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Diffusional kurtosis and diffusion tensor imaging reveal different time-sensitive stroke-induced microstructural changes

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ABSTRACT

Background and Purpose: Diffusion magnetic resonance imaging (dMRI) is a promising, clinically feasible imaging technique commonly used to describe white matter changes following stroke. We investigated the sensitivity of dMRI to detect microstructural alterations in grey matter following sensorimotor cortex (SMC) stroke in adult male rats.

Methods: The mean diffusivity (MD) and mean kurtosis (MK) of peri-lesion motor cortex (MC) was compared to measures in the contra-lesional forelimb area of SMC at 2h, 24h, 72h or 25d post-surgery. MD and MK were correlated to the surface densities of glia, dendrites and axons.

Results: Peri-lesional MK was increased at 72h and 25d post-stroke, while MD was no longer different from contra-lesion SMC at 24h post-stroke. There was a significant increase in the density of glial processes at 72h post-stroke in peri-lesional MC, which correlated with peri-lesional MD.

Conclusions: These data support that MK and MD provide different but complimentary information regarding acute and chronic changes in peri-lesional cortex. Glia infiltration is associated with pseudonormalization of MD in the peri-lesion MC at 72h post-lesion; however, this association is absent 25d post-lesion. These data suggest that there are likely several different, time-specific microstructural changes underlying these two complimentary diffusion measures.

INTRODUCTION

Each year, ~795,000 Americans suffer a stroke and ~400,000 will suffer long-term disability¹, predominately motor impairments². Brain areas that are connected, via overlapping arterial territory and/or are part of the same neural networks, undergo time-dependent structural and functional changes following ischemia (e.g.,³) and often undergo experience-dependent regeneration⁴⁻⁸. Peri-lesional motor areas are involved in spontaneous and experience-driven functional recovery⁵⁻⁷ and treatments that enhance motor recovery result in greater neural plasticity in these regions⁹⁻¹². Currently, there are relatively few tools available for clinicians to non-invasively investigate these changes in peri-lesional motor areas acutely or chronically. The ability to evaluate and understand these time-dependent microstructural changes occurring in the peri-lesional regions may lead to more individually tailored treatments. Diffusion magnetic resonance imaging (dMRI) is a promising and clinically feasible tool, but too little is known about the exact relationships between dMRI and the underlying tissue microstructure across the time-span of stroke recovery.

Diffusion tensor imaging (DTI) and diffusional kurtosis imaging (DKI) are sensitive to microstructure changes in the brain¹³⁻¹⁶. DTI assumes water diffusion to be Gaussian and, therefore, is unable to completely characterize tissue microstructure. DKI is a clinically feasible dMRI method that accounts for diffusional non-Gaussianity, which can reveal more about the heterogeneity and complexity of CNS tissue^{13, 16-19} and thus is a compliment to standard DTI. The application of dMRI techniques in surviving peri-lesion grey matter, previously shown to undergo stroke-related changes, has been under investigated. A clearer understanding of the relationships between peri-lesional cortical dMRI metrics, tissue biophysics, and morphological alterations following stroke is needed.

In this study, we investigated the sensitivity of dMRI to reveal changes in peri-lesional remaining motor cortex (MC) acutely and more sub-chronically after a unilateral, focal endothelin-1 (ET-1) induced stroke of the forelimb area of the sensorimotor cortex (Fl-SMC). We examined the acute post-lesion time point 2h after surgery, a time point that captures the early stages of ET-1 induced vasoconstriction and reduced blood flow, and 24h post-ischemia, a time when blood flow has been reported to return to control levels following ET-1 induced stroke²⁰. Previously, changes in dMRI following a middle cerebral artery occlusion (MCAo) in rats revealed that diffusional kurtosis at 72h was elevated while diffusion tensor measures were no longer different from the non-lesion homologous brain region at this time-point¹⁴. Thus, we included this sub-acute time-point for comparison across studies, in keeping with recommendations from STAIR²¹. Finally, we explored sub-chronic effects of stroke on dMRI measures 25d following stroke, in areas known to support recovery of function^{5, 10-12}.

Similar to previous reports (i.e.,¹⁶), we found that diffusional kurtosis (mean kurtosis; MK) continues to be elevated in peri-lesion areas, in this case the remaining motor cortex, compared to the non-lesion Fl-SMC acutely and sub-chronically (25 days) following unilateral, SMC lesions. On the other hand, the mean diffusivity (MD) of peri-lesion remaining MC was only significantly elevated acutely during vasoconstriction (2h post-surgery) and then was no longer different from contra-lesion SMC at 24h, 72h or 25d post-lesion. Our data revealed that an increase in glia infiltration into the remaining peri-lesion cortex at 72h was significantly related to MD in peri-injury cortex. These data suggest that the pseudo-normalization of MD early after stroke in the peri-lesion cortex may be in part due to the increased diffusibility of water in astrocytes.

MATERIALS AND METHODS

Animals

Long Evans male rats (n=33, 3-4 months old) received food and water *ad libitum* and were kept on a 12:12 h light:dark cycle. Rats were randomly assigned to one of four groups that underwent MRI scans and were euthanized at 2h (n=7), 24h (n=8), 72h (n=9), and 25 days (n=9) post-lesion. Two animals were excluded from the study, due to poor tissue extraction (n=1) and dMRI artifacts (n=1). All work was done in accordance with the Medical University of South Carolina Animal Care and Use Committee guidelines.

Surgical Procedures

All animals were scanned prior to stroke and then at 2h, 24h, 72h or 25d post-lesion. Animals were anesthetized with isoflurane/air (4-5% for induction/1-3% for maintenance) for all MRI scans. In the acute stroke animals sacrificed at 2h, 24h or 72h, unilateral ischemic lesions were induced via infusion of ET-1 (American Peptide, Inc) into layer V of the left forelimb area of the sensorimotor cortex (Fl-SMC) through four holes drilled at 0.5 mm posterior, 2.5 mm anterior, and 3.5 and 4.6mm lateral to Bregma²². One μl of ET-1 ($0.2\mu\text{g}/\mu\text{l}$ in sterile saline) was injected into each hole via a Hamilton syringe (lowered to 1.5mm DV), at a rate of $1\mu\text{l}/2$ min.

For the 25d post-lesion group (these animals were included from a different study), animals received a cocktail of ketamine (110 mg/kg) and xylazine (70mg/kg). Craniotomy was performed at 0.5 mm posterior and 1.5 mm anterior to bregma and 3.0–5.0 mm lateral to midline, and then dura was gently retracted. Four μl ($0.2\mu\text{g}/\mu\text{l}$ in sterile saline) of ET-1 was applied directly on the brain surface at approximately $1\mu\text{l}/\text{min}$, with a 2 min wait between each $1\mu\text{l}$ of ET-1 application.

After the final application of ET-1 in both sets of surgeries, the brain was left undisturbed for 5 min and then the holes or craniotomy was covered with gel film and UV cured dental

acrylic. **These coordinates for ET-1 application reliably produce sensorimotor forelimb deficits and cause damage to the forelimb SMC overlap region^{10,11,22}.**

Diffusion Magnetic Resonance Imaging

MRI scans were acquired pre-injury (day 0) and at 2h, 24h, 72h, or 25 days post-injury using a 7T/30 Bruker BioSpec (Billerica, MA) animal scanner. A two shot spin-echo echo planar imaging (EPI) diffusion sequence with 30 diffusion-encoding directions and 4 b-values (0, 650, 1300, 2000 s/mm²) was used. Other imaging parameters were: TR/TE=4750/32.5ms, FOV=30mmx30mm, matrix=128x128, in plane resolution=0.23x0.23x1mm³, $\delta/\Delta=5/18$ ms, and number of excitations (NEX)=2. 19 axial slices with no gap were collected with a slice thickness=1mm.

Data presented are for MD and MK corresponding to the apparent diffusion and kurtosis coefficient, respectively, averaged over all directions¹⁷. Diffusion and diffusional kurtosis tensors were calculated using Diffusional Kurtosis Estimator (DKE), a publically available in-house software²³.

Multi-slice regions-of-interest (ROIs) were manually drawn in the A) infarct core (**Fig 1A**), B) the homologous region in the contra-lesional SMC, C) the peri-lesion layers II/III and V of the remaining motor cortex (**Fig 2A**), and in the contra-lesion layers II/III and V of the FI-SMC. The peri-lesion ROIs were drawn on three contiguous MRI slices (1mm thick) and was inclusive of layers II/III and V.

MD and MK results were analyzed and presented as a ratio of post-lesion/pre-lesion values, allowing for comparison and more comprehensive visual representation of stroke related changes in dMRI of both hemispheres compared to their pre-lesion values (**Fig 1 & 2**).

Additionally, this allowed us to reduce inter-animal differences between groups of animals. Pre

versus post-lesion MD and MK results show similar patterns of changes as those of post-lesion/pre-lesion ratios (data not shown). The actual MD and MK values, however, were used to investigate the relationships between peri-lesion remaining cortex and microstructural changes in glia, dendrites and axon densities in these regions.

Tissue Processing

Following MRI scans at 2h, 24h, 72h, or 25 days post-injury, animals were deeply anesthetized with pentobarbital (Euthasol, 100mg/kg, IP) and perfused with 0.1M phosphate-buffer (PB) and 4% paraformaldehyde. Six serial rostral to caudal sets of 50 μ m coronal sections were produced using a vibratome and stored in cryoprotectant. Three sets of sections were processed for immunohistochemistry (IHC) to ascertain post-injury morphological changes in the surface density of glia processes, dendrites, and axons in the peri-lesion MC.

Briefly, as described previously²², free-floating sections were processed for IHC. Tissue was incubated for 48 hours in one of the following primary antibodies: glial fibrillary acidic protein (GFAP) for glia (1:800 rabbit polyclonal; Dako), microtubule protein 2 (MAP2), for dendrites (1:500 mouse monoclonal; Sigma-Aldrich), and for axons, Pan-Axonal Neurofilament Marker (SMI-312; 1:2000 mouse monoclonal; Covance). Sections were incubated in peroxidase-linked avidin-bioin complex (ABC kit) for 2 hours. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) with nickel ammonium sulfite (NAS) intensification. All animals were included in each batch of IHC processing and each batch included negative control sections without primary antibody. For the 25d subset of animals, tissue was only processed for GFAP immunohistochemistry to explore changes in post-lesion glia and to relate these findings to dMRI measures.

Microstructure Quantification

The cycloid grid intersection method²⁴ was used to determine the surface density (Sv) of GFAP, MAP2 and SMI-312 immuno-reactive (IR) processes. For each antibody, we sampled tissue that was represented in three adjacent MRI slices that included the FI-SMC. While we did not directly co-register ROIs and IHC section, we did carefully select tissue regions based upon clear cytoarchitectural features of the tissue and lesion boundaries. Data were obtained in three adjacent coronal sections (~600µm apart) which included peri-lesion MC and contra-lesional FI-SMC (i.e., between approximately +1.2 and -0.26mm anterior/posterior relative to Bregma)²⁵. Cycloid arcs (ImageJ) were overlaid on light microscopic images, taken at 100x oil immersion (final magnification=1,400X), of four sample regions per hemisphere, two adjacent sets (~250µm) in layers II/III and two set in layer V beginning at approximately 250µm medial to the lesion core (towards midline). Each IR process that crossed an arc was counted. The surface density was calculated using the formula $S_v = 2(I/L)$, where I is the total number of intersections and L is the sum of the cycloid arc lengths.

Statistical Analysis

All data are reported as averaged group means with ±S.E.M. One-way analysis of variance (ANOVA) was used to test for time-point differences using SPSS software. Multiple comparisons were corrected using Bonferroni post-hoc analyses to further explore lesion time point differences. Pearson's linear correlations were used to determine relationships between changes in the density of glia, dendrites and axons with post-lesion dMRI metrics. The significance level was $\alpha=0.05$.

RESULTS

MD and MK of lesion core and homologous contra-lesion SMC

MD was significantly different in the lesion core compared to the contra-lesion homologous cortex ($F_{3,29}=22.547$, $p\leq 0.01$). Similar to previous reports¹⁴, MD in the lesion core was significantly reduced compared to the contra-lesion homologous cortex at 2h ($p\leq 0.01$) and 24h ($p\leq 0.01$) post-stroke (**Fig 1B**), but there were no longer significant differences at 72h and 25d post-lesion.

Similar to previous findings^{14, 16}, MK remained significantly elevated across all time-points in the lesion core ($F_{3,29}=31.194$, $p\leq 0.01$) compared to contra-lesion SMC (**Fig 1C**) at 2h, 24h, 72h, and 25d post-lesion (p 's ≤ 0.01). There were no significant differences between post-lesion time points.

MD and MK of peri-lesion remaining MC and contra-lesion FI-SMC

At 2h post-lesion (mean= $.95\pm.02$), MD was subtly but significantly decreased in remaining peri-lesion MC compared to non-lesion FI-SMC (mean= $.99\pm.01$; **Fig 2B**; $p\leq 0.05$). At 24h, 72h, and 25d post-lesion, peri-lesion MD was no longer significantly different from the non-lesion FI-SMC.

On the other hand, MK remained elevated in the remaining MC days and weeks after injury compared to the contra-lesional FI-SMC. Peri-lesion MK in the remaining MC was significantly increased compared to contra-lesion FI-SMC at 72h ($p\leq 0.05$) and 25 days ($p\leq 0.05$) post-lesion and there was a non-significant tendency at 24h post-lesion ($p=0.054$; **Fig 2C**).

Surface density of glia processes (GFAP immunoreactivity)

There was a significant effect of time after injury on the surface density of GFAP-IR processes in peri-lesion motor cortex ($F_{3,29}=26.822$, $p\leq 0.01$). At 72h, there was a significant increase in GFAP positive processes compared to contra-lesion FI-SMC ($p\leq 0.001$) and compared

to acute (2h, $p \leq 0.01$; 24h, $p \leq 0.01$) and sub-chronic (25 days ($p \leq 0.01$) post-lesion time-points (**Fig 3B**).

Additionally, there was a significant increase in the density of glia processes in the non-lesion FI-SMC at 72h compared to 2h ($p \leq 0.05$). In the contra-lesion SMC, glia infiltration was elevated acutely compared to the sub-chronic 25d post-lesion time ($p' s \leq 0.01$).

Surface density of dendrites (MAP2 immunoreactivity)

There were no significant differences between peri-injury MAP2 surface densities at any acute time-point compared to the non-lesion FI-SMC ($p' s \geq 0.05$).

Surface density of axons (SMI-312 immunoreactivity)

There were no significant differences between peri-injury surface densities of SMI-312 at any acute time point compared to the non-lesion FI-SMC ($p' s \geq 0.05$).

Correlation of dMRI with morphological characteristics of peri-lesion remaining MC and contra-lesion FI-SMC.

Although MD in the peri-lesional remaining MC was no longer significantly different from contra-lesional FI-SMC, GFAP surface density at 72h time point was highly correlated with peri-lesional MD ($r=0.897$, $p \leq 0.001$). The return of MD to pseudonormalized levels acutely after ischemia may be due to microstructure changes related to increased glia infiltration and an increase in water diffusion through these astrocytes. There was also a significant, but weak, correlation overall between MAP2 surface density in the peri-lesion remaining MC and peri-lesion MD ($r=0.462$, $p \leq 0.05$, data not shown).

DISCUSSION

These studies provide further evidence that MK and MD reveal different but likely complimentary information about microstructural changes following stroke. Similar to previous

findings¹⁴, MD in the lesion core was reduced at 2h and 24h post-lesion, but was no longer significantly different from the contra-lesion SMC at 72h or 25 days post-lesion. On the other hand, MK of the lesion core remained significantly different from contra-lesional homotopic SMC for weeks following stroke. These findings are similar to those reported following unilateral MCAo in rats and likely reflect, as others have reported in different models, changes in water diffusion associated with edema²⁶, axon beading²⁷, and demyelination²⁸. These early changes in MD and MK in the lesion core are also likely due to other, as of yet unidentified microstructural changes. We were unable to address the underlying cause of these changes because there was rarely lesion tissue remaining 72h and 25 days after injury.

In these studies, we also sought to determine if following a focal, unilateral ET-1 induced ischemic insult to the SMC, alterations in microstructure were detectable using dMRI in the perilesional remaining MC, an area known to undergo acute and chronic changes following injury and experience-dependent changes. We observed that in the perilesional remaining MC, MK remains elevated for days and weeks after stroke, at time points when MD has pseudonormalized. Our data also indicate that elevations in MK are not likely due to glia infiltration or acute disruption of the overall density of axons and dendrites in perilesion cortex. It is likely, but yet unexplored in this stroke model, that these ongoing elevations in MK are related to axon beading, microglia upregulation or other microstructural changes. Further studies are underway to investigate these other likely microstructural changes that underlie the time-specific changes in MK and MD.

Interestingly, the surface density of glia and dendritic processes in perilesional cortex was correlated with perilesional MD. Infiltration of glia cells into the perilesional remaining motor cortex was robustly elevated at 72h and was more strongly correlated with MD at this time

point. It is possible that the “renormalization” of MD at 72h actually is indicative of glia infiltration at this acute time period. **Glia, specifically astrocytes**, expresses aquaporin-4 making them highly permeable to water, leading to increased water diffusion. After ischemic injury, **glia** infiltration can lead to greater edema; when aquaporin-4 is knocked out, there is reduced edema²⁹. Thus, the increase in glia infiltration in the peri-lesional MC at 72h following stroke is one explanation for why MD is increased (to more normalized levels) at 72h post-lesion. However, glia are no longer increased 25 days after lesion, and thus other, as of yet unidentified microstructural changes in peri-lesion cortex are likely responsible for the pseudonormalized MD measures weeks after stroke. Further study is needed on the full time-course of these changes in microstructure and their relationship to dMRI.

The correlation between peri-lesion MD and surface density of dendritic processes is weak, but may reflect acute lesion induced dendritic remodeling. Further investigation is needed to understand this relationship. The water permeability rates are unknown in dendritic processes and these rates likely depend on shape and size. Water permeability may be changing following ischemic damage, leading to MD detecting these changes but likely do not capture all of the changes occurring in peri-lesion microstructure.

Axonal remodeling after stroke has previously been demonstrated³⁰, is linked to behavioral recovery³⁰, and has been associated with changes in dMRI measures, primarily in white matter tracts²⁸. In the present study, we were interested in whether dMRI could capture axonal degeneration in cortical grey matter. However, we found no significant changes following stroke in the density of axons in remaining motor cortex and no significant correlation with dMRI measures. It is possible that dMRI is not sensitive enough to pick up acute stroke changes in axon density. Further studies are needed.

Together these data indicate that DKI measures are sensitive to changes in tissue properties of acute and chronic peri-lesion remaining MC; however, this is likely not due to alterations in the surface density of glia processes, dendrites, and axons. On the other hand, glia infiltration into the remaining peri-lesion MC may underlie the normalization of diffusion properties measured by MD at 72h post-lesion, but likely there are also other structural changes responsible for the apparent normalization of MD during the more chronic post-stroke period.

In summary, this work is one of the first to characterize morphological changes following ET-1 induced ischemic stroke and to relate these findings to changes in dMRI metrics. These data further support that diffusional kurtosis and diffusion tensor measures provide different but complimentary information regarding acute and chronic changes in peri-lesion cortex following stroke. There are likely several different microstructural changes underlying these two complimentary diffusion measures and we need to further investigate these time-sensitive, region specific changes.

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DISCLOSURES

Joseph A. Helpert is listed on the DKI patent: System, Method and Computer Accessible Medium for Providing Real-Time Diffusional Kurtosis Imaging (US Patent # 8,811,706 B2, August 19, 2014).

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Figure Legends:

Figure 1. dMRI of lesion core and contra-lesion homologous SMC. **A.** Representative ROIs (blue) around lesion core (left) and the contra-lesional homotopic SMC (right). **B.** At 2h and 24h post-lesion, mean diffusivity (MD) was significantly reduced compared to the contra-lesional hemisphere (**p's ≤ 0.01). **C.** Mean kurtosis (MK) remained elevated in the injury core

compared to contra-lesion hemisphere at each time-point (**p's ≤ 0.01). Dotted line indicates ratio of 1.0 or equal to pre-lesion baseline.

Figure 2. dMRI of peri-lesion remaining MC and contra-lesion FI-SMC. **A.** ROIs (red) were drawn for the peri-lesional layers II/III and V in remaining motor cortex (left) and where compared to contra-lesional FI-SMC (right) on b2000 images. **B.** MD in peri-lesion cortex was significantly reduced at 2h post-lesion compared to the contra-lesion hemisphere (*p ≤ 0.05). **C.** MK was increased in peri-lesion cortex compared to contra-lesional FI-SMC at 72h and 25d post-lesion (*p ≤ 0.05). Dotted line indicates ratio of 1.0 or equal to pre-lesion baseline.

Figure 3. Surface density of glia. **A.** Representative 100X image of glia density at 72h post-lesion in the peri-lesional area (scale bar=50 μ m). **B.** The surface density of glial processes was increased at 72h in the lesion hemisphere (\dagger p ≤ 0.001) compared to all time points. There was also an increase in glia response at 72h compared to 2h in the non-lesion hemisphere (*p ≤ 0.05). In the non-lesion SMC, glia infiltration was elevated at 25d post-lesion compared to all other times (p's ≤ 0.01). **C.** GFAP surface density at 72h time-point, when there is the greatest glia infiltration into the peri-lesion cortex, was highly correlated with peri-lesional MD (r=0.897, \dagger p ≤ 0.001).