Investigation of Angiogenesis following CART peptide Treatment in Transient Ischemic Rat Stroke Model Using Susceptibility-Weighted Imaging

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Introduction:
Cocaine- and amphetamine-regulated transcript (CART) is a peptide that was found in various brain regions, including striatum, hippocampus, and cortex. It is involved in several important physiological responses in energy metabolism and neural injury [1]. The expression of CART is upregulated in the brain after focal cerebral ischemia in rats. Although enhanced neuroregeneration and behavioral recovery following CART therapy in rat stroke models have been shown recently [1], studies using a noninvasive imaging method to evaluate the cerebral angiogenesis after CART treatment are limited. Susceptibility-weighted imaging (SWI) has been increasingly used in human studies of cerebral vascular malformations with improved visualization due to its high sensitivity in detecting blood oxygen level-dependent phase effects between venous and the surrounding brain parenchyma [2]. In the present study, we applied the SWI technique in a rat stroke model to quantitatively investigate cerebral angiogenesis after stroke with or without CART treatment.

Materials and methods:

Animal preparations. A total of 30 male Sprague-Dawley rats (250-350 g) were included in this study. Bilateral common carotids were ligated with nontraumatic arterial clips first and the right middle cerebral artery was ligated with a 10-O suture to generate focal infarction in the cerebral cortex. The ligature and clips were then removed after 90-min ischemia to allow reperfusion. All animals received either CART (CART55–102, 0.1nmol/10mL) or saline delivered into the nostrils of each rat at a dose of 40 mL on day 3 after stroke and then 20 mL daily for another 6 to 9 days.

MRI scans. All animals were subjected to serial MRI measurements at 0 (pre-stroke), 2, 10 and 25 days, respectively, after stroke using a Brucker 9.4T animal MRI scanner. Rats were imaged using T2-weighted imaging (T2WI), diffusion-tensor imaging (DTI) and SWI under 1.8% isoflurane anesthesia in air / O_2 (80:20). T2WI were acquired with FOV = 3.2 cm^2, matrix size = 128×128 (zero-filled to 256×256), TR/TE = 2750/ 13.3 ms, and 23 slices with 1 mm thickness. DTI were acquired with a spin echo single-shot echo planar imaging sequence, 19 slices with 1mm thickness, FOV = 3.2 cm^2, matrix size = 96×96, TR/ TE = 9500/ 38 ms. Thirty diffusion-weighted images along independent orientations (b=1000 s/mm^2) and 1 baseline image (b=0) were acquired for each slice, and the acquisition was repeated three times to improve signal-to-noise ratio. Measurements of SWI used a three-dimensional gradient-echo imaging sequence with gradient flow compensation in three directions [4]. The acquisition matrix was 256×256×128 for a 32x32x20 mm^3 imaging volume, TR/ TE=30/ 10 ms, and flip angle=15^0.

Image processing and analyses. Rats were divided into the CART-treated and control groups according to similar lesion size measured from T2WI on day 2. Lesion volumes on day 10 and 25 were quantified relative to that on day 2 (rLV). For the DTI data, fractional anisotropy (FA) was derived using the dTV software (University of Tokyo Hospital, Tokyo, Japan). On FA maps, a region-of-interest (ROI) was manually drawn within the perilesional hyperintense region on each of the lesion-containing slice [3]. For comparison, an additional ROI was placed in contralateral white matter. After normalizing signal from the perilesional region to the contralteral white matter, the FA ratio was compared between the CART and control groups. SWI maps were calculated using the in-house developed Matlab script based on the previous study [4]. A ROI was first manually selected on the fine linear structure within the perilesional boundary of the ipsilateral cortex. An automatic segmentation method based on the fuzzy c-means clustering (FCM) algorithm was then applied to remove the possible intra-observer variation [5]. A six-class FCM algorithm was applied to the selected ROI to identify the first four cluster classes with hypointensities representative of the venous structure and the other two cluster classes with hyperintensities thought to represent the non-venous tissues adjacent to the venous structure. An additional ROI with similar size was placed in a contralateral area for comparison. Change of SWI signal intensity was expressed as a ratio of lesion-to-contralateral area. Comparisons of rLV, FA, or SWI ratios between CART and control animals across all stages were analyzed using a two-way ANOVA with repeated measures. A p value < 0.05 was considered statistically significant.

Histology. To evaluate the vascular activity, α-smooth-muscle actin (α-SMA) immunohistochemistry [6] was applied in the sacrificed animals and the number of α-SMA-positive vessels were counted in the penumbra of lesioned cortex.

Results:

The lesion volume on day 2 after stroke determined from T2WI for the CART-treated and control groups were 204.72±25.39 $mm^3$and 203.67±31.83 mm^3, respectively. There was a time (p<0.0001) and a treatment (p<0.0001) dependent reduction in rLV as revealed in the ANOVA analysis. There was a significant interaction between treatment and follow-up time points (p=0.003). Post-hoc analysis showed that CART significantly reduced rLV on 10 and 25 days after stroke as compared with control animals (p=0.004 and p=0.02 for 10 and 25 days, respectively, Fig. 1). Absolute FA values in the contralateral white matter area were not significantly (p > 0.05) altered before CART treatment on day 2 (CART: 0.74± 0.18 vs. control: 0.74±0.20) as well as after CART treatment on day 10 (CART: 0.74± 0.12 vs. control: 0.75±0.08) and day 25 (CART: 0.74±0.18 vs. control: 0.74±0.09). FA ratio in the perilesional cortex was significantly enhanced by CART treatment (p=0.0005) or follow-up time (p<0.0001, two-way ANOVA). There was a significant interaction between treatment and follow-up time (p=0.0006). CART significantly increased FA on days 10 and 25 days after stroke (p=0.004 and p=0.002 for 10 and 25 days, respectively, post-hoc test, Fig. 2). Quantitative SWI characterization after stroke is presented in Figure 3. Ten days after stroke onset, the linear hypointense area identified in CART-treated animals rapidly decreased and achieved the minimal value at 25th day. In contrast, the value in control animals showed a relatively gradual decrease within 25th day after stroke. ANOVA revealed a time (p<0.0001) and a treatment (p=0.0001) effects in the SWI data. There was also a significant interaction between treatment and follow-up time points (p=0.02). The averaged SWI signal intensity values related to angiogenesis exhibited significant differences between treated and control rats from 10 days (CART: 0.64±0.22 vs. control: 0.85±0.3, p=0.02, Post-hoc) to 25 days (CART: 0.58±0.21 vs. control: 0.81±0.19, p=0.003, Post-hoc) after stroke. Representative SWI data from the two CART-treated and two control rats are shown in Fig. 4 (left). The low intensity linear structures shown in the perilesional boundary in SWI images were clearly present for CART-treated rats. Control rats also showed low intensity linear structure around the perilesional boundary, however, the signal intensity is relatively weaker as compared with the CART-treated animals. Supportive data with enhanced density of α-SMA immunoreactivity are presented in Fig.4 (right).

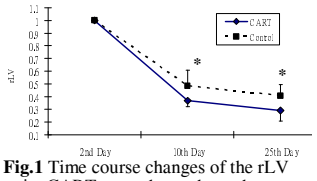

Fig.1 Time course changes of the rLV ratio. CART-treated rats showed significant reduction in lesion volume at 10 and 25 days after stroke (*P < 0.05).

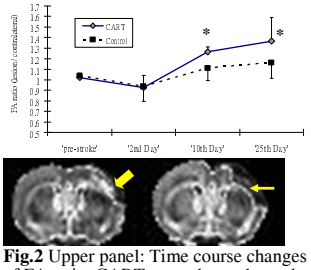

Fig.2 Upper panel: Time course changes of FA ratio. CART-treated rats showed significantly higher FA values at 10 and 25 days after stroke (*P < 0.05). Lower panel: Typical FA maps from a CART-treated (left) and a control (right) animals at 25th day after stroke onset.

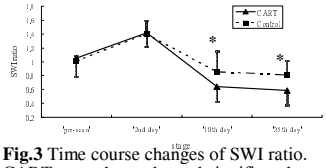

Fig.3 Time course changes of SWI ratio. CART-treated rats showed significantly enhanced hypointensity in SWI at 10 and 25 days after stroke (*P < 0.05).

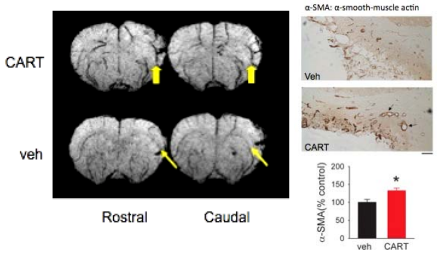

Fig.4 (Left) Representative SWI from two CART- and two vehicle-treated animals. CART-treated animals showed enhanced hypointensity (arrow heads) compared with control animals (arrows). (Right) CART-treated animals showed increased density of α-SMA immunoreactivity in the perilesional boundary compared with control animals (*P < 0.05).

Discussion and conclusions:

In this study we used SWI to evaluate changes of vascular activity following CART treatment in a rat stroke model. The CART-treated animals showed enhanced SWI values in areas surrounding the infarction, compared with the control groups. Our data demonstrated that SWI identified cerebral angiogenesis after stroke in rats, and CART treatment enhanced angiogenesis compared to the saline treated rats. We further showed that animals treated with CART showed reduced rLV and higher FA values in areas surrounding the infarction. These imaging findings are consistent with previous report that intranasal CART treatment facilitates neuroregeneration in stroke brain [1]. Our previous studies have demonstrated that CART administration increased synaptic connections and showed improvements in a skilled reaching test [1]. Taking together, the present study demonstrates the feasibility of quantitative SWI as a noninvasive maker to reflect the level of angiogenesis and possibly to monitor the progress of stroke recovery.

References: 1. Luo et al.. J Cereb Blood Flow Metab. 2013: 33:300-10. **2.** Pinker et al.. AJNR Am J Neuroradiol. 2007:28:1280-6. **3.** Liu et al.. Neuroimage. 2011;56:280-9. **4.** Haacke et al., Magn Reson Med. 2004; 52:612-8. **5.** Emblem et al., J Magn Reson Imaging. 2009; 30:1-10. **6.** Schneider et al., Stroke. 2007;38(4):1320-8. **Acknowledgement:** This work was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health.