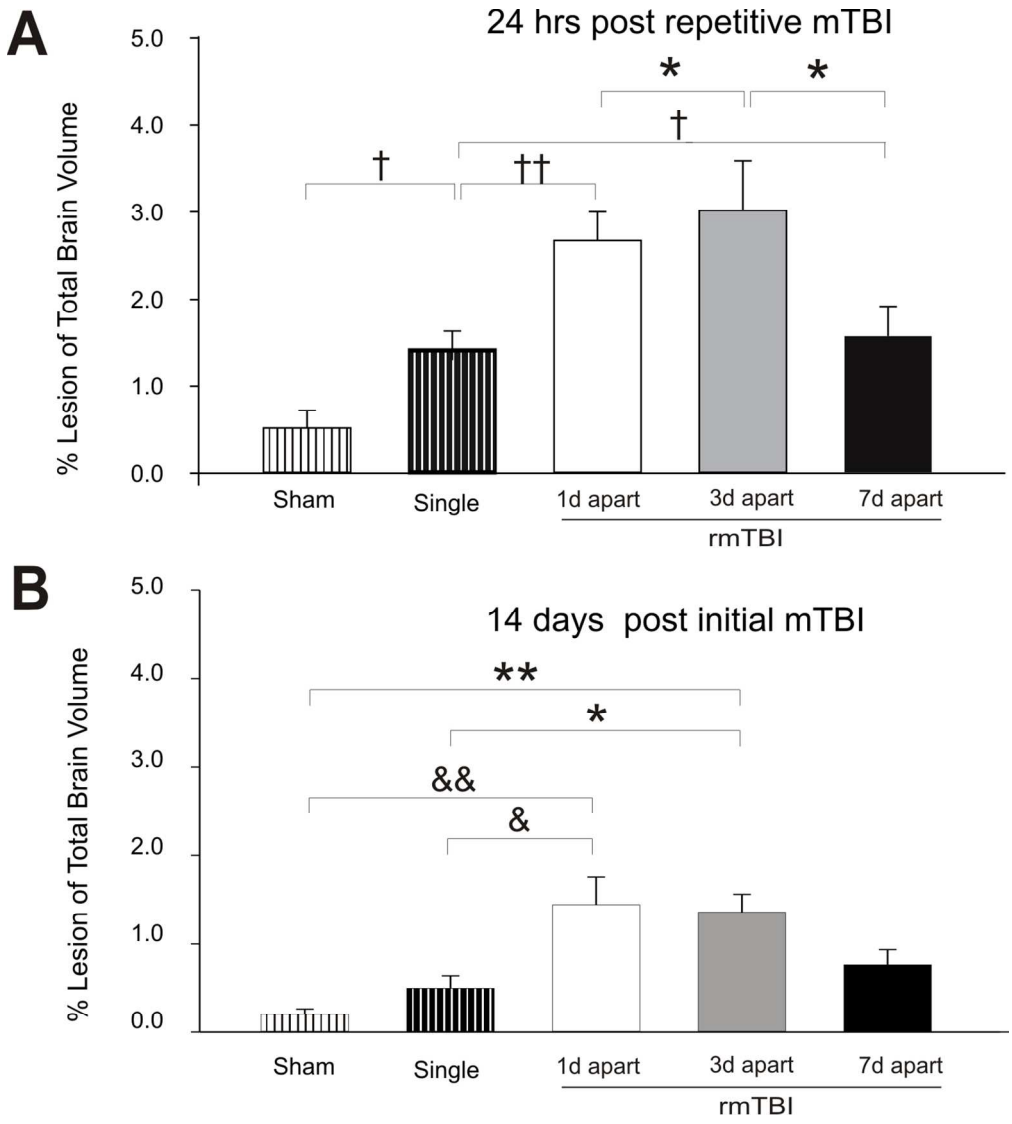

Fig. 3 T2WI-derived lesion volumes after rmTBI. Significant increases in cortical lesion volumes 24 hrs after rmTBI (A) were still present 14 days after the initial impact (B). $^{*}p<0.05$, $^{**}p<0.01$ vs rmTBI 3d apart; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs Single; $^{\&}p<0.05$, $^{\&\&}p<0.01$ vs rmTBI 1d apart.
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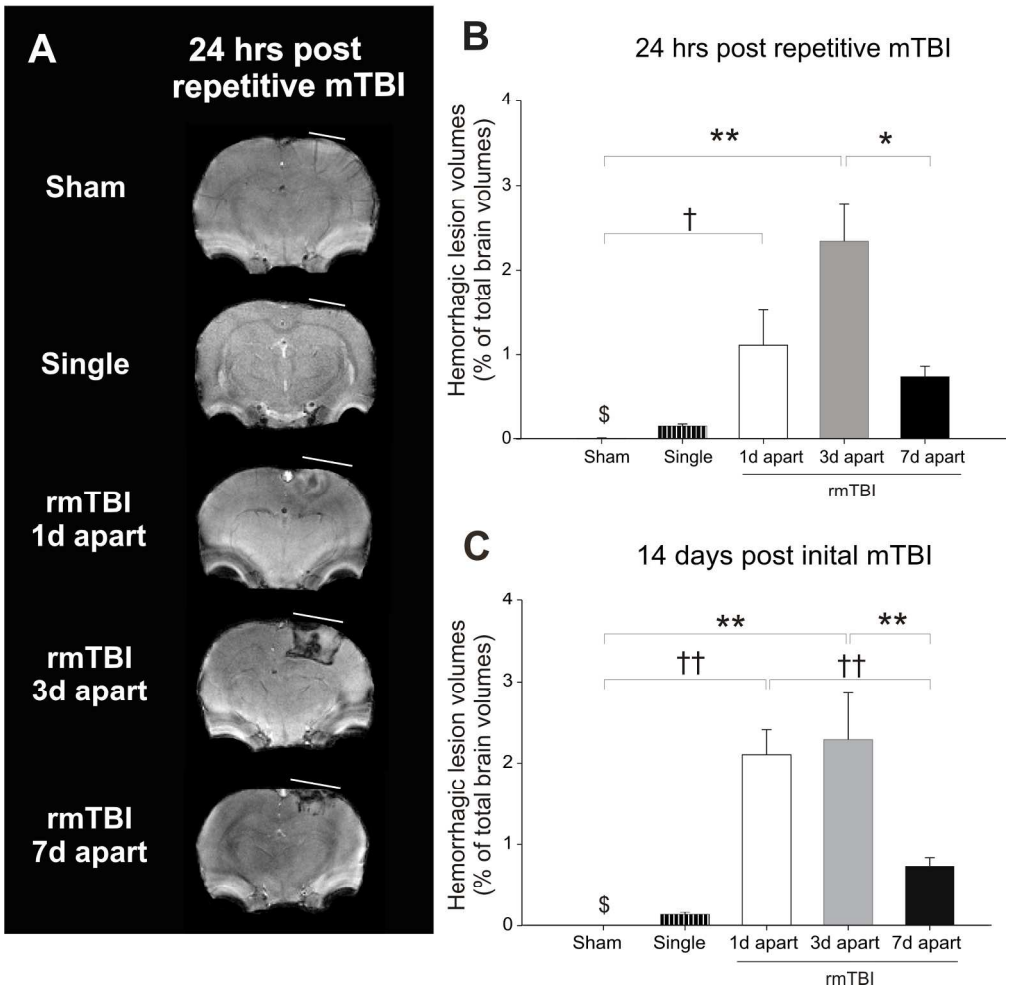

Fig. 4 SWI-derived hemorrhagic lesion following rmTBI. Representative SWI images revealed that rmTBI resulted in greater hemorrhage (hypointensity) within the traumatized brain tissue 24 hrs after injury (A). Quantifying lesion volumes revealed that the significant increases in hemorrhagic lesion volumes 24 hrs after rmTBI (B) were persistent to 14 days after the initial impact (C). The hemorrhagic vulnerability was most prominent in animals receiving rmTBI 3 days apart. $^{*}p<0.05$, $^{**}p<0.01$ vs rmTBI 3d apart; $^{+}p<0.05$, $^{++}p<0.01$ vs rmTBI 1d apart. Sham hemorrhage volumes were less than 0.05% ($). Lines indicate location of craniotomy and CCI.
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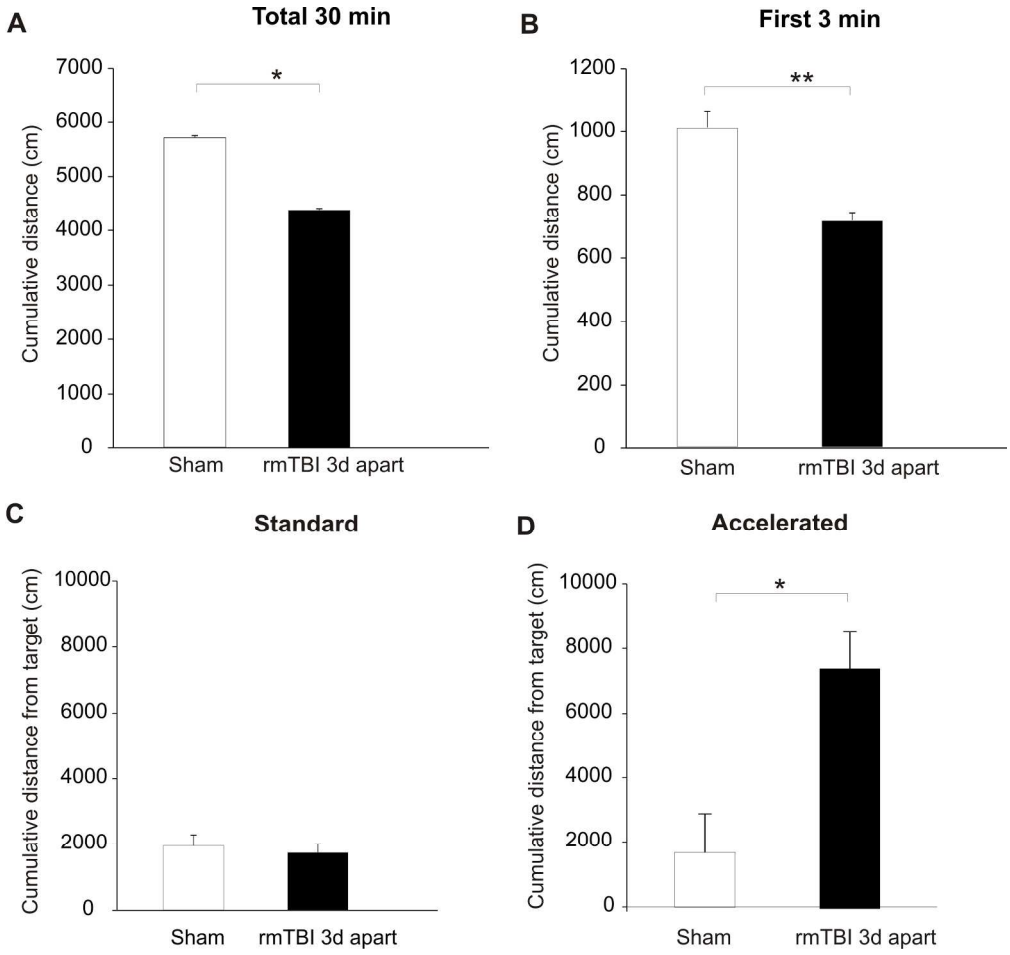

Fig. 5 Open field and water maze tests in rmTBI 3d apart animals at 30 days after the initial mTBI. A) The overall distance traveled over 30 min (10 blocks of 3 min) in the 3d apart rmTBI group was significantly less compared to shams; B) The distance traveled in the first 3 min, a marker of exploratory behavior in a novel environment, was also significantly decreased in rmTBI animals compared to shams; C) No differences were observed using the standard spatial learning water maze protocol; D) An accelerated water maze protocol that increased cognitive demands revealed spatial learning deficits in spatial memory in rmTBI animals compared to sham animals. $^{*}p<0.05$, $^{**}p<0.01$ vs Sham.
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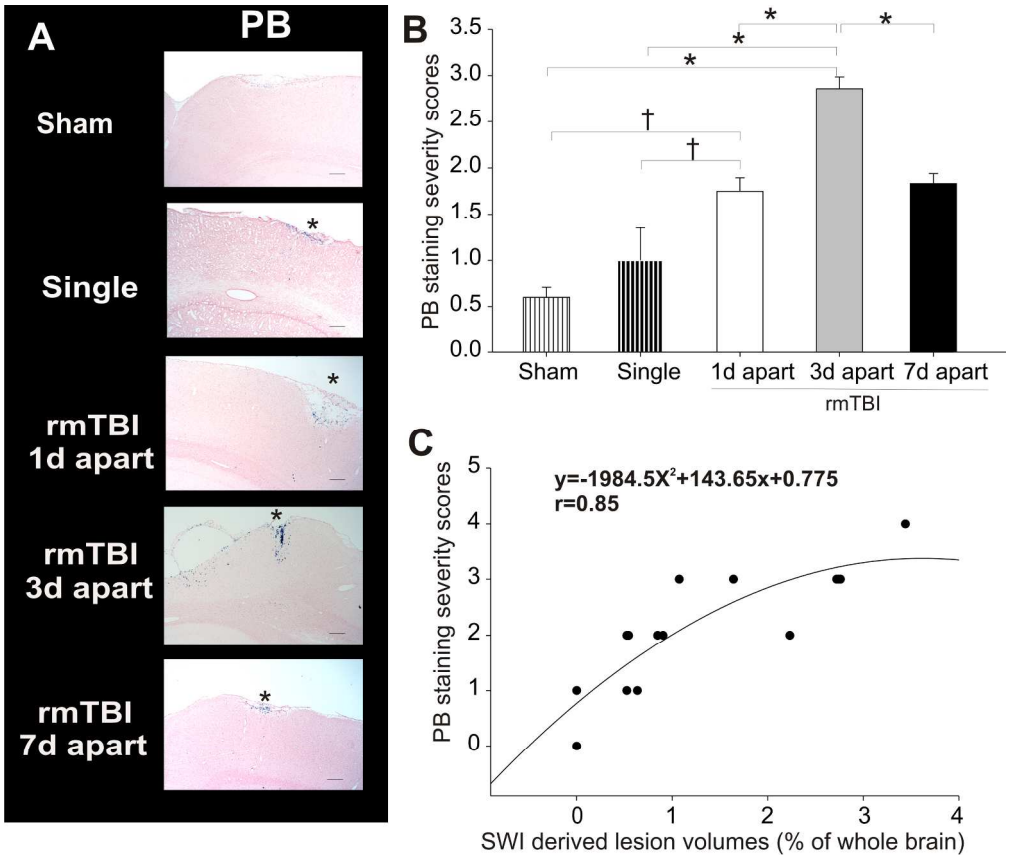

Fig. 6 Evaluation of iron deposition in tissues at 14 days after the initial mTBI. A) Representative microphotographs of Prussian Blue (PB) staining. There was increased lesion size and iron deposition in brains subjected to rmTBI at either 1 or 3 days apart; B) Scoring of PB stained sections revealed that rmTBI resulted in significantly greater iron staining (extra-vascular blood), compared to sham or single mTBI; C) SWI-derived hemorrhagic lesion volumes (%) significantly ($p=0.002$) correlated with histologically identifiable-iron deposition, which validated the sensitivity of SWI as an in vivo MRI biomarker for hemorrhage following rmTBI. $^{*}p<0.05$ vs rmTBI 3d apart, $^{\dagger}p<0.05$ vs rmTBI 1d apart and rmTBI 7d apart.
224x190mm (300 x 300 DPI)

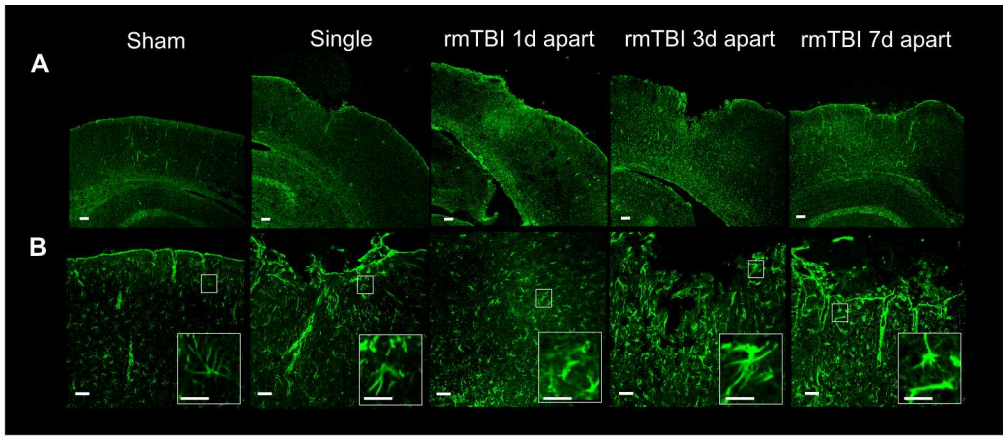

Fig. 7 Representative microphotographs of GFAP staining 14 days post initial mTBI. A) Increased staining intensity within the ipsilateral cortex after rmTBI revealed astrocytic gliosis; cal bar=200 µm; B) Higher magnification illustrates the morphological changes of the astrocytes; cal bar=50 µm. Hypertrophic astrocytes were more evident in rmTBI 3d apart (see inserts, cal bar=25 µm).
249x108mm (300 x 300 DPI)

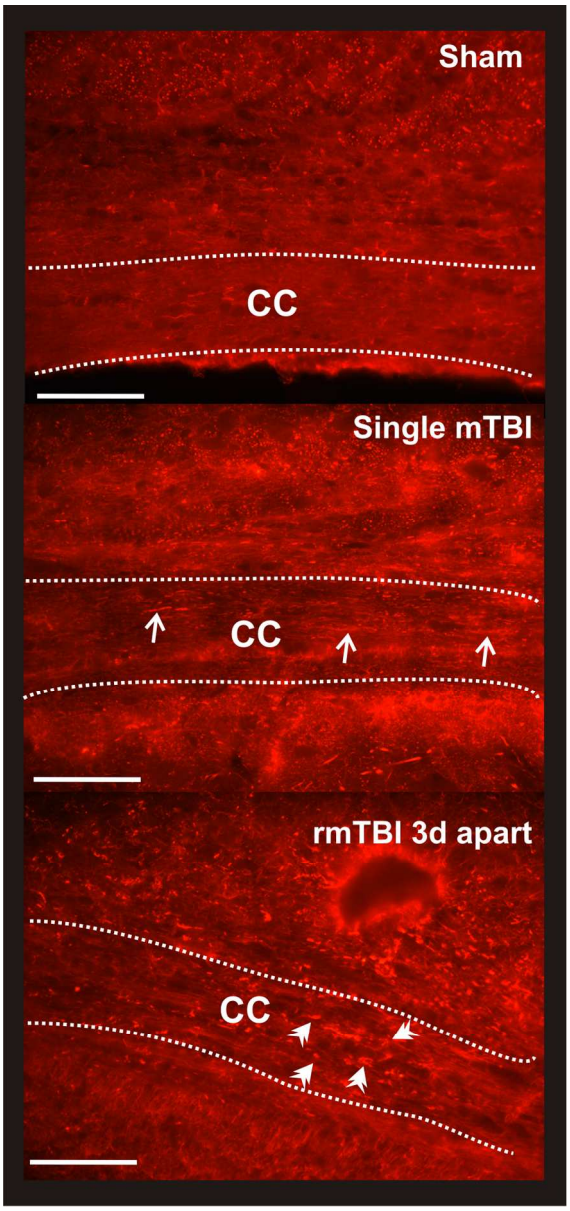

Fig. 8 Representative photomicrograph showing traumatically injured axons within the ipsilateral corpus callosum (CC). Compared to shams, a single mTBI resulted in NF200 accumulation in axons (arrows) and subtle decrease in NF200 immunoreactivity, suggesting axonal swelling and possible loss of axonal microstructures. NF200 accumulation in axonal bulbs (arrowheads) was observed in the rmTBI 3d apart. Cal bar=20μm.
83x179mm (300 x 300 DPI)