Laboratory Investigation

Does the Apparent Diffusion Coefficient Value Predict Permanent Cerebral Ischemia/Reperfusion Injury in Rats?

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Rationale and Objectives: Variation in tissue damage after cerebral ischemia/reperfusion (I/R) can cause uncertainty in stroke-related studies, which can be reduced if the damage can be predicted early after ischemia by measuring the apparent diffusion coefficient (ADC). We investigated whether ADC measurement in the acute phase can predict permanent cerebral I/R injury.

Materials and Methods: The middle cerebral artery occlusion model was established using the intraluminal suture method to induce 60 minutes of ischemia followed by reperfusion in rats. T2-weighted images and diffusion-weighted images were obtained at 30 minutes and 24 hours after ischemia. Neuronal cell survival was assessed by neuronal nuclei (NeuN) immunofluorescence staining. The correlation between relative ADC (rADC) values at 30 minutes and I/R injury at 24 hours after ischemia was analyzed. Magnetic resonance imaging results were confirmed by histologic analysis.

Results: The correlation between rADC values at 30 minutes and 24 hours was strong in the ischemic core and peri-infarct region but moderate in the anterior choroidal and hypothalamic region. Histologic analysis revealed that the correlation between rADC values at 30 minutes and the number of NeuN-positive cells at 24 hours was strong in the ischemic core and peri-infarct region but moderate in the anterior choroidal and hypothalamic region. Furthermore, there was a strong positive correlation between the sum of rADC values of three regions at 30 minutes and the infarct volume at 24 hours.

Conclusion: ADC measurement in the acute phase can predict permanent cerebral I/R injury and provide important information for the evaluation of ischemic stroke.

Key Words: Apparent diffusion coefficient; Permanent cerebral ischemia/reperfusion injury; Prediction.

INTRODUCTION

Tissue damage following ischemic stroke is heterogeneous, and it is difficult to predict individual outcomes early after ischemia. An improved accuracy in predicting outcomes at an early stage is useful for clinical decision making in acute stroke management. Diffusion-weighted imaging (DWI), in which the image contrast is based on the water apparent diffusion coefficient (ADC), is the most frequently used method in clinical acute stroke studies (1,2). However, the evolution of the initial ADC value depends on various conditions, such as the duration of ischemia and the degree of occlusion or reperfusion (3). Some tissues with an early ADC decline can recover, whereas some do not (1,4). Despite the uncertainty of differentiating irreversible tissues from reversible tissues, DWI is widely used for the initial diagnosis of acute stroke in clinical practice (5).

The suture middle cerebral artery occlusion (MCAO) model in rats is widely used to produce focal ischemia in preclinical ischemic stroke studies, allowing the investigation of stroke pathophysiology and new therapeutic approaches (6,7). Despite the standardization of experimental stroke models, final infarct sizes after MCAO vary considerably in size and distribution. Previous studies have demonstrated that the site of filament insertion (8), anatomical variations in the origin of the MCA (9,10),

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and efficacy of reperfusion (11) are factors that could contribute to the variability of the final infarct size. These factors result in uncertainty in the study of stroke pathophysiology and in the evaluation of new therapies for stroke. If permanent brain damage caused by these factors can be predicted early after ischemia, it may help reduce the uncertainty in stroke-related research.

The purpose of this study was to evaluate the usefulness of the DWI as an early predictor of permanent cerebral ischemia/reperfusion (I/R) injury in rats. Quantitative ADC values were measured to compare acute ADC changes after ischemia with permanent tissue damage caused by focal cerebral I/R injury.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the guidelines of the National Institutes of Health. All of the procedures were performed following approval by the Institutional Animal Care and Use Committee of Asan Medical Center (IACUC Number: 2016-14-283). All surgeries were performed under isoflurane anesthesia. All of the animals were carefully monitored by trained individuals who can assess animal pain-related behavior. Euthanasia was planned if the animal exhibited continuous pain-related behavior; however, no animals exhibited severe pain-related behavior during the experimental period.

Transient Middle Cerebral Artery Occlusion (tMCAO)

Male Sprague-Dawley rats were used in the experiments (8 weeks old; weight, 280−310 g; Orient Bio, Pyeongtaek, Republic of Korea). All animals were individually housed in standard plastic cages and maintained on a 12 hours light-dark cycle (lights on at 08:00 A.M.) at an ambient temperature of 24.0°C−25.0°C with free access to food and water.

A tMCAO rat model of I/R injury was established for analysis. In brief, to surgically generate transient middle cerebral ischemia, the animals were initially anesthetized with 5% isoflurane and maintained with 2%−3% isoflurane during the procedure. Based on a previously described method of intraluminal vascular occlusion (12), MCAO was performed for 60 minutes to induce ischemia, and the occlusion was relieved to induce reperfusion. In particular, an 18−20 mm 4-0 suture thread (Ethylon surgical monofilament polyamide; Ethicon, Livingston, United Kingdom) with a fire-polished tip (diameter, 0.38−0.40 mm) was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA (Fig 1). After 60 minutes, the inserted intravascular thread was removed gently.

A total of 31 tMCAO models were established to analyze the correlation between acute ADC changes and cerebral I/R damage. The aim of this study was to obtain significant results ($p < 0.05$) with sufficient power (90%) to detect a correlation coefficient of at least 0.5. Therefore, the minimum required sample size for this study was 31 as calculated by G-power program version 3.1 (13,14). The formula for calculation was based on a one-tailed test. If death occurred during the follow-up period, additional rats were included to meet the sample size number.

Magnetic Resonance Imaging (MRI)

MRI was performed using the 7 Tesla Bruker PharmaScan 70/16 MRI system (Bruker BioSpin, Ettlingen, Germany). A 72 mm birdcage volume coil was used for excitation, and a

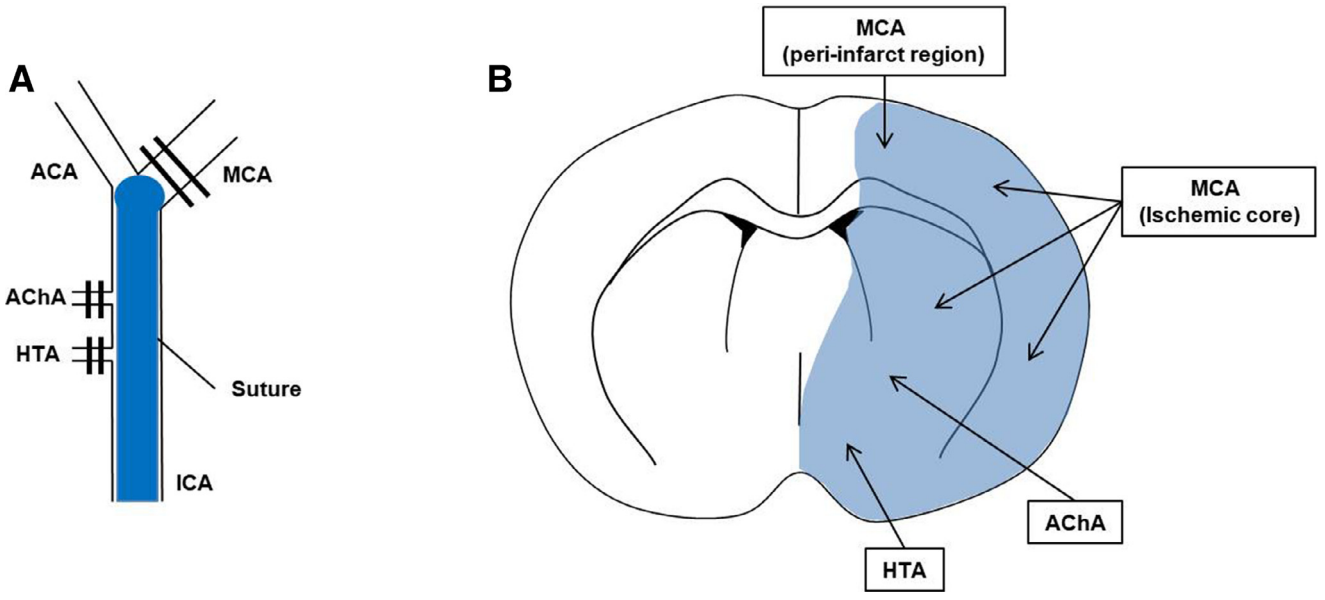

Figure 1. Diagram of the MCAO model using the intraluminal suture method. (a) Blood supply was blocked by the suture. (b) Regions of ischemic brain injury caused by the suture. ACA, Anterior cerebral artery; AchA, Anterior choroidal artery; HTA, mypothalamic artery; ICA, internal carotid artery; MCA, middle cerebral artery.

surface coil served as the receiving coil. All animals were anesthetized through a mask via the spontaneous inhalation of 2.0%–2.5% isoflurane in a 1:2 mixture of O_2:N_2O. Respiration was monitored, and the rats were maintained under normothermic conditions at 37.5°C ± 0.5°C using a heating bath circulator (CW-05G Heated Circulating Water Bath; MIDSCI, St. Louis, MO). MRI data were obtained at 30 minutes and 24 hour after ischemia.

The MRI protocol included T2-weighted images (T2-WIs) and spin-echo echo-planar diffusion-weighted images (SE-EPI-DWIs). T2-WIs were acquired with a TurboRARE sequence (TR, 4000 ms; TE, 33 ms; rare factor, 8; averages, 1; field of view, 25 × 25 mm, matrix, 256 × 256; slice number, 16; slice thickness, 1.0 mm, no gap). The SE-EPI-DWI parameters were as follows: TR, 3000 ms; TE, 17.18 ms; averages, 3; segments, 4; field of view, 25 × 25 mm; matrix, 96 × 96; slice number, 16; slice thickness, 1 mm, no gap; b-values, 0 and 1000 s/mm^2). A diffusion gradient was applied along the readout, phase encode, and slice directions. Quantitative ADC maps were calculated by least-squares fit using the Image Processing Tool of Paravision 6.0.1 software (Bruker Biospin, Ettlingen, Germany). Respiratory gating was used for SE-EPI-DWI acquisition, and the total scan time was <10 minutes.

MRI Analysis

All MRI data were assessed by an observer blinded to the group information. MRI analysis was conducted using ImageJ software (http://rsbweb.nih.gov/ij/; National Institutes of Health, Bethesda, MD). The correlation between the ADC in the early phase and I/R injury was evaluated by comparing the ADC values at 30 minutes after ischemia with both the ADC values and infarct volume at 24 hours after ischemia. First, ADC values were measured in three different regions on an ADC map. The regions of interest were located in the ischemic core (IC), peri-infarct region (PIR), and anterior choroidal and hypothalamic region (AHR) of the ipsilateral hemisphere and contralateral hemisphere (Fig 2). Thereafter, the relative ADC (rADC) value was calculated as a ratio (ipsilateral value/contralateral value). Second, the total infarct volume at 24 hours after ischemia was measured on T2-WI scans using the 2D volumetry technique, which involves the summation of the infarct volume measured from each slice (15).

Histologic Analysis

Neuronal cell survival in the I/R injury region was assessed by neuronal nuclei (NeuN) immunofluorescence staining. In

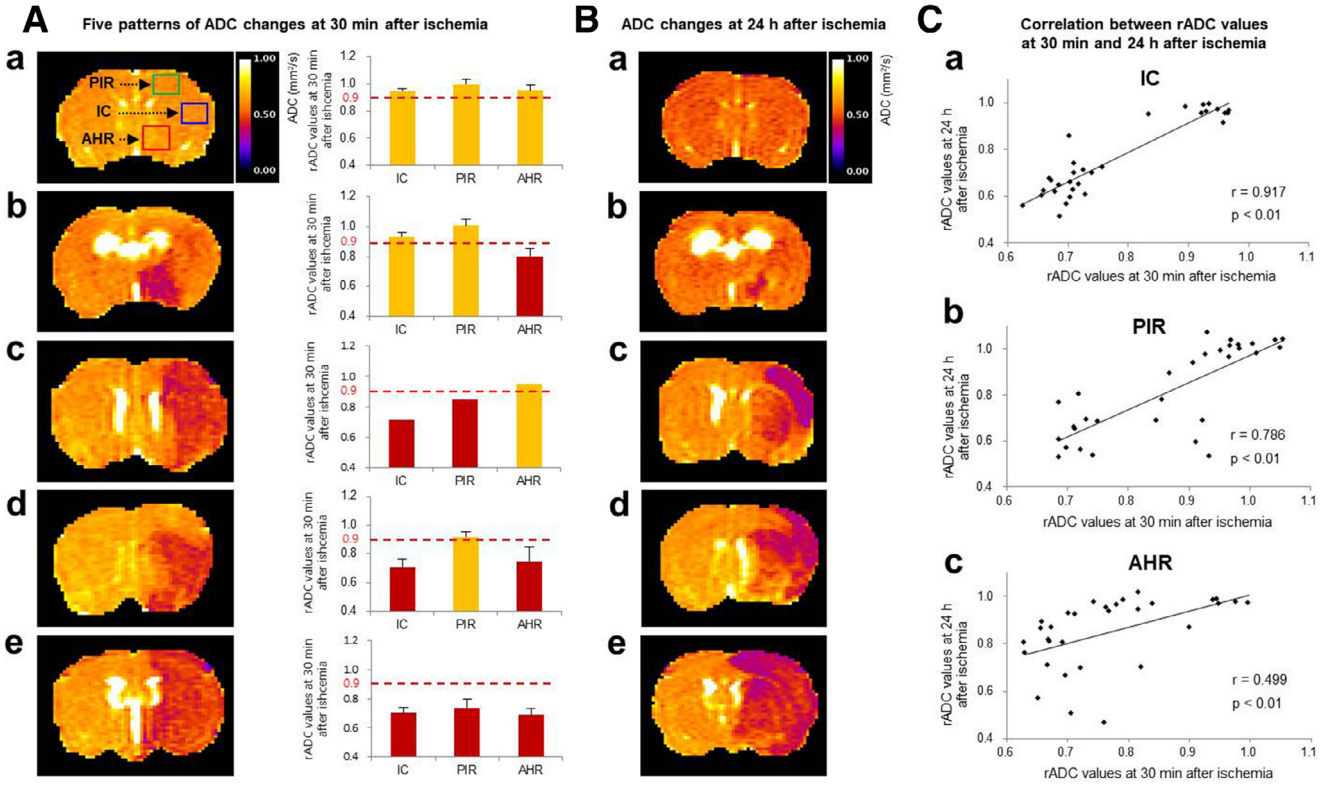

Figure 2. Correlation between rADC values at 30 minutes and 24 hours after ischemia. (a) ADC values measured in the ipsilateral region (IC [*blue square*], PIR [*green square*], and AHR [*red square*]) as well as the contralateral region at 30 minutes after ischemia. Changes in the rADC values of the five patterns were observed at 30 minutes after ischemia. (b) ADC maps at 24 hours after ischemia compared to the ADC maps at 30 minutes after ischemia. (c) Correlation between rADC values at 30 minutes and 24 hours after ischemia in the IC (r = 0.917, $p < 0.01$), PIR (r = 0.786, $p < 0.01$), and AHR (r = 0.499, $p < 0.01$). ADC, apparent diffusion coefficient; AHR, anterior choroidal and hypothalamic region; IC, ischemic core; PIR, peri-infarct region; rADC, relative ADC. (Color version of figure is available online.)

brief, whole brain tissues were harvested at 24 hours after I/R modeling and immediately fixed with 4% paraformaldehyde. The fixed brain tissues were then sectioned coronally (thickness, 3 μm) and mounted on prechilled glass slides coated with poly-L-Iysine. Immunofluorescence staining was performed using paraffin-embedded block sections to quantify the number of NeuN-positive cells (16). In brief, the slides were subjected to deparaffinization, hydration, blocking of the nonspecific binding immunoglobulin, NeuN primary antibody staining (1:1000; Millipore, Billerica, MA), secondary antibody staining (FITC for avidin D detection; DAPI for counterstaining), dehydration, and mounting.

The NeuN-stained sections were examined using the Perkin Elmer Vectra 3.0 Automated Quantitative Pathology Imaging System (magnification 200 ×; Perkin-Elmer, Waltham, MA) and photographed. The number of NeuN-positive cells was counted with ImageJ (v1.46r.) in three randomly chosen fields of the IC, PIR, and AHR.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using SPSS version 13.0 software (SPSS, Chicago, IL). Correlation analysis was performed to calculate the Pearson correlation coefficients between (1) rADC values at 30 minutes and 24 hours after ischemia, (2) rADC values at 30 minutes and the number of NeuN-positive cells at 24 hours after ischemia, and (3) the sum of rADC values of three regions at 30 minutes and the infarct volume at 24 hours after ischemia. Differences with a p value of less than 0.05 were considered statistically significant.

RESULTS

tMCAO Modeling

The mortality rate in tMCAO modeling was 18.4% (7 out of 38 rats undergoing surgery to induce ischemia). The seven rats were excluded from the final analysis because of intraoperative death ($n = 2$) and postoperative death ($n = 5$). Among the 31 surviving rats, five patterns of ADC changes were observed at 30 minutes after ischemia. First, ADC values in the IC, PIR, and AHR showed little difference between the ipsilateral and contralateral regions ($n = 5$, rADC values of the IC, PIR, and AHR >0.9) (Fig 2a). Second, ADC reduction mainly occurred in the ipsilateral AHR ($n = 5$, rADC values of the AHR <0.9) (Fig 2a). Third, ADC reduction was primarily observed in the ipsilateral IC and PIR ($n = 1$, rADC values of the IC and PIR <0.9) (Fig 2a). Fourth, ADC reduction was mainly observed in the ipsilateral IC and AHR ($n = 7$, rADC values of the IC and AHR <0.9) (Fig 2a). Lastly, ADC reduction was observed in all three ipsilateral regions ($n = 13$, rADC values of the IC, PIR, and AHR <0.9) (Fig 2a). The first and second patterns indicated model failure because there were no ADC changes in the IC and PIR. However, all rats were analyzed to examine the correlation between acute ADC changes and cerebral I/R damage.

Correlation Between Acute ADC Changes and Cerebral I/R Injury

Figure 2b shows the ADC maps at 24 hours after ischemia compared to the ADC maps at 30 minutes after ischemia. Figure 2c shows the correlation between rADC values at 30 minutes and 24 hours after ischemia in the IC, PIR, and AHR. A strong positive correlation between rADC values at 30 minutes and 24 hours after ischemia was observed in the IC ($r = 0.917$, $p < 0.01$) and PIR ($r = 0.786$, $p < 0.01$); however, the correlation between these values was moderate in the AHR ($r = 0.499$, $p < 0.01$).

MRI results were validated by histologic analysis; the correlation between rADC values at 30 minutes after ischemia and the number of NeuN-positive viable cells at 24 hours after ischemia was strong in the IC ($r = 0.825$, $p < 0.01$) and PIR ($r = 0.733$, $p < 0.01$) but moderate in the AHR ($r = 0.546$, $p < 0.01$) (Fig 3). NeuN-positive cells were clearly observed at 200 × magnification in each area. The blue dye indicates the nuclei, and the yellow dye indicates NeuN-positive cells.

The T2-WIs in Figure 4a show five representative infarct region change patterns at 24 hours after ischemia compared with the ADC maps at 30 minutes after ischemia in Figure 2a. Figure 4b shows the correlation between the sum of rADC values at 30 minutes after ischemia and the infarct volume at 24 hours after ischemia. There was a strong negative correlation between the two values ($r = -0.794$, $p < 0.01$). The infarct region on T2-WIs at 24 hours after ischemia and the low ADC region at 30 minutes after ischemia were almost identical.

DISCUSSION

In this study, we found that ADC measurement in the acute phase can predict permanent cerebral I/R injury in rats. Although the correlation between rADC values at 30 minutes and 24 hours after ischemia was moderate in the AHR, there was a strong positive correlation between these values in the IC and PIR, which was validated by histologic analysis. In addition, the sum of rADC values measured in the IC, PIR, and AHR at 30 minutes after ischemia showed a strong negative correlation with the infarct volume at 24 hours after ischemia. The findings may provide a method for predicting permanent brain damage after 60 minutes of ischemia followed by reperfusion and would contribute to stroke-related studies such as stroke pathology studies and new therapeutic assessments.

A comparison of ADC values at 30 minutes and 24 hours after ischemia showed a strong correlation in the following order: IC, PIR, and AHR, and histologic analysis showed similar results. The results indicated that ADC changes between 30 minutes and 24 hours after ischemia due to reperfusion were large in the following order: AHR, PIR, and IC. Reperfusion for the treatment of acute stroke can reduce brain injury by salvaging the reversibly damaged penumbra tissue; however, it can also exacerbate brain damage in some patients (17–20). In this study, at 30 minutes after ischemia, the reversible and irreversible regions

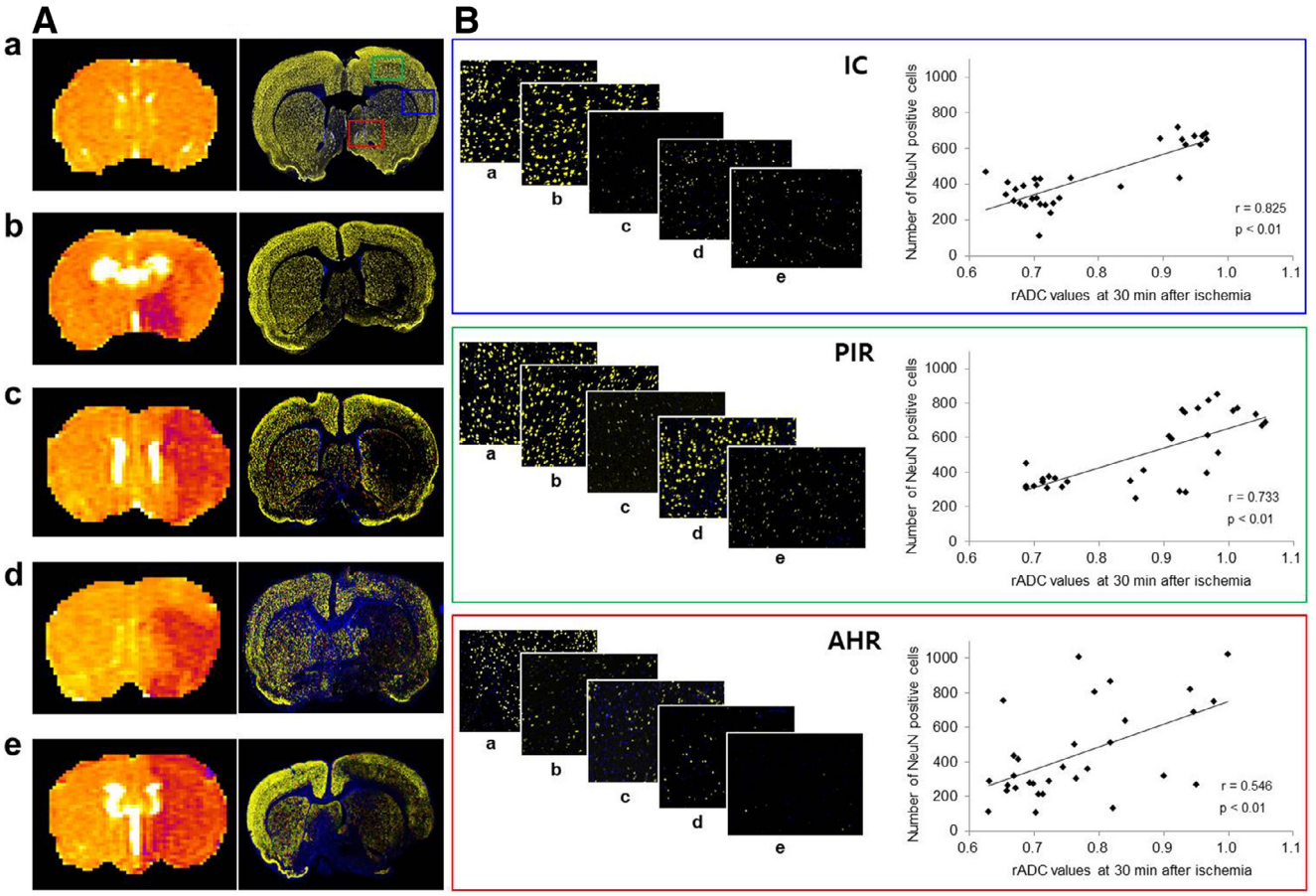

Figure 3. Correlation between rADC values at 30 minutes after ischemia and the number of NeuN-positive cells at 24 hours after ischemia. (a) ADC maps at 30 minutes after ischemia and NeuN-stained images at 24 hours after ischemia. (b) Representative photomicrographs of NeuN-stained cells in the ipsilateral IC (*blue square*), PIR (*green square*), and AHR (*red square*) (magnification 200 ×) and the correlation between rADC values at 30 minutes and 24 hours after ischemia in the IC ($r = 0.825$, $p < 0.01$), PIR ($r = 0.733$, $p < 0.01$), and AHR ($r = 0.546$, $p < 0.01$). (Color version of figure is available online.)

were not distinguishable on the ADC map; however, ADC changes due to reperfusion were different in the three regions at 24 hours after ischemia. This may affect cerebral I/R injury prediction based on ADC values at the initial stage after ischemia. Nevertheless, there was a strong correlation between the sum of rADC values at 30 minutes after ischemia and the infarct volume at 24 hours, suggesting that the initial ADC value after ischemia in the three regions could be used as a predictor of I/R injury.

In addition, five patterns of ADC changes were observed at 30 minutes after ischemia, and these patterns were mostly maintained on ADC maps and T2-WIs at 24 hours. First, ADC changes were not observed on ADC maps. Second, ADC reduction mainly occurred in the AHR. These two patterns indicated the failure of MCAO modeling because of inaccurate filament insertion due to anatomical deformation of the MCA or incorrect filament position (8–10). Third, ADC reduction was primarily observed in the IC and PIR. Fourth, ADC reduction was mainly observed in the IC and AHR. Lastly, ADC reduction was observed in all three regions. These three patterns indicated that ADC values may vary in the PIR and AHR based on the reduction of the ADC value in the IC when establishing a transient MCAO model using the suture method.

Recently, a study has reported a technique in which only the MCA is occluded without the occlusion of the anterior choroidal and hypothalamic arteries by adjusting the suture coating length (21). Although this method can induce ADC changes only in the MCA region early after ischemia, whether this change also occurs in the PIR and IC should be determined. In this study, we observed that the initial ADC changes in the IC and PIR were strongly correlated with ADC changes and neuronal survival at 24 hours after ischemia. Therefore, monitoring the changes in the initial ADC after ischemia and selecting models in which ADC changes are constant in the IC and PIR will reduce the uncertainty in stroke-related research.

There are two well-known methods for confirming MCAO in an experimental stroke study. First, the laser Doppler flowmetry method can be used to confirm whether regional cerebral blood flow (rCBF) is reduced by 20%~30% compared to the baseline after ischemia (22,23). Second, MCAO can be identified by assessing brain function through behavioral tests such as the rotarod, adhesive removal, and mNSS tests. Changes in the rCBF and behavioral tests after ischemia have been reported to be associated with the final infarct regions (24–26); however, there are also contrasting results. Taninishi et al. recently reported that a

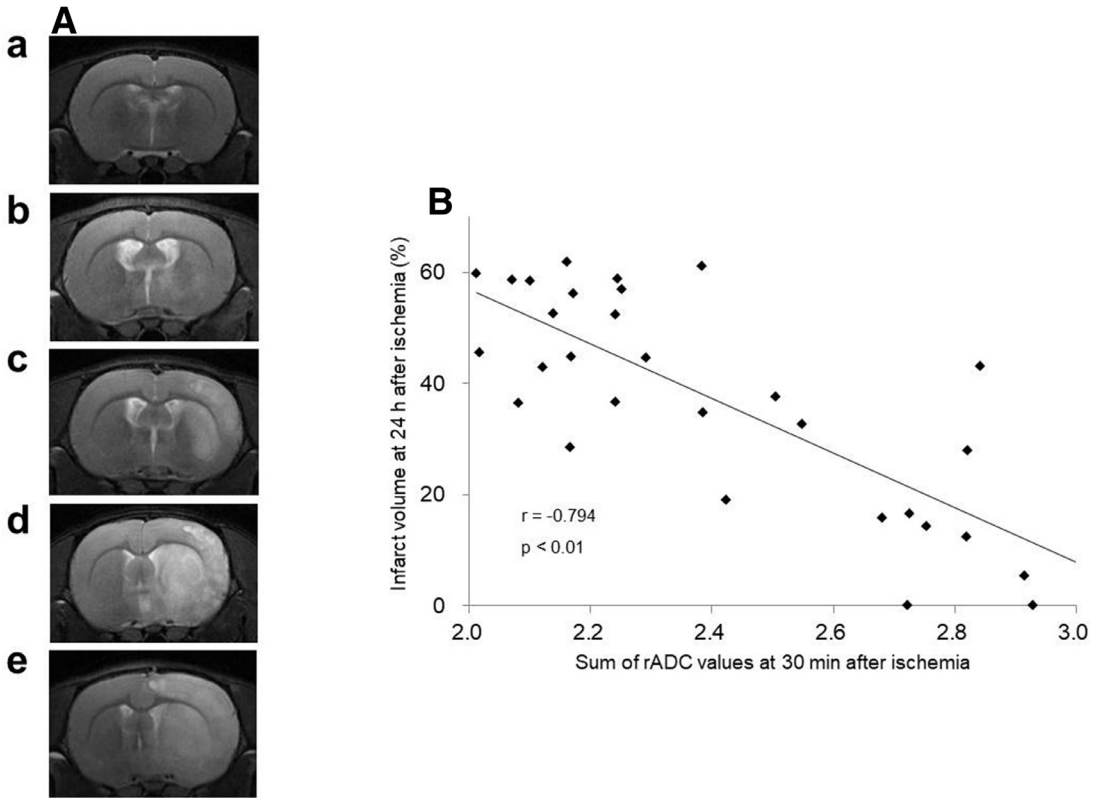

Figure 4. Correlation between the sum of rADC values of the IC, PIR, and AHR at 30 minutes after ischemia and the infarct volume at 24 hours after ischemia. (a) T2-WIs of the five representative infarct region change patterns at 24 hours after ischemia. (b) Correlation between the sum of rADC values of three regions at 30 minutes after ischemia and the infarct volume at 24 hours after ischemia ($r = -0.794$, $p < 0.01$).

decline in the rCBF is not identical to histologic infarction (27). Furthermore, several studies have revealed a low correlation between the infarct volume and poststroke behavioral tests (28,29). These findings demonstrated the potential for uncertainty in experimental stroke studies. In the present study, acute ADC changes were correlated with permanent I/R injury. Therefore, it would be helpful to reduce the uncertainty in the study.

There are some limitations in the translation of our results to human stroke patients. We set 30 minutes after ischemia as the time for early ADC changes and 60 minutes as the reperfusion time. However, time intervals can be completely different in human stroke patients. In a previous study, acute stroke patients had various reperfusion times with IV tissue plasminogen activator or IA endovascular treatment even though the window time for each treatment has been well established (IV Tissue plasminogen activator within 4.5 hours and IA thrombectomy within 6 hours) (30). Furthermore, acute stroke patients are admitted to hospitals at various times after symptom onset. Some patients may have an unclear onset time, including those with wake-up stroke. ADC values can change over time with the evolution of acute ischemic stroke lesions. The severity of ischemic stroke including the infarct core volume before revascularization may affect reperfusion injury. Therefore, the severity of ischemic stroke, which represents the initial infarct core volume and collateral flow, could be an important confounding factor in the translation of our results to human stroke. In human stroke, the stroke mechanism varies based on the TOAST classification (31). This is one of the barriers in the translation of the results from the MCAO model established using the intraluminal suture method.

Although acute ADC values after ischemia were effective in predicting permanent brain injury after 60 minutes of ischemia and reperfusion, we could not determine whether these predictions were effective at other ischemic times. Therefore, additional studies are needed to determine if these predictions are valid for different ischemic times. In addition, MRI devices for small animals are not universally available. Nevertheless, MRI provides valuable data for experimental stroke research.

CONCLUSION

ADC measurement in the acute phase of ischemic stroke could be an important predictor of permanent brain damage caused by focal cerebral I/R injury in rats. Furthermore, permanent brain injury prediction based on early ADC changes after ischemia may help reduce the uncertainty in stroke pathology studies and new therapeutic assessments.

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