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Investigation of Post-Mortem Magnetic Resonance Imaging for the Detection of Intraneural Hemorrhage

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Purpose: Our over-arching hypothesis is that three-dimensional (3D) post-mortem magnetic resonance imaging (PMMR) can be optimized to enable visualization of intraneural hemorrhage at a resolution useful for future application to the assessment of the cervical spine in infants and small children. This basic research study proceeded in three phases: I) we created anatomically correct pediatric phantoms and assessed the detection sensitivity of currently available magnetic resonance imaging coils; II) we optimized PMMR spatial resolution and image contrast of several candidate PMMR image acquisition protocols, and used these image acquisition protocols to establish a basic knowledge of the MR appearance of nerve hemorrhages at different stages of hemoglobin degradation; and III) we tested the optimized PMMR image acquisition protocols using a rabbit model of intraneural hemorrhages of varying antemortem ages: hyperacute (~1 hr), acute (1 day), early subacute (7 days) and late subacute (2 weeks). These investigations are designed to provide PMMR protocols that are optimized and tested for detecting intraneural hemorrhage, for future application to deceased human subjects.

Project Subjects: Whole rabbit blood, preserved with heparin or citrate and shipped overnight on ice, was obtained from Sierra for Medical Science (Whittier, CA). Fresh bovine or porcine spinal cord tissue was obtained as a by-product of meat production, on the day of slaughter, from Western Way Custom Meats (Moriarty, NM). Intact Long Evans laboratory rats (approx. 500 g) were obtained (after CO_2 euthanasia) and utilized for image protocol optimization. The use of post-mortem blood, tissue, and intact rats was conducted under a tissue protocol (#15-200300-T-HSC) approved by the University of New Mexico Health Sciences Center IACUC. Thirty intact male New Zealand White rabbits (NZW;

Orcytolagus cuniculus) approximately 3 kg each (range: 3.10kg - 3.71kg; mean 3.38kg), were obtained from a commercial vendor and underwent a 7 day quarantine. They were individually housed in an indoor animal facility (12:12h light:dark cycle, 30-70% RH, 16- 22C), where they received ad libitum access to water and were fed Teklad 2031C Certified Global High Fiber Rabbit (Envigo Bioproducts, Inc., Madison, WI). Live rabbit procedures were conducted by Lovelace Respiratory Research Institute (Albuquerque, NM), an AALAC-accredited facility under an IACUC-approved protocol (#FY17-015). All procedures and husbandry were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals 8th Ed.*, Animal Welfare Act Regulations, LRRI Institutional Animal Care and Use Committee policies, and current Office of Laboratory Animal Welfare recommendations.

Project Design, Methods, and Data Analysis: All MR studies were conducted using a Siemens Sonata 1.5 Tesla MR scanner.

Phase I. Assess the sensitivity of available MR detection coils, singly and in combination, using pediatric imaging phantoms representing pediatric subjects ages 3 mo, 6 mo, and 12 mo. We constructed phantoms and used them to determine which MRI detection coil or coil combination has the best sensitivity over a volume covering the pediatric cervical spine and how to best position the pediatric subject in the optimum sensitive volume. Phantom construction began by selecting high resolution, thin slice, postmortem CT data representing normal 3, 6 and 12 month old infants (Protocol:1.0 mm slice thickness, 0.5 mm slice spacing, soft tissue algorithm, performed on a 16 slice Philips Brilliance Big Bore CT scanner). These data sets were converted into a 3D isosurface, surface shaded display (Settings: pixel value 500, decimate resolution 0.50, smooth iterations 20) and exported into object file format (.obj) using the open source DICOM viewer OsiriX (OsiriX 64 bit, version 5.0.1, Pixmeo). The object file was imported into Rhinoceros (version 5.0, McNeel North America, Seattle, WA) and converted to stereolithography format (.stl). The resulting polygon mesh was cropped to the head and neck, cleaned of extraneous objects and patched to fill any gaps. Transverse head measurements of the mesh were compared with measurements from the original data sets to ensure fidelity. Axial CT images from the original data sets were placed within the mesh at anatomic landmarks, allowing a virtually built cylinder

to be positioned within the mesh at the location of the cervical spine and vixed with virtually constructed struts. The inside of the cylinders were built to conform to the outer dimensions of a typical 50 ml centrifuge tube. Meshes were repaired automatically using functions within Rhinoceros and then Magics 18 (version 18.02, Materialise, Leuven, Belgium) software, and exported in .stl format into the 3D printer software Catalyst EX (Stratasys, Eden Prairie, MN). The final infant phantoms were printed in ABS plastic using a Dimension 1200es 3D printer (Stratasys, Eden Prairie, MN),

To determine the best receiver coil or coil combination to maximize image quality for in vivo small animal work (this project) and human infant studies (future projects), we performed MRI on human infant phantoms containing a 50 ml centrifuge tube of an MR-visible gel (with MR imaging properties similar to nerve tissue) at the location of the cervical spine. The gel-containing phantoms were imaged using two basic MR acquisition protocols – a 2D slice-selective Spin Echo sequence and a 3D high-resolution gradient-recalled echo sequence, both with 5 mm slice thickness. For each of the 32 different coil/phantom/position combinations tested, each of the 2 protocols was performed 4 times (+/- channel receiver normalization, and +/- manual adjustment of the frequency and transmitter settings prior to the scan) for a total of 256 measurements. In addition to imaging, photography was used to document the configuration and relative position of the coils and phantoms. To measure detection sensitivity of each configuration, the signal-to-noise (SNR) ratio was measured from the resulting MR image data as follows. Using the Mean Curve data analysis utility on the Siemens MR scanner, each image was displayed and a circular region-of-interest (ROI) was drawn within the boundaries of the gel phantom, in the image slice corresponding to the center of the gel phantom. A second circular ROI was drawn in the same image slice in a region containing air (background). The mean signal amplitude, standard deviation of the signal amplitude, and area of each ROI were recorded. The SNR for each configuration was computed as the mean signal intensity of the gel phantom ROI (signal) divided by the standard deviation of the background ROI (noise) - the standard method for computing the SNR in MR image [1].

<u>Phase II. Optimize 3D high-resolution MR imaging protocols to maximize sensitivity for detecting blood in neural tissue.</u> Extravasated blood essentially acts as a contrast agent in MRI, and it is

well known from the clinical literature that hemorrhage of different antemortem ages yields different contrast, relative to normal tissue, due to changes in the chemical and physical state of hemoglobin as it degrades [2]. In Phase 2 we used several in vitro approaches to develop and validate imaging protocols to optimize detection of blood preparations in normal nerve tissue. We also determined the MR imaging properties of different in vitro blood preparations as a function of temperature. Finally we used the optimized protocols to verify the detection of small volumes (~20 microliters) of blood in tissue samples. For all in vitro and ex vivo experiments, sample temperature was varied (4-35^{o}C) using an incubator and maintained during MR imaging using water bottles, ice packs, or isothermal warming pads, as needed. Sample temperature was measured using an MR-compatible Omega FOB-400 fiber optic thermometer.

First, imaging phantoms were constructed using agarose gel doped with $CuSO_4$ (to yield a gel with MR properties mimicking post-mortem neural tissue) mixed with whole animal blood (fresh hemorrhage) or ferritin (old hemorrhage); however, it was unclear that the mixing of blood with gel adequately simulated the appearance of blood in neural tissue, which has a different structure than the gel. We next attempted to inject blood directly into fresh neural tissue (bovine or porcine spinal cord); however, the elasticity of the tissue caused an unknown fraction of the injected blood to seep back out of the needle track. We therefore chose fresh, intact, deceased lab rats as subjects for optimizing imaging protocols, given that all of the relevant tissue types are present (nerves and blood, as well as fat and muscle), and the consistent availability of samples (lab rats are retired weekly from other investigators' animal protocols). Imaging rats enabled the radiologist to qualitatively assess, for each imaging protocol, overall tissue contrast, visibility of small anatomic structures, image artifacts, and signal-to-noise (SNR). Quanitative criteria for each acquisition protocol were: 1) covering a volume of at least $(6\ cm)^3$, suitable for a human infant C-spine, 2) voxel dimensions of 1 mm or less, and a 3) total scan time < 30 minutes. Many user-specified acquisition parameters were varied (one at a time) for a range of pulse sequences, and the impact on image quality due to each adjustment was assessed by the radiologist. Several iterations of parameter adjustments were undertaken to arrive at the final 4 protocols tested in Phase 3. The Table below describes the final protocols and lists several key acquisition parameters.

Pulse Sequence Name	Description	Phase Encode Direction	Fat Suppression	Repetition Time, TR (ms)	Echo Time, TE (ms)	Flip Angle, FA (deg)	Number of Averages	Total Scan Time (min:sec)	Voxel Size, read x phase x slice (mm)	Field of View, read x phase x slice (mm)
Lt2_fi3d_cor(AXIAL)	Axial 3D T2-weighted gradient echo	R>>L	none	22	8.8	40	2	12:59	0.5 x 0.5 x 1	140 x70 x60
Rt2_tse3d_sag (AXIAL)	Axial 3D T2-weighted turbo spin echo	R>>L	fat saturation	3000	117	150	1	26:29	0.5 x 0.5 x 1	140 x 70 x 60
Dt1_fl3d_we_cor_(AXIAL)	Axial 3D T1-weighted gradient echo	R>>L	water excitation	25	11.6	25	6	23:08	0.5 x 0.5 x 1	140 x 70 x 60
Ft1_mpr_slabselect_1mmAX	Axial 3D T1-weighted inversion-prepared gradient echo	R>>L	none	1750	4.38	15	6	24:32	0.5 x 0.5 x 1	140 x 70 x 72
t1_tse_axial	Axial 2D T1-weighted turbo spin echo	R>>L	none	664	10	180	3	11:29	0.6 x 0.6 x 2.5	160 x 80 x 65
T2 ANAT COR TSE 3mm	Coronal 2D T2-weighted turbo spin echo	R>>L	none	2000	89	180	4	4:04	0.5 x 0.5 x 3	120 x 71 x 48
T2 ANAT SAG TSE 3mm	Sagittal 2D T2-weighted turbo spin echo	A>>P	none	3000	89	180	4	4:30	0.5 x 0.5 x 3	120 x 120 x 78

We then developed wet laboratory protocols for treating fresh whole animal blood to produce different hemoglobin states (oxyhemoglobin, deoxyhemoglobin, intracellular methemoglobin, and extracellular methemoglobin), corresponding to hemorrhage of various ages (hyperacute, acute, early sub-acute, and late sub-acute, respectively). We used two different preservatives (citrate and heparin) to determine whether any MR parameters depended on the choice of preservative, and we compared clotted and un-clotted blood. All methods used to treat the blood involve minimal chemical additives (by volume), such that the hemoglobin concentration was maintained within the normal range of whole blood. Briefly, fresh blood contains oxyhemoglobin within intact red blood cells. Fresh blood was deoxygenated by pouring the blood repeatedly between two sample vials under a dry nitrogen (oxygen-free) atmosphere. Fresh blood was oxidized using a small addition of sodium nitrite (0.1 M $NaNO_2$, 90 microliters per 1 milliliter of blood) to produce intracellular methemoglobin [3]. The deoxygenation and oxidation prodecures were verified by the observed color change; fresh, oxygenated blood is bright red, deoxygenated blood is a darker shade of red, and oxidized blood turns brown. Finally, oxidized whole blood (containing intracellular methemoglobin) was frozen at -20^{o}C and thawed to rupture the red blood cells and release methemoglobin to the extracellular environment. We verified the release of methemoglobin from red blood cells in frozen/thawed preparations of oxidized blood by monitoring settling of red blood cells under gravity. Intact red blood cells settle within a few hours resulting in a cell-dense layer at the bottom and a layer of clear serum on top. By contrast, extracellular methemoglobin

released from ruptured red blood cells remains well-mixed with serum indefinitely – no settling was observed even after several days.

The above preparations (~2 ml samples in capped, o-ring sealed vials) were used to study the temperature dependence of the magnetic resonance properties of whole blood containing hemoglobin in different states, representing various ages of hemorrhage. Imaging was performed to determine image contrast (using a variety of acquisition protocols). The relaxation times T1, T2 and T2* were measured by varying TR (SE sequence), TE (SE sequence), or TE (GE sequence), respectively, plotting signal intensity vs. TR (TE), and fitting the result with an exponential function. We observed that the relaxation time T2 (and hence the contrast observed in T2-weighted imaging) depended on whether the intact blood cells remained well-mixed with serum. Therefore, an additional set of samples was prepared with the hematocrit (i.e., red cell volume fraction) varied from 0% (pure serum) to 100% (completely packed red cells) in increments of 20%, to determine the effect of the red blood cell concentration on T2.

We finally developed a method to incorporate a known volume of blood into ex vivo neural tissue, without leakage. The method involves freezing 1 cm thick disks of neural tissue (bovine or porcine spinal cord) and using a set of calibrated punches (designed for leather work) to create cylindrical voids of various known sizes in the center of each tissue disk. The voids were then sealed on the bottom by affixing a thin disk of plastic (transparency film) to the tissue using cyanoacrylate glue. The void was then filled with a known volume of whole blood (oxy, deoxy, intracellular met, or extracellular met), serum, or ferritin solution (which mimics chronic hemorrhage) using a calibrated microliter pipettor. The filled void was then sealed at the top with a plastic disk and cyanoacrylate glue. Essentially, this procedure created sealed "test tubes" with a very small internal diameter, ~ 1mm, and very thick walls, ~1 cm, made of neural tissue. The neural tissue disks containing the blood inclusions were then placed in wells and covered with saline to prevent drying/shrinking of tissue during MR imaging. The tissues loaded with deoxygenated blood were prepared under a nitrogen atmosphere and covered with deoxygenated saline and sealed to prevent re-oxygenation during imaging. MR imaging of these samples

was performed over a range of relevant post-mortem temperatures using the imaging protocols developed for use in Phase 3, described above.

Phase III. Test optimized PMMR intraneural hemorrhage detection protocols in a rabbit model of nerve root hemorrhage. The aim of Phase 3 was to create such an animal model to validate the PMMR imaging protocols developed in Phases 1 and 2. A rabbit model of human pediatric intraneural bleeding was designed with the intent of enabling controlled creation and in vivo aging of hemorrhages, enabling systematic study. We developed an US guided needle injection technique to inject small volumes of fresh, autologous blood directly into or immediately adjacent to the cervical nerve sheaths or nerve roots in the cervical spines of live rabbits. We used a high resolution cardiac US probe with optimized depth and gain settings determined during initial testing. The rabbits were injected with blood in a side lying position, after shaving the injection field. The probe was held slightly oblique to the cervical spine, with the needle introduced laterally (straight up and down with respect to the operator). Although our in vitro work indicated we might visualize as little as 20 ul, we chose to inject 200 ul (= 4 drops) of autologous blood, based on the appearance of the blood collections at dissections of pilot study rabbits and concern that smaller amounts would be too difficult to inject reliably, especially if clotting occurred.

For the experimental study animals, we prepped the injection site and performed an US guided injection of autologous blood under anesthesia, in either the left or right nerve root at either C2/3 or C3/4, with a dry needle insertion (opposite side, either C2/3 or C3/4) as a control. In total, 24 rabbits underwent the US guided procedure. 5 rabbits were humanely euthanized immediately (hyperacute phase), with staggered survival of the remaining rabbits: 5 at one day (acute phase), 5 at one week (early subacute phase) and 5 at two weeks (late subacute phase). In each of the four survival cohorts, a single uninjected rabbit was included to serve as a control. During the survival period the animals were closely monitored for complications. No complications were noted in any animals. After humane euthanasia, the animals were immediately transported for post-mortem MR imaging. After receiving the animals, they were positioned for scanning immediately, before rigor mortis set in. Positioning was prone, haunches folded underneath to center and stabilize the body, forepaws extended and chin placed between the forepaws (to

stabilize the head and neck), with a small disposable pad under the neck to straighten the cervical spine. A vitamin E tablet was taped to the right side of the neck to confirm laterality in the imaging. All animals underwent post-mortem MR imaging at ambient temperature (18^{o}C), using the following acquisition sequences: 2D Axial T1-weighted TSE and 2D Coronal and Sagittal T2-weighted TSE imaging for overall anatomy and localization for setting the field of view, followed by high resolution 3D sequences (T2-w TSE, T2-w FISP, T1-w FLASH, T1-w MPRAGE). The 3D sequences are described above.

Following imaging, the cervical spines were dissected en bloc and formalin fixed. Tissue blocks were sent to Tricore Reference Laboratories (Albuquerque, NM) for preparation of histology specimens (tissue sectioning, mounting, and staining). The histology was reviewed by a board-certified neuropathologist to assess for the presence hemorrhage and, if present, the appearance and location. Radiological interpretation of MR images was performed by a board-certified radiologist using Osirix (v 5.0.1, 64 bit, Pixmeo SARL, Bernex, Switzerland). The initial reads were blinded, followed by a second unblinded review after reference to the presence and location of the blood injection (or dry needle puncture) and histological findings.

Study Findings:

Phase I. Using the pediatric phantoms, we found that the 8-Channel Head Coil with "normalize on" results in superior signal-to-noise (SNR), by a factor of 2 or more, compared to any other available combinations of coils and receiver settings, for all phantoms (3 mo, 6 mo, 12 mo). Another significant finding is that the variation in SNR related to phantom size was small compared to the variation related to the choice of coil and receiver settings. Due to the superior performance of the 8 Channel Head coil, using "normalize on" and "manual pre-scan" with each phantom positioned as low as possible within the coil volume, the 8 Channel Head coil was used for subsequent tissue and post-mortem animal scanning.

Phase II . Over 600 measurements of T1, T2, and T2* were obtained from in vitro samples containing hemoglobin in various states (normal hematocrit) or varying hematocrit (fresh oxygenated blood), over a range of temperatures. Differences in T1 and T2 observed from hemorrhage of different

ages, reported in the clinical MR literature [1], were recapitulated in these laboratory-prepared in vitro samples. Oxy- and deoxy-hemoglobin showed much longer T1 values relative to intra- and extracellular methemoglobin. Oxy- and extracellular methemoglobin show much longer T2 and slightly longer T2* values relative to the T2 and T2* values of deoxy-and intracellular methemoglobin at the same temperature. Overall, T1 and T2 both increased with increasing temperature, and T2* did not show a clear dependence on temperature. T1 and T2 increased strongly with increasing hematocrit in fresh, oxygenated blood, but again T2* showed no clear dependence on hematocrit in the same set of samples. Using our optimized high-resolution 3D PMMR protocols, reliable detection of 20 microliters of whole blood in ex vivo neural tissue was demonstrated for all hemoglobin states.

Phase III. Hemorrhage was observed at histology in 3 of 5 subjects in both the hyperacute and acute groups, at variable locations (perineural, intraneural, intramuscular and connective tissue), but not in the early and late subacute groups. In the 6 positive cases, there were level discrepancies and sided-ness discrepancies, with respect to the recorded injection site locations. The lack of positive findings in controls suggests these observations of hemorrhage are real; no hemorrhage was detected in any controls by histology or imaging, suggesting that observed hemorrhages were related to the US guided autologous injection and not handling, dissection, or other artifact. On PMMR imaging, heterogeneous signal was noted in the superficial paraspinal musculature in 2 of 5 of the early subacute cases, but this region was outside the histology field of view and could not be confirmed. No other positive findings were noted at imaging, even following a second unblinded review with knowledge of the histology findings.

Given the demonstrated ability of these same protocols to detect a small volume of blood (20 microliters) in neural tissue in vitro, the overall lack of concordance between imaging and histology in the rabbit study suggests that the resulting “hemorrhages” produced in the rabbit cervical nerves were too small in volume or too diffuse in spatial extent, possibly owing to redistribution and dilution of the injected blood by the cerebral spinal fluid (CSF) surrounding the nerves during the interval between injection and euthanasia. While the injected blood volume (200 microliters) was known, the volume of blood retained at the injection site is unknown. The inconsistent positive results at histology (in rabbits

injected in known locations) are suggestive of redistribution of the blood due to CSF flow and intradural communication, which would explain hemorrhage found at non-injected levels, at contralateral locations, or not found. In general, visualization of anatomic structures during the ultrasound (US)-guided injection of blood into the rabbit cervical nerves was difficult. A higher resolution US transducer and/or purpose-made US needles may improve the reliability of injection and are recommended in any future work using this model. Overall, however, we conclude that injection of blood in rabbit cervical nerve roots is not a sufficiently reliable model to mimic human neural injuries. In the future, we will apply these imaging protocols to human infant decedents with known or suspected cervical trauma; we will then compare the PMMR and pathology findings in cases with actual human injuries produced by real injury mechanisms.

Implications for Criminal Justice Policy and Practice in The United States: PMMR provides soft tissue information, non-invasively, that is not available by standard x-ray or computed tomography (CT) and may be useful for assessing nerve injury [*e.g.*, 4-8]. While this study did not attempt to evaluate intraneural hemorrhage as an indicator of the mechanism of death in cases of neurotrauma, the methods we developed have the potential to be used for such a study. In future studies, we or others (preferably large, multi-center studies) could apply these tools to assess intraneural bleeding in the evaluation of both accidental and inflicted trauma. Application of these methods to the noninvasive evaluation of extracranial nerve injury has the potential to redefine our understanding of the pathophysiology of a number of traumatic lesions, including the so-called "shaken baby syndrome" [*e.g.*, 9-13]. By rigorously characterizing the post-mortem MR imaging appearance of blood of various ages, these results will have broad potential application to evaluating blood observed by PMMR in other organs/tissues, resulting from other pathologies or injuries. Lastly, we hope that by improving the capabilities of MR for detecting nerve injury with high resolution and good contrast in the forensic setting, our colleagues in the clinical arena will be motivated to more vigorously engage in research to improve and validate cervical spine assessment protocols appropriate for clinical application to cases of suspected neurotrauma in living subjects [14].

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