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The Evolution of Traumatic Brain Injury in a Rat Focal Contusion Model

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Abstract

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Author Contributions

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Serial magnetic resonance imaging (MRI) facilitates the *in vivo* analysis of the intra- and intersubject evolution of traumatic brain injury (TBI) lesions. Despite the availability of MRI, the natural history of experimental focal contusion lesions in the controlled cortical impact (CCI) rat model has not been well described. We performed CCI on rats and MRI during the acute to chronic stages of cerebral injury to investigate the time course of changes in the brain. Female Wistar rats underwent CCI of their left motor cortex with a flat impact tip driven by an electromagnetic piston. *In vivo* MRI was performed at 7 T serially over six weeks post-CCI. The appearance of CCI-induced lesions and lesion-associated cortical volumes were variable on MRI, with the percent change in cortical volume of the CCI ipsilateral side to the contralateral side ranging from 18% within 2 hours of injury on Day 0 to a peak of 35% on Day 1 and a trough of -28% by Week 5/6, with an average standard deviation of $\pm 14\%$ at any given time point. In contrast, the percent change of ipsilateral side to contralateral side cortical volumes in control rats was not significant ($1\pm 2\%$). Hemorrhagic conversion within and surrounding the CCI lesion occurred between days 2 and 9 in 45% of rats with no hemorrhage noted on initial scan. Furthermore, hemorrhage and hemosiderin within the lesion were positive for Prussian blue and highly autofluorescent on histological examination. Although some variation in injuries may be technique related, the divergence of similar lesions between initial and final scans demonstrates the inherent biological variability of the CCI rat model.

Keywords

traumatic brain injury; controlled cortical impact; MRI; hemorrhage; autofluorescence

Introduction

Traumatic brain injury (TBI) afflicts 1.7 million people in the U.S. per year, and is also a hallmark injury of the wars in Iraq and Afghanistan (1–3). Despite preclinical successes, translational effective neuroprotective or neuroregenerative strategies to treat TBI remain elusive except for symptomatic treatments such as reduction of elevated intracranial pressure (4). Several animal models have been developed for the experimental investigation of TBI (5). In a clinical population, TBI can result from one or more mechanisms of injury (i.e., blast, acceleration-deceleration, blunt trauma, penetrating trauma), often complicated by polytraumatic injuries to multiple organ systems (6). Of the current animal models for TBI, no single model encompasses all the injury types and the heterogeneity observed clinically with TBI.

Prior to MRI, the study of TBI in experimental animals was limited to endpoint analysis, with characterization of the lesion by immunohistochemistry (IHC), necessitating the use of additional cohorts of animals at various times post injury. Serial MRI allows investigators to monitor the evolution of experimental TBI lesions *in vivo*, and permits inter- and intrasubject comparisons (7–14). Although longitudinal MRI studies of the lateral fluid percussion (LFP) model in the rat have been reported (10–13,15,16), the natural history of focal contusion over time in the controlled cortical impact (CCI) rat model has not been as well characterized by MRI. MRI techniques have been utilized to investigate cerebral blood volume in the CCI model (14,17) within the first 5 hours (18) to a week (10) after injury.

However, none of these studies has fully investigated the evolution of the CCI lesion over the acute to chronic time course with the description of changes in cortical volume and microenvironment in the surrounding parenchyma. The purpose of this study was to examine by MRI and histology the extent of the variability of moderate to severe CCI in the rat with respect to lesion size changes in cortical volumes and evolution of hemorrhage. In the present study, young adult female Wistar rats underwent CCI to model moderate to severe focal contusions, and were then followed by MRI in the acute to chronic stages after injury. Radiological pathological correlations and behavioral testing were performed.

Experimental

Animals

All experiments were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. All studies were approved by the Animal Care and Use Committees at our institutions. Female Wistar rats age 8 to 12 weeks (200 to 250 g) were obtained from Charles River Laboratories (Wilmington, MA) (n=37). Rats were housed in pairs under 12 hour light/dark cycles in temperature-controlled conditions, and fed standard rodent laboratory chow and water *ad libitum*.

Controlled Cortical Impact (CCI) Model

The rat CCI model (19–21) was performed using an electromagnetic CCI device (Impact One stereotaxic impactor, myNeuroLab.com/Leica Microsystems, Richmond, IL) (22). Rats were anesthetized via nosecone with a mixture of 2–3% isoflurane and 97–98% oxygen for all procedures. Rats were placed in a stereotaxic frame for CCI. A rectal temperature probe was inserted, and body temperature was maintained with a circulating water warming pad. After scalp incision, a 6 mm craniotomy was performed over the left motor cortex (2.5 mm left lateral and 1.0 mm anterior of Bregma) using a micromotor drill with a dental bit. CCI was delivered with a 5 mm flat impactor tip at a velocity of 5 m/s, depth of 2.0 mm, and dwell time of 100 msec. Post-injury, the bone flap was replaced and sealed with dental acrylic cement. The scalp incision was closed with 4.0 nylon sutures. Rats were placed in warming cages and closely observed during recovery from anesthesia, then returned to their cages until further assessment. All TBI injuries for a given cohort of rats were performed by the same surgeon.

Behavioral Testing and Statistical Analysis

The neurological severity score-revised (NSS-R), which is a modification of several pre-existing NSS examinations (23–27), was performed as described (28). In brief, the NSS-R evaluates motor, sensory, and reflex function with ten tests. For each test, a normal response scores zero, moderate impairment scores one, and complete impairment scores two. The total score for an individual animal on the NSS-R ranges from 0 (completely normal) to 20 (most impaired). Rats underwent NSS-R evaluation at baseline and on days 1, 14, and 28 after injury (n=17). Data were analyzed with a one-way analysis of variance (ANOVA) and subsequent post-hoc Tukey, using Prism version 5.0c for Macintosh (GraphPad Software, San Diego, California, USA, www.graphpad.com), with a two-tailed *p* value of <0.05 considered significant.

MRI

In vivo MRI of the brain was performed on a 7 T Bruker Biospec (Billerica, MA) using a 40 mm inner diameter quadrature volume coil (Doty Scientific, Columbia, SC). For the first cohort of rats, MRI was performed on days 2, 9, and 30 post-CCI (n=23). To obtain information at other time points, depending on scanner availability, a second cohort of rats underwent MRI on day 0 (within an hour after CCI) or 1, day 3 or 4, Week 1 (day 7, 8, or 9), and week 4, 5, or 6 post-CCI (n=14). Seven healthy control rats were also scanned to determine variability in brain volumes. For comparison between the two cohorts, MRI scans were grouped as follows: initial scan (day 0, 1, or 2), Week 1 scan (day 7, 8, or 9), and final scan (week 4, 5, or 6). During MRI, physiological monitoring was performed on all animals. T2- and T2*-weighted images were acquired using a multi-echo spin echo or gradient echo sequence, respectively, to obtain images with high signal to noise and contrast by magnitude averaging the individual echo images. T2-weighted images were acquired with the following parameters: repetition time (TR)= 3500ms, effective echo time (TE) = 38.5 ms (10 echoes, 7 ms echo spacing), resolution of 112×112×500 μm^3 , and T2*-weighted 3D images were acquired with the following parameters: TR = 60ms, effective TE = 14.9 ms (6 echoes, 4.25 ms echo spacing), resolution of 117 μm isotropic, and 15° flip angle. *Ex vivo* MRI was performed on excised paraformaldehyde perfused brains using a 7 T Bruker vertical bore magnet with a 10 mm inner diameter birdcage coil. Brains were immersed in susceptibility matching fluid (Fomblin, Solvay Solexis, Inc., West Deptford, NJ) and imaged with a 3D multi-echo gradient echo sequence: TR = 200 ms, effective TE = 17.5 ms (6 echoes, 5 ms echo spacing), field of view = 3.0×1.5×1.25 cm^3 , 512×256×128 matrix, 4 averages, 30° flip angle.

Immunohistochemistry

Paraformaldehyde perfused brains were placed in a sucrose gradient, then transferred to optimum cutting temperature (OCT) media and frozen in liquid nitrogen. Cryosections were cut at 10 μm thick in the coronal plane, mounted on glass slides and stored at -20°C. Representative sections underwent hematoxylin and eosin (H&E) staining using standard methods. For Prussian blue staining, brain sections were washed in deionized water then stained in 10% potassium hexacyanferrate (II) trihydrate (Sigma #P3289, St. Louis, MO) and 10% hydrochloric acid for 20 minutes at room temperature. Sections were then counterstained in Nuclear Fast Red Stain (NFS500, ScyTek Laboratories, Logan, UT) for 4 minutes. Slides were then dehydrated with ethanol and xylene prior to coverslip mounting in Permount (Fisher Scientific, Pittsburgh, PA).

For detection of autofluorescence, after rehydration and washing with phosphate buffered saline (PBS), brain slices were mounted in ProLong Gold antifade with DAPI (Invitrogen, Carlsbad, CA). Slides were visualized with a light and fluorescent microscope (Axioplan Imaging II; Zeiss, Oberkochen, Germany) and Axiovision software (Zeiss, Oberkochen, Germany).

Imaging and Statistical Analysis

Cortical and lesion volumes were determined using MEDx software (Medical Numerics, Germantown, MD). In brief, automated edge detection software was used to strip the skull

from the T2-weighted MRI. The corpus callosum was identified and demarcated on T2w images and line was then drawn at the corpus callosum, and all brain structures beneath were removed. The identified white matter in the corpus callosum was also removed. The medial longitudinal fissure was then used to separate the TBI hemisphere from the contralateral hemisphere, and cortical volumes measured on both lesion side and contralateral side (Figure 1 depicts how the cortical volumes and lesion volumes were determined). Whole hemispheric volumes were not used in this study because of partial volume effects that would limit the ability to detect or would minimize changes resulting from CCI. When cyst formation occurred, these hyperintense volumes of interest were excluded from cortical volume measurements. To demarcate the lesion volumes a segmentation algorithm (FAST – FMRIB Automated Segmentation Tool v3.1, University of Oxford, United Kingdom) was applied to the T2-weighted MRI of CCI cortex in order to automatically delineate and determine the number of voxels that would have signal intensity consistent with cerebral spinal fluid from gray and white matter. Repeated cortical volume measurements were performed in 5 rats multiple times (n=5) to determine the coefficient of variation (COV) in the technique (BKL). To test correlation between percent changes in ipsilateral TBI versus contralateral volumes on different days, Pearson's correlation coefficient was calculated using Microsoft Excel for Macintosh 2011 (Microsoft, Redmond, Washington, USA), with a p value of <0.05 considered significant.

For qualitative analysis of hemorrhage, two independent observers with MRI experience (LCT, JAF) graded each day 2, 9, and 30 MRI in random order for the presence or absence of hemorrhage in and around the CCI lesion in order to determine how often hemorrhagic transformation occurred. For quantitative analysis of hemorrhage, the region of T2* signal voids were manually traced on T2*-weighted 3D gradient echo images. The observer (LJ) was blinded to the time point (day 2 or 9). The volume was calculated for each animal at each time point. A paired t-test was used to compare the signal void volumes between Day 2 and Day 9, with a p value of <0.05 considered significant using Microsoft Excel for Macintosh 2011 (Microsoft, Redmond, Washington, USA). A separate analysis was performed as described above to determine whether any additional change in hemorrhage occurred between Day 9 and Day 30.

Results

The evolution of the moderate to severe CCI is depicted in Figure 2A, demonstrating the time course of comparable averaged slices from all rats from the registered MRIs on a given day. On serial MRIs, cortical lesions consistently extended over 12 to 14 out of 30 total 500 μm thick sections through the whole brain.

Figure 2B plots the mean and standard deviation of the percent change in cortical volume from CCI to contralateral side on days post-injury. Figure 2C graphs the mean and standard deviation in CCI lesion volumes (mm^3) over time, which mirrors the pattern of changes seen in Figure 2B. Analysis of control rats indicated that there were minimal differences in left to right cortical volumes (mean $\pm$ standard deviation (SD): $-1 \pm 2\%$) prior to CCI injury (Table 1). MRI performed immediately following injury demonstrated an acute increase in CCI cortex compared to contralateral cortex on Days 0 through 4 secondary to increased swelling

of the cortical lesion (percent change TBI/contralateral cortical volumes: Day 0=18%±13%; Day 1=35%±10%; Day 2=26%±11%; Day 3/4=17%±12%). Over time, the absolute cortical volume on the CCI side decreased, with the percent change in TBI versus contralateral cortical volumes changing from a mean of -1%±13% at 1 week after TBI to a -8%±14% loss by Week 4 and a -28%±11% loss by Week 5/6. The initial increase in mean percent ratio is consistent with acute peri-lesion swelling from vasogenic and cytotoxic edema and inflammation, subsequently leading to axonal/neuronal loss and cortical thinning resulting in hemispheric atrophy, ventricular enlargement and/or cerebrospinal fluid cyst formation, as indicated by the decreased mean percent ratio by Week 4.

The MRI appearance and volume of CCI-induced lesions on initial scan, Week 1 scan, and final scan were variable as presented in Figure 3. For the first cohort of rats, there was no significant correlation between the percent change of CCI side cortex volume to contralateral side cortex volume on initial scan to subsequent exams at the middle time point and the final MRI time points (mean±SD: Cohort 1: initial=26±11%; middle = -2±14%; final = -10±14%) by Pearson's correlation (initial versus middle Pearson's $r = -0.39$, $p = 0.12$; initial versus final Pearson's $r = -0.25$, $p = 0.34$) (Figures 3A and B, top). In the second cohort of rats, there was a correlation between percent change in cortical volumes of CCI side versus contralateral side (Cohort 2: initial=24±15%; middle=2±13%; final= -19±17%) on the initial and middle scan (Pearson's $r = 0.71$, $p < 0.01$), but not between the initial and final scan (Pearson's $r = 0.40$, $p = 0.15$). For both cohorts of rats, there was, however, a statistically significant correlation between percent change of the CCI side versus the contralateral side on the middle scan versus the final scan (Cohort 1: Pearson's $r = 0.76$; $p < 0.01$; Cohort 2: Pearson's $r = 0.76$; $p < 0.01$) (Figure 3B, bottom).

Lesions that had similar cortical volumes on T2-weighted MRI on initial scan could vary dramatically by final scan (Figure 3C). The <10% loss observed on final MRI at week 4, 5, or 6 was seen in 42% of animals having both initial and final scans. The 10 to 20% loss pattern observed on final scan was seen in 19% of animals. The >20% loss pattern observed on final scan was seen in 39% of rats. The coefficient of variation of percent change CCI cortical volume/contralateral cortex measurements was 2.0%.

Behavioral analysis demonstrated a statistically significant increase (one way ANOVA $p < 0.05$) in the NSS-R relative to baseline (NSS-R = 3.4±0.3) on Day 1 (NSS-R = 7.3±0.4) and Day 14 (NSS-R = 5.5±0.5) after injury (Figure 4). By Day 28 post-injury, NSS-R (4.7±0.4) was not different from baseline values ($p > 0.05$). The NSS-R on Day 1 was significantly different from the days 14 and 28 NSS-R values ($p < 0.05$). There was no statistical correlation between NSS-R and MRI lesion volume at comparable time points (NSS-R Day 1 and MRI Day 2; NSS-R Day 14 and MRI Day 9; NSS-R Day 28 and MRI Day 30) (data not shown).

While 29% of rats displayed hemorrhage (i.e., hypointense voxels on T2*-weighted images) by the first scan after injury in the first cohort of rats (with scans on days 2, 9, and 30), 45% of rats with no evidence of hypointense voxels at Day 2 developed hemorrhage by Day 9 (Figure 5). Rats that were negative for hemorrhage on T2*-weighted MRI on Day 9 did not develop hypointense voxels by Day 30. All rats in the second cohort had some evidence of

hemorrhage on initial scan (day 0 or 1) and were excluded from further analysis of hemorrhagic conversion. To further quantify hemorrhage, T2*-weighted signal voids from all rats in the first cohort with MRI scans on both days 2 and 9 were manually traced and measured by an investigator blinded to day and rat. A statistically significant increase in hemorrhage-related T2*-weighted signal voids was seen, with $3.8 \pm 4.4 \text{ mm}^3$ on Day 2 and $7.0 \pm 4.0 \text{ mm}^3$ on Day 9 ($p=0.006$) (Figure 5B). There was no change in T2*-weighted signal voids on Day 30 ($4.4 \pm 5.0 \text{ mm}^3$; $p=0.1$) compared to Day 9. These regions of hemorrhage on the CCI lesion side demonstrated Prussian blue positive cells, and fluorescence microscopy at 10 days post-CCI revealed autofluorescence of Prussian blue positive cells in the cortex (Figure 6).

Discussion

The major findings of this study were as follows: 1) variation occurs in the natural history of CCI lesions and cortical loss based on MRI as a result of the same impact parameters; and 2) hemorrhage can evolve on MRI between days 2 and 9 following CCI. In the current study, serial MRI was performed over 6 weeks following moderate to severe CCI in female rats over the left motor cortex, with observation of a wider variation in lesion volume, cortical loss and evidence of hemorrhage than was previously reported in this model.

The CCI model of TBI has been previously described as highly consistent when looking at endpoint analysis (22). Onyszchuk et al. reported a high correlation between MRI and histological lesion cavity volumes at 14 days post-injury in the mouse CCI model (29). Twenty-four hours after CCI in the mouse there were T2-weighted hyperintensities around and within the injury site that diminished by 48 hours, consistent with acute edema and its resolution (29,30). By 7 days, a ring of T2 signal hypointensity surrounded a hyperintense central area that converted entirely to a region of high signal intensity by Day 14 post-injury (29). These lesion-associated T2 hyperintense cavities persisted up to at least 2 months in mice (31).

The temporal evolution of CCI lesions by MRI in the rat has not been as well described. Three studies have used MRI to track lesion volumes after CCI in rats over time (14,17,32), two of which also focused upon cerebral blood flow while the third analyzed white matter changes by fractional anisotropy (32). Cross-sectional MRI studies performed at different time points out to 14 days in rats following moderate CCI revealed large intersubject variability in lesion size for similar impact settings (14). Serial MRI in a CCI rat model demonstrated T2 hyperintense lesions that increased in size one day after injury that subsequently decreased in volume out to 6 weeks while ventricular volumes increased (17). These results were similar to findings in the CCI mouse model (29,30). None of these studies formally addressed how consistent or variable the cortical injuries were by MRI.

The temporal evolution of other TBI models, such as lateral fluid percussion (LFP) in rats, has shown similar variability in lesion volume with time as compared to the CCI model. While Kharatishvili et al. observed correlation between 3 days post-LFP T2-weighted MRI lesion volumes and endpoint histology in male rats, there was significant variation in lesion volumes with overlap within their moderate and severe injury groups (11). Immonen et al.

examined LFP in the rat with serial MRI up to 11 months post injury, and reported degrees of intersubject variability in lesion severity and size despite similar initial injury parameters (13). These authors subsequently divided the injured animals into three different subgroups (i.e., mild, moderate or severe lesions) based on MRI at 23 days post-injury (13).

In contrast to other studies that performed CCI posterior to Bregma in a cortical area over the hippocampus, the current study targeted the left frontal motor cortex for the CCI lesion. This location was chosen because an injury to the motor cortex might display more obvious neurological findings. In the current study, the behavioral results (Figure 4) revealed an increase in the NSS-R within 1 week after injury, predominantly on the basis of the motor cortex injury. However, the rats rapidly compensated for the left motor cortex neurological impairment with normalization of the NSS-R by Week 4 after injury. No correlations were observed between NSS-R and MRI lesion volumes at comparable time points in these experiments. Due to the neurological recovery 4 weeks post-CCI, we evaluated the percent change of TBI to contralateral cortical volumes as a potential MRI outcome measure. A possible limitation of using the percent change of the CCI to contralateral cortex is the lack of sensitivity to changes in the uninjured volumes.

In the present study, where initial scans were performed on Days 0 to 2 post-CCI, the standard deviations in the mean percent changes in TBI/contralateral cortical volumes were approximately 14%. The percent change in the ratio of cortical volume obtained from the initial MRI scans did not correlate with the ultimate lesion loss at 29 to 42 days post-injury. The craniotomy procedure itself can produce mild changes on MRI out to 14 days post-injury, with mean lesion volumes ranging from 2 to 10 mm³ (28). The size of the craniotomy-associated lesions is a small percentage (10 to 15%) of the lesion volumes produced by the CCI surgery itself as seen in the present study. Although the early variation (MRI on Days 0 to 2) in CCI lesions may be attributed to differences in surgical technique or technical variability inherent in the Leica CCI impactor device, the divergence of similar lesions in rats with initial, middle, and final time point MRI scans post-CCI clearly demonstrates the inherent biological variability in this model. Similar variability has been reported in the rat LFP model (11,13). The biological variability needs to be further evaluated and is observed in both sexes of rats used in LFP and CCI studies (11,13,14).

In the current study, gonadally intact female rats were used. Approximately half of the world's human population is female, and TBI affects both men and women (1). In contrast, the vast majority of published experimental brain injury studies, including the few that have utilized MRI as an outcome measure, focus exclusively on male animals. We chose to study intact female rats to balance the experimental TBI MRI literature. The use of randomly cycling intact female rats most closely mimics the premenopausal human female population, in which there is no control over when TBI might occur.

We recognize that potential differences in ovarian hormone levels based on the phase of the estrus cycle may be contributing some additional variation in this study. Female rats housed in a room together, even in separate cages, can synchronize their estrous cycles (33). Combined with the short length of the rat's estrus cycle (averaging 4.5 days in length (34,35)) and limitations on MRI access, ensuring that all intact female rats are at the same

phase of the estrous cycle at the time of injury and/or scanning was difficult. In future studies the possible role of changes in ovarian hormone levels on MRI outcomes could be investigated by surgically or chemically ovariectomizing female rats with and without subsequent supplementation of estrogen and/or progesterone.

The fluctuating ovarian hormone levels seen during the cycle of the female rat during its reproductive years can affect the response to brain injury (36,37). Progesterone can reduce neuronal injury and edema in both focal (38–41) and diffuse injury (42) in TBI models. Whether or not progesterone or estrogen has any effect on the appearance of lesions by MRI is unknown. Some of the variation seen on initial MRI in this study may be related to differences in ovarian hormone levels in the gonadally intact female rats. Although the variation in progesterone levels may have resulted in different responses to injury on the Day 0 MRI, it would be an unlikely cause of similarly appearing lesions on Day 2 that ultimately resulted in the variability in cortical volumes observed on MRI at later time points. The outbred Wistar rat was used in this study and may have also contributed to the variability seen in the response to injury over time. The use of inbred strains would minimize genetic differences but would be less clinically relevant. It should be noted that large variability in MRI lesion volumes were observed in male rats in the LFP model using identical impact parameters (11,13) raising the question that the variability in lesion volumes observed in penetrating TBI models is due to gender differences.

The degree of variation in lesion volume observed in this cohort of animals has implications for the design of future studies using the CCI model in which MRI metrics such as lesion volume or cortical preservation would be used as an outcome measure or biomarker for novel cellular or drug therapies. Based on the standard deviations across all time points for the percent difference in TBI/contralateral cortex (14%), with an alpha of 0.05 (power of 0.80), and the ability to detect 10 to 20% difference as a result of treatment, future studies would require a minimum of 9–43 subjects per arm for two-treatment parallel group design (http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html), with additional animals required in case of technical difficulties (i.e., anesthesia deaths, extreme weight loss, or seizures requiring euthanasia of the animals). The number of animals per treatment arm needed to detect this difference is larger than most animal studies investigating treatment effects (of drugs or stem cells versus placebo, for example) in stroke studies (43). A survey of the TBI field demonstrates a similar preponderance of small subject numbers in recent preclinical trials (for example, see (17,32,44–47)). These findings suggest that most experimental studies reporting positive neuroregeneration results in response to treatment would be underpowered based on MRI metrics (43,48,49). Moreover, researchers should consider applying intention-to-treat analyses in these studies (49), as currently done in clinical research. In addition, as noted in the Cole et al. study (28), craniotomy itself can injure the surface of the brain, so investigators need to be sure to include the appropriate control groups (i.e. CCI plus treatment and CCI plus placebo for treatment studies; naïve control versus CCI and/or sham depending on study of other factors) for their particular study design.

Prior studies have noted that hemorrhage can occur after the initial injury in TBI models (12,50–54). MRI detected intracerebral hemorrhage within hours after injury in 89–100% of

rats following CCI (14,17). Similar findings have been observed after LFP with hemorrhage being detected in 21–100% of rats (15). An assumption on the basis of endpoint analysis for the above studies was that the degree of hemorrhage remained static after the initial insult. In the current study, in the first cohort of rats, the evolution of intraparenchymal hemorrhage was observed between days 2 and 9 in 45% of rats without previous evidence of intracerebral blood after the initial MRI. In the present study, we are unable to address whether the degree of hemorrhage seen on initial scan changes over time because we excluded animals with evidence of intracerebral bleeding on initial MRI scan from further quantitative analysis of hemorrhage. The development of hypointense regions on T2*-weighted images over time was reminiscent of observations reported after ischemic stroke (55), and is consistent with IHC observation of progressive secondary hemorrhage in a rat contusion model (56,57). This finding suggests that the delayed hemorrhage in the penumbra of the traumatic CNS injury may result from secondary ischemia from edema or damaged/friable capillaries (55–57). Clinically, hemorrhagic conversion after TBI has not been well documented by MRI; however hemorrhagic progression after contusion (HPC) in the acute care setting has been described on the commonly used computerized tomography (56,58–61).

The possibility of intraparenchymal hemorrhagic evolution during the first week after experimental TBI has implications for interpreting results from studies tracking superparamagnetic iron oxide nanoparticle (SPION) labeled cells by MRI (17,32,62–64). Intracerebral hemorrhage and hemosiderin associated in CCI lesions have similar properties on T2 and T2*-weighted imaging, and result in Prussian blue positive staining within cells (65). Serial MRI has been used to investigate the homing of SPION labeled cells used as possible treatment in experimental TBI (17,63,64). Recently Li et al. reported that SPION-labeled cells showed persistent T2 and T2* hypointensity over time while old hemorrhage does not (17). This result is problematic in comparison to clinical situations, as chronic hemosiderin deposition in superficial siderosis demonstrates persistent T2 hypointensity with associated Prussian blue positive cells (66–69). T2 hypointensities were also observed in the cerebral microbleeds associated with risk of stroke recurrence (70) and in cerebral amyloid angiopathy (71). The persistence of hypointense voxels on T2 and T2*-weighted images has also been demonstrated to occur more often in experimental animals that reject labeled stem cell transplants (72), complicating the interpretation of MRI cell tracking studies using SPION-labeled cells. The current study underscores the need for a control group as well as for obtaining serial MRI in order to determine if new hypointense voxels on T2*-weighted images after TBI are due to hemorrhage and microbleeds versus the migration of magnetically labeled cells into the brain following an intravenous injection. More importantly, cell therapy experiments have utilized Prussian blue and immunohistochemistry to identify SPION-labeled cells within the lesion. As we have demonstrated in the absence of any cell therapy, hemorrhagic products within the lesion stain for Prussian blue. Moreover, these same foci were highly autofluorescent at the 488 nm excitation wavelength and could be misconstrued as positively stained exogenous cells. Therefore, in CNS injuries that result in hemorrhage, numerous potential confounds are present that need to be appropriately controlled for and examined in order to interpret the results from cell therapy studies. The use of T1 contrast agents such as gadolinium chelates (73–75) or gadolinium

nanoparticles (76–78) for the labeling of cells in MRI tracking experiments would be an alternative approach to the use of SPION-labeled cells in diseases such as TBI where hemorrhage may be a confounder.

Conclusions

This study documented variability in the natural history on MRI of TBI lesions induced by similar initial CCI over time along with the delayed intracortical hemorrhagic conversion of contusions that were hemorrhage negative on initial MRI scan between days 2 and 9 after injury. Inclusion of serial MRI in studies to track lesion evolution over time would be invaluable to better understand the response to injury, rather than solely focusing on endpoint analysis.

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Abbreviations

CCI	controlled cortical impact
H & E	hematoxylin and eosin
HPC	hemorrhagic progression after contusion
IHC	immunohistochemistry
LFP	lateral fluid percussion
NSS-R	Neurological Severity Score – Revised
PBS	phosphate buffered saline
SD	standard deviation
SEM	standard error of mean
SPION	superparamagnetic iron oxide nanoparticle
TBI	traumatic brain injury

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Figure 1.

To analyze MRI for cortical and CCI lesion volumes, the skull was first stripped away from the image using automated edge detection software. A line was then drawn at the corpus callosum, and everything beneath the line was removed. The corpus callosum was also then removed. The medial longitudinal fissure was used to separate the TBI and contralateral hemispheres. A segmentation algorithm (FAST – FMRIB’s Automated Segmentation Tool v3.1; <http://www.fmrib.ox.ac.uk/>) was applied only to the TBI side.

Figure 2.

The percent volume of injured side versus contralateral side changes over time after CCI. A) T2-weighted images were spatially registered to a common space and averaged among all available animals for each time point. The time course demonstrates the evolution of the injury over time. Substantial edema was apparent in the acute phase of injury, followed by an evolution to cortical atrophy in the subacute to chronic phase. B) Quantitative results pooled across all animals demonstrate an increase in the volume of the injured side relative to the uninjured side in the acute phase, consistent with injury related edema. As the edema resolves, cortical atrophy occurs on the injured side, with loss of approximately 10% on the lesion side versus the contralateral side. C) Lesion volumes (mm³) not including the cystic volume in injured animals mirror changes in cortical volume. Day 0 scans were obtained 1

hour post-injury. Week 1 includes all MRI obtained on days 7, 8, or 9. The number of animals represented by each time point is presented in Table 1.

Figure 3.

Experimental TBI lesions with similar initial degrees of cortical injury and edema on initial time point MRI can appear markedly different on final scan. A) The variability seen in cortical volumes on initial scan increases by middle and final scans. The left graph displays the first cohort of rats, which had their scans on days 2, 9, and 30. The right graph depicts the results from the second cohort of rats (initial scan on day 0 or 1, middle scan on day 7 or 8, and final scan on day 29, 35, or 42). The lines on the scatter plot represent the mean $\pm$ SD for each day's percent cortical volume on the CCI side versus the contralateral side. B) A scatter plot of the percent change in cortical volumes of the CCI versus contralateral sides on initial scan versus final scan shows no correlation in either cohort of rats (Cohort 1: Pearson's $r = -0.25$; $p = 0.34$; Cohort 2: Pearson's $r = 0.40$; $p = 0.15$) (top). The percent change in cortical volumes on the middle scan versus the final scan is correlated (Cohort 1: Pearson's $r = 0.76$; $p < 0.01$; Cohort 2: Pearson's $r = 0.76$; $p < 0.01$) (bottom). C) Marked variation can be observed in the appearance of similar injuries over time. The T2-weighted images, which had similar degrees of cortical injury on initial scan, are organized into three groups by the percent cortical loss observed by MRI at final scan: $> -10\%$; < -10 to -20% ; $< -20\%$. The three representative rats depicted here all came from the first cohort of rats. Similar observations were made in rats from the second cohort.

Figure 4.

Behavioral testing of rats demonstrates a statistically significant initial increase in the Neurological Severity Score – Revised (NSS-R) on days 1 and 14 after injury, which subsequently decreases by Day 28, at which point there was no statistical difference from baseline values (* = $p < 0.05$ by one-way ANOVA with post-hoc Tukey). The error bars for the NSS-R indicate the standard error of the mean, in accordance with behavioral testing standards.

Figure 5.

Hemorrhagic conversion can occur in the days after initial CCI. A) Animals with little or no evidence of hemorrhage by MRI at 2 days after CCI can develop more hemorrhage by Day 9, as assessed by T2-weighted MRI and T2*-weighted 3D gradient echo MRI. B) Quantification of hemorrhage in manually traced T2* signal voids on days 2 and 9. Each grey line represents an individual rat's T2* signal void on days 2 and 9. The black line indicates the mean and standard deviation values of all measured rats on those days.

Figure 6. Potential confounding effects of hemorrhage in CCI lesions detected by MRI, Prussian blue, and fluorescence microscopy. A) *In vivo* T2*-weighted MRI at 9 days post-CCI depicts a region of hypointensity indicative of hemorrhage (arrow); B) *Ex vivo* T2*-weighted MRI depicts the similar pattern at 10 days post-injury; C) Hematoxylin and eosin stain of the slice matched section; D) Prussian blue staining in the regions shown in A demonstrates multiple staining foci that are solely the consequence of hemorrhage; E) Hemorrhagic products within the injured tissue at 10 days post-CCI are highly autofluorescent in the FITC channel (green) as shown in an antibody-naïve section rehydrated with PBS then counterstained with DAPI (blue).

Table 1

Percentage change in the CCI side versus contralateral side cortical volumes over time.

Time Point	% Change CCI Side/Contralateral Side $\pm$ SD	Number of rats (n)
Baseline	-1 ± 2	7
Day 0	18 ± 13	9
Day 1	35 ± 10	5
Day 2	26 ± 11	23
Day 3/4	17 ± 12	12
Week 1 (Days 7, 8, 9)	-1 ± 13	37
Week 4 (Days 29, 30)	-8 ± 14	27
Week 5/6 (Days 35, 42)	-28 ± 11	9