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ORIGINAL ARTICLE

Reperfusion plus Selective Intra-arterial Cooling (SI-AC) Improve Recovery in a Nonhuman Primate Model of Stroke

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Abstract

Early reperfusion is increasingly prioritized in ischemic stroke care, but outcomes remain suboptimal. Therefore, there is an urgent need to find neuroprotective approaches that can be combined with reperfusion to maximize efficacy. Here, the neuroprotective mechanisms behind therapeutic hypothermia were evaluated in a monkey model of ischemic stroke. Focal ischemia was induced in adult rhesus monkeys by placing autologous clots in the middle cerebral artery. Monkeys were treated with tissue plasminogen activator (t-PA) alone or t-PA plus selective intra-arterial cooling (SI-AC). Serial MRI scans and functional deficit were evaluated after ischemia. Histopathology and immunohistochemistry analysis were performed after the final MRI scan. t-PA plus SI-AC treatment led to a higher rate of MRI tissue rescue, and significantly improved neurologic deficits and daily activity scores compared with t-PA alone. In peri-infarct areas, higher fractional anisotropy values and greater fiber numbers were observed in models receiving t-PA plus SI-AC. Histological findings indicated that myelin damage, spheroids, and spongiosis were significantly ameliorated in models receiving SI-AC treatment. White matter integrity was also improved by SI-AC based on immunochemical staining. Our study demonstrates that SI-AC can be effectively combined with t-PA to improve both structural and functional recovery in a monkey model of focal ischemia. These findings provide proof-of-concept that it may be feasible to add neuroprotective agents as adjunctive treatments to reperfusion therapy for stroke.

Key Words Stroke · recovery · hypothermia · white matter · diffusion tensor imaging · rhesus monkey

Di Wu, Yongjuan Fu and Longfei Wu contributed equally to this work.

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Introduction

Stroke remains the leading cause of long-term disabilities worldwide. Motor disabilities are usually the most debilitating for patients [1, 2]. Although great benefits have been found in patients receiving clot-retrieval treatments, only half of them achieve a good clinical outcome [3]. Recently, a novel strategy of combing reperfusion and neuroprotection has been advocated to improve outcomes [4, 5]. Both preclinical and clinical data have proven the feasibility of this novel combination therapy, but its efficacy is still under debate in the clinic [5, 6]. A preclinical study has proposed that a time window of neuroplasticity opens following a stroke, during which the greatest gains in recovery occur [7]. However, we do not know if a combination strategy used in the acute stage of stroke will lead to long-term clinical benefit in a higher order brain, or what the mechanism behind this efficacy may be, especially in nonhuman primates (NHPs).

Hypothermia protects against trauma or stroke-induced cell death in oligodendrocytes, loss of myelin, and functional impairments in neuronal circuitry in rodent models [8, 9]. Mild hypothermia has also been found to reduce white matter injury and improve neurobehavioral functions in rodent models of middle cerebral artery occlusion (MCAO) [10]. Retained white matter tracts as shown on non-invasive imaging have been associated with improved motor outcomes in patient studies [11]. DTI analysis allows for evaluation of the underlying structural integrity of cerebral white matter tracts and serves as a potential biomarker for tracking and predicting motor recovery. However, limited data regarding the molecular and histopathological changes after ischemic stroke in patients makes it difficult to attain a mechanistic understanding of stroke recovery. In a previous translational study, we discovered that selective intra-arterial cooling (SI-AC) treatment reduced infarct volumes in the acute stage and improved neurologic deficits in the chronic stage in standardized NHP models with complete or partial reperfusion [5]. As a model that closely simulates human physiology, NHP study will provide better insight into cellular sequential steps and potential mechanisms based on imaging and histological changes in cerebral parenchyma after focal ischemia.

The Stroke Treatment Academic Industry Roundtable X meeting has proposed to restudy and repurpose previous neuroprotective agents as well as study and develop new neuroprotective agents as adjunctive treatments to reperfusion therapy [12]. Therefore, this study was designed to explore the long-term benefits of the novel combination therapy on stroke recovery and potential mechanisms based on MRI imaging and image-directed biopsy in a rhesus monkey model of ischemic stroke.

Materials and Methods

Animals A total of 14 adult male rhesus monkeys (*Macaca mulatta*), aged 7 to 11 years old and weighing 7.2 to 10.6 kg, were used in this study. All animals were screened and were free of Tuberculosis, Shigella, Salmonella, Helminths, Ectoparasites, Entamaebahistolytica, and B virus. Monkeys were caged individually in stainless steel cages in the same room. They were fed with commercially prepared monkey food twice daily with fruits and unrestricted water supply. This study was approved by the Animal Use and Care Board of the Institute of Laboratory Animal Sciences, Capital Medical University. All experiments were also in compliance with national guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals [13].

Anesthesia, Endovascular Operations, and Postsurgical Management Full descriptions of experimental procedures were described in our previous reports [5, 14, 15]. In brief, animals were fasted for 12 h prior to the induction of anesthesia and maintained intravenously with propofol. A Prowler-10 micro-catheter (Codman) with a SilverSpeedTM −10 Hydrophilic micro-wire was introduced into the guiding catheter and navigated to the distal end of M1 segment of the right MCA. Next, a 10-cm-long autologous clot was transferred into micro-catheter and flushed into the end of M1 segment with 2 mL saline, marking the initiation of ischemia. Postsurgical management was described previously to minimize pains and sufferings of models.

IA Thrombolysis and SI-AC Alteplase (Boehringer Ingelheim Limited, 1.1 mg/kg) for IA thrombolysis was based on established protocols, in which the full t-PA dose was infused to the middle cerebral artery through the micro-catheter at 2.5 h after clot placement [5]. Based on reperfusion states, SI-AC was randomly assigned to models in each reperfusion state, including complete, partial, and no reperfusion. All 14 models with partial reperfusion, as defined by complete recanalization at the main trunk of M1-MCA without recanalization at one M2-MCA branch, were included in this study. So, animals with partial reperfusion were assigned to receive SI-AC or no SI-AC in a random way. Administration of SI-AC was described in our previous study. In brief, a total of 100 mL of iced lactated Ringer's solution (0–4 °C) was infused into the MCA vessel vascular distribution over 20 min (5 mL/min) via the micro-catheter [5, 14]. The reperfusion states were the same as those after t-PA thrombolysis based on DSA images (Supplement Fig I). At 24 h after ischemia, MRA images also showed impaired perfusion at the branches of the middle cerebral artery (Supplement Fig II).

MRI Scanning MRI scanning was performed on a Magnetom Trio MRI Scanner (3.0 T; Siemens AG, Siemens Medical

Solutions, Erlangen, Germany). MRI sequences and parameters were reported in our previous study [5]. MRI images at baseline, then at 7 days and 30 days after ischemia onset. Based on recent reports, DWI images obtained 1 day after ischemia were defined as "originally abnormal region", and T2-Flair images at 30 days were defined as "final infarct size" [16–18]. Tissue rescue was operationally defined as (Volume $_{DWI}$ − Volume $_{T2}$)/Volume $_{DWI}$. We also analyzed the color-coded fractional anisotropy (FA) eigenvectors after Diffusion tensor tractography (DTI) to identify pathophysiological changes in the affected white matter [11, 19, 20]. Baseline DTI data could not be obtained for 4 animals, so a total of 10 monkeys were included in the functional imaging analysis. In brief, the DiffusionKit package (downloaded from http://diffusion.brainnetome.org), a light, one-stop, cross-platform solution for dMRI data analysis, was used to perform data processing, modeling, and visualization [11].

We selected a symmetric region of interest at internal capsule levels from both sides, which generally matched no-tissue rescue areas in this study. We then calculated the number and volume of fibers through ROIs. We also calculated and created FA eigenvector based color maps. FA values were calculated at the dorsal region of the internal capsule in both the ipsilateral (affected) and contralateral WM.

Neurological Assessments Neurologic deficit was assessed by a standardized score as previously reported at 1, 7 and 30 days after ischemia [21]. Of the 100 points possible, 28 are assigned to consciousness, 22 to the sensory system, 32 to the motor system, and 18 to skeletal muscle coordination. From a total of 100 points, 0 corresponds to normal behavior and 100 to severe neurological impairment. We also used a primate version of Rankin Scale (pRS) for ranking neurological dysfunction in monkeys following a stroke, which was similar to a modified Rankin Scale (mRS) for stroke patients [22]. In brief, pRS is a simple scale with only 6 levels, similar to the widely used modified Rankin Scale. It is designed to assess impairments in activities of daily living in monkey models. Functional deficit can be easily classified into none (category 0), slight (categories 1–2), moderate (category 3–4), and severe disabilities (category 5) based on pRS. pRS score was evaluated based on behavior video at 30 days after ischemia and the percentage in each score was calculated. Assessment was performed by two experienced observers.

Histological Analysis At the study end point (30 days after ischemia), animals were euthanized (overdose of sodium pentobarbital) and their brains removed and immediately fixed in 10% formalin. A custom-designed matrix was used to correctly orient the brain, to allow cutting of 2.5-mm coronal blocks. Sections were evaluated blindly for evidence of neuropathology using light microscopy under the most significant visual field and scored on a scale of 0 (none), 1 (mild, pathological features accounting for less than 25% of the section), 2 (moderate, pathological changes accounting for 25 to 50% of the section), 3 (severe, pathological changes accounting for 50 to 75% of the section), or 4 (most severe, pathological features accounting for more than 75% of the section) according to two previous papers [23, 24]. These tissues were processed with embedding paraffin wax. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E). Eight-micrometer-thick sections were cut and stained with Luxol fast blue (LFB). Selected sections were also immunohistostained using mouse monoclonal antibodies against neurofilament protein (NF 2F11; Zymed, San Francisco, CA, USA; dilution 1:100). A double-labeling immunofluorescence study was also performed with rabbit polyclonal antibodies against myelin basic protein (MBP, 1:100) and mouse monoclonal antibody antineurofilament (NF 200 kDa, clone RT97; Millopore, USA; dilution 1:400).

Statistical Analysis Statistical analysis was performed with SPSS for Windows, version 21.0 (SPSS, Inc.). Repeated-measures ANOVA was used to measure the effects of SI-AC on repeated variables. Independent sample *t* test was adopted to make a comparison between two groups. $P < 0.05$ was defined as statistically different.

Results

SI-AC Treatment Led to Remarkable Tissue Rescue and Mitigated Neurologic Deficit

As described in our previous report, we developed a novel thrombus-thrombolysis model in rhesus monkeys, which revealed improved outcomes when chemical thrombolysis was performed in combination with SI-AC [5]. Major physiologic parameters, including blood pressure, heart rate, respiration rate, and pulse oximetry during experiments, were stable during the ischemia and reperfusion treatment in both groups. In further analysis, we found notable infarct sizes in the middle cerebral artery-supplied regions based on DWI images at 1 day after ischemia in all models (Fig. 1A). Interestingly, two distinct patterns were appreciated when compared to MRI images at 30 days; areas with tissue rescue, with an abnormally high signal at day 1 but normal at day 30, and areas without tissue rescue, which displayed abnormally high signal at both 1 and 30 days. Tissue rescue areas were primarily noted in the cortex and no-rescue areas were primarily in the internal capsule. Importantly, a significantly higher rate of tissue rescue was appreciated in the t-PA plus SI-AC group than when t-PA was given alone (Fig. 1A).

Next, we resorted to two kinds of neurological scores to evaluate functional deficit during a 30-day observation period [21, 22]. Those models treated with SI-AC plus t-PA

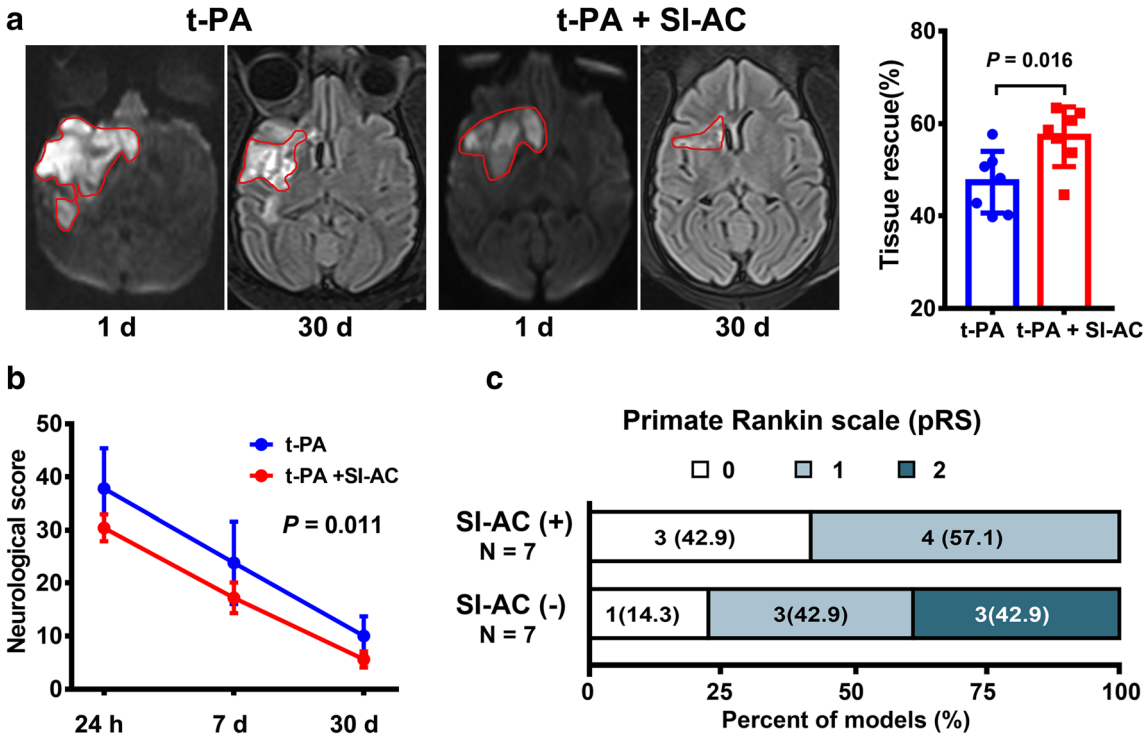

Fig. 1 Analysis of infarct evolution and dysfunctions in models. (**A**) DWI (1 day) and T2-Flair images (30 days) identified infarct site in models with only t-PA and those with t-PA plus SI-AC treatment. A higher tissue rescue rate was observed in models with t-PA plus SI-AC treatment. (**B**) Neurologic function was significantly improved in models receiving both t-PA and SI-AC treatment during a 30-day observation period. (**C**) Models receiving t-PA plus SI-AC treatment achieved a higher percentage of primate Modified Rankin (0–1) than those with t-PA alone at 30 days after ischemia. Data are mean ± SD, $N = 7$ per group, independent t test or two-way repeated-measures analysis of variance

exhibited a better neurologic score ($P = 0.011$, two-way repeated-measures analysis of variance) throughout the observation period (Fig. 1B). We also resorted to a novel pRS for monkey models, similar to the widely used mRS in patients, to evaluate impairments in activities of daily living. All models receiving t-PA plus SI-AC treatments achieved a better functional outcome of pRS score 0 to 1 at 30 days after the onset of ischemia, while only 4 animals (57.1%) receiving t-PA alone achieved pRS score 0 to 1 (Fig. 1C), suggesting a better preservation of daily function.

SI-AC Treatment Mitigated Pathological Changes

To evaluate the histopathologic changes present in regions evaluated by MRI, hematoxylin and eosin (H&E) staining was performed in both tissue rescue and no-rescue areas. In no-rescue areas, a general hypocellularity was appreciated, which was consistent with MRI findings. Rescue areas, in contrast, displayed preservation of cellularity and organization of neuron, fibers, and glia (Fig. 2A).

Pathologic features of no-rescue areas were then more closely evaluated [23, 24]. In both groups, numerous pathologic processes were identified, including tissue rarefaction, myelin loss, vascular hyperplasia, spongiosis, spheroids, macrophage infiltration, and astrocytosis. However, myelin degeneration, axonal swelling (spheroids), spongiosis, and macrophage infiltration (gitter cells) were more pronounced in models treated with only t-PA thrombolysis than those receiving the combination therapy (Fig. 4 B and C). No significant differences in tissue rarefaction, vascular hyperplasia, or astrocytosis were appreciated (Supplement Fig III).

Anisotropy Analysis of Infarct and Peri-infarct Areas

DTI analysis allows for evaluation of the underlying structural integrity of brain white matter tracts and serves as a potential biomarker for tracking and predicting motor recovery. DTI analysis was utilized to evaluate cerebral diffusivity in infarcted regions. FA values for the ipsilateral (affected) and contralateral white matter were calculated for both groups (Fig. 3A). In both groups, FA values dropped at 7 days after a stroke, but recovered toward baseline (before ischemia) values at 30 days after surgery. At all time points evaluated, FA values for the ipsilateral hemisphere were increased in the SI-AC arm when compared with the t-PA-only group ($P = 0.031$, Fig. 3B). No significant difference in FA values was observed in the contralateral hemisphere between the two groups (Fig. 3B).

Peri-infarct areas represent foci of functional recovery in rat stroke models. In this study, peri-infarct areas were primarily

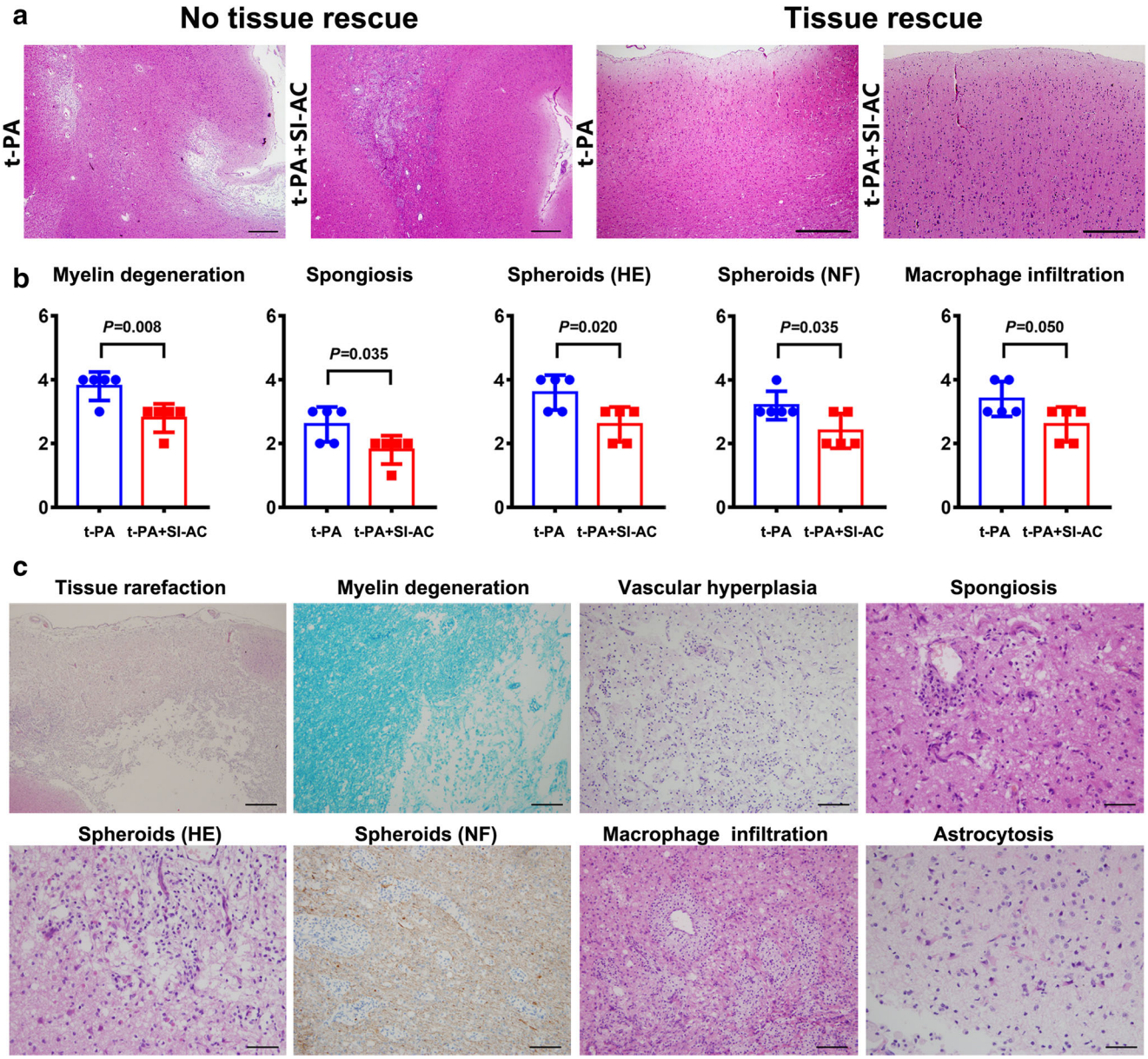

Fig. 2 Histological features and evaluation. (**A**) HE staining of the specimens in both rescue and no-rescue areas. Bar = 500 μm. (**B**) Pathological features were compared between two groups. (**C**) Necrosis and cavitation (tissue rarefaction) was observed on H&E staining (Score 4, bar = 500 μm), myelin degeneration on luxol fast blue staining (Score 4, bar = 100 μm), vascular hyperplasia on H&E (Score 4, bar = 100 μm), spongiosis on H&E (Score 3, bar = 50 μm), spheroids on H&E (Score 3, bar = 50 μm) and on neurofilament (NF 2F11) staining (Score 3, bar = 50 μm), macrophage infiltration on H&E (score 4, bar = 100 μm), and astrocytosis on H&E (score 2, bar = 50 μm). Data are Mean + SD, $N = 5$ per group

at the level of the internal capsule. So, anterior commissure (AC) was selected as a guide and the dorsal region of the internal capsule as the target region in both ipsilateral and contralateral sides (spherical radius 4.0 mm) to measure fiber number and length. We resorted to DiffusionKit for fiber tracking and statistical analyses in both ipsilateral and contralateral hemispheres. Focal ischemia/reperfusion led to a reduced fiber number and length at 7 days after ischemia in both groups, and a partial recovery of fiber number and length at 30 days after ischemic stroke (Fig. 3C). Two-way repeated-measures ANOVA indicated that SI-AC treatment only improved the decrease of fiber number in ipsilateral hemisphere when compared with the t-PA group throughout the 30-day observation period ($P = 0.032$); however, SI-AC treatment did not affect fiber length in the ipsilateral hemisphere between the two groups ($P = 0.426$, Fig. 3D). Fiber number and length were slightly increased at 7 days after ischemia in the contralateral hemisphere in both t-PA and t-PA plus SI-AC groups, but SI-AC treatment did not provide further improvement (Supplement Fig IV and V).

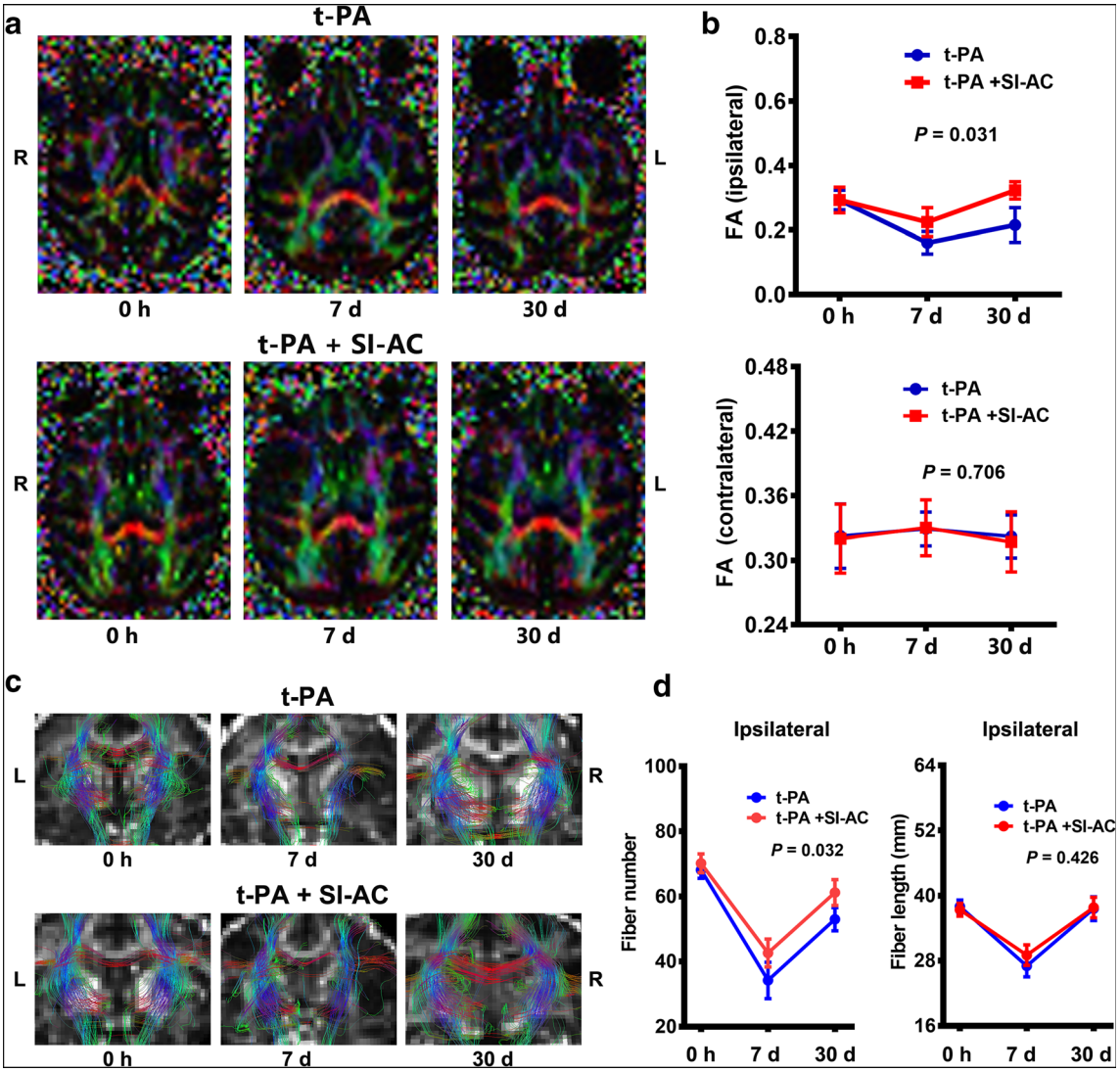

Fig. 3 DTI analysis of anisotropy. (**A**) In both groups, color-coded fractional anisotropy (FA) maps depicted territorial changes in the infarct areas after ischemia. (**B**) SI-AC treated models exhibited a significantly ($P = 0.031$) higher FA values compared to t-PA-only models on ipsilateral side. (**C**) The analysis of fiber number and length was completed with DiffusionKit. (**D**) SI-AC treatment mitigated fiber loss on the ipsilateral side, but SI-AC treatment did not affect fiber length in the ipsilateral side. Data are mean ± SD, $N = 5$ per group, two-way repeated-measures analysis of variance

SI-AC Treatment Promoting White Matter Integrity

The effect of SI-AC on white matter integrity was assessed by immunostaining with two white matter markers, NF200 (a marker of axons) and MBP (a marker of myelin sheaths) [8, 10]. As shown in Fig. 4A, both tissue rescue areas and no-rescue areas were selected for staining. SI-AC treatment significantly increased MBP/NF200 rate when compared with those with t-PA alone in the no-rescue area (Fig. 4B and C). But, the MBP/NF200 rate was not significantly different between the two groups in the rescue area (Fig. 4D). These results indicated that SI-AC reduced the serious myelin damage due to focal cerebral ischemia in the white matter area.

Discussion

This translational study indicated that the combination of SI-AC with chemical thrombolysis promoted structural and functional recovery in a NHP stroke model. The combination therapy led to remarkable tissue rescue and improved neurological function. In parallel, we found neuronal network improvements and differentially expressed molecular factors in white matter, which were also proven in pathological and immunochemical results. A new strategy of neuroprotectants in conjunction with reperfusion therapy may help to promote stroke recovery.

The present findings are significant for several reasons. First, they provided evidence to a new strategy of combining neuroprotectant with reperfusion therapy on promoting stoke

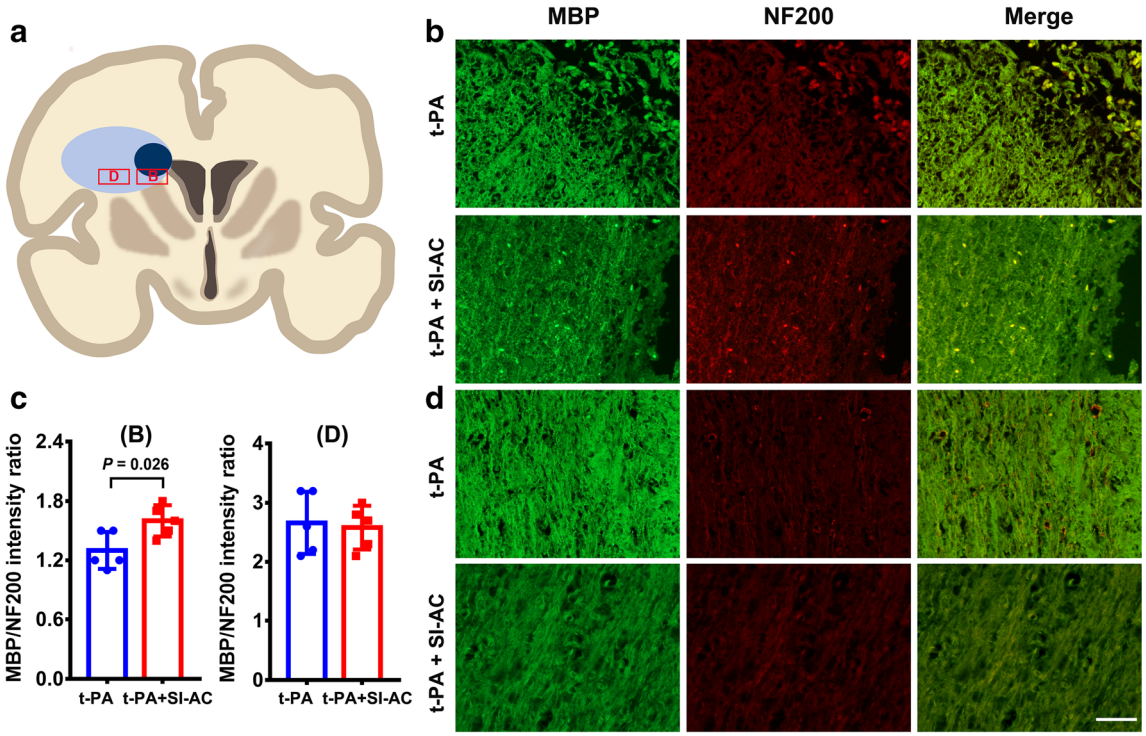

Fig. 4 SI-AC treatment attenuated demyelination in the no-tissue rescue area. (**A**) Schematic of no-rescue (**B**) and tissue rescue (**D**) areas. B. Immunostaining of MBP and NF-200 in the no-rescue area of the ipsilateral hemisphere 30 days after cerebral ischemia injury. (**C**) Quantification of the relative ratio of MBP to NF-200 immunostaining intensity in no-rescue and rescue areas. D. Immunostaining picture in the tissue rescue area. Bar = 50 μm in both (**B**) and (**D**) pictures. Data are mean ± SD, $N = 5$ per group

recovery in a translational NHP model. Experimental data in animal models suggested that numerous neuroprotectants reduced the brain's vulnerability to ischemia, while none had been successfully translated to phase III trials [25, 26]. These failures have been attributed to preclinical models of transient ischemia but inadequate reperfusion in previous clinical trials, lacking long-term functional evaluations [27]. So, it is advocated in the era of reperfusion that neuroprotectant plus reperfusion therapy may enhance neuroprotective benefits when reperfusion is achieved in patients receiving endovascular thrombectomy [28]. For example, recently published ESCAPE-NA1 trial hinted that an improved functional independence was achieved in patients receiving nerinetide plus endovascular thrombectomy despite a failure of primary endpoint [6]. In this regard, we observed that this new strategy was effective in enhancing stroke recovery in NHP models based on MRI images and functional scores. In addition, a better reperfusion might account for an important mechanism of neurological benefits associated with SI-AC treatment. In a previous study, a significantly reduced infarct volume was found in ischemic rats with a local saline infusion at body (37 °C) temperature compared with that in rats without the infusion [29].

Two kinds of evidence, including imaging analysis and pathological features, further indicated functional recovery in models receiving SI-AC treatment. Previous clinical studies found that MRI reversal in the acute stage was associated with neurological improvement and favorable clinical outcomes in patients receiving intravenous thrombolysis and endovascular treatment [18, 30]. Additionally, FA values of DTI analysis provided independent prediction of motor outcome for stroke patients [31]. The dynamic changes that FA values decreased at the acute stage of stroke and increased at the chronic state were observed in the affected white matter in NHP models of focal ischemia [32]. However, histological findings and cellular and molecular processes, responsible for plastic remodeling after stroke, were only explored in experimental model studies, such as crucial mediators for angiogenesis (VEGF, HIF, and EPO), neurogenesis and gliogenesis (VEGF and BDNF), axonal regrowth (CK2, GAP-43), myelination (Netrin-1, SHH, Sox17), and synaptogenesis (IGF-1, CXCL12, and CCL2) [33]. But, it was difficult to make a biopsy to explore mechanisms in patients. NHP models have great advantages in translational studies, such as a bigger brain volume and higher percentage of WM, and possible pathological analysis [34]. In this study, we observed a tissue rescue from day 1 to day 30 based on MRI images. We also found that a further improved FA values and pathological results during the recovery stage in this study.

Functionally, white matter plays an important role in neural signal transmission and communication within the brain.

White matter injury is an inevitable complication of ischemic stroke and an important factor of sensorimotor and cognitive impairment [35, 36]. Our previous studies found that mild focal hypothermia treatment in the acute stage significantly promoted white matter integrity 28 days and improved neurobehavioral function in both mouse and rat [10, 37]. We also found that focal hypothermia mitigated functional impairments in white matter based on compound action potentials in mouse [8]. Results in this study further confirmed previous findings in a higher order brain. In mechanism, hypothermia had been shown to promote oligodendrocytes maturation in hypoxic-ischemic neonatal rat brains [38]. It was reported that hypothermia promote the differentiation of induced oligodendrocyte progenitor cells into mature oligodendrocytes in adult mice [10, 39]. In this study, long-term protective effects in a high-order brain may be attributable to preserved integrity of white matter. A decreased inflammatory level, preserved blood brain barrier, improved polarization of microglia and macrophages, and less histopathologic damages at the acute stage may lead to a better preserved white matter, and then finally improved neurologic functions [8, 40, 41].

This study had several limitations. Firstly, we only focused on no-tissue rescue areas to explore molecular and histopathological changes after SI-AC treatment. Going forward, we would benefit from a greater focus on the neuroprotective mechanisms of SI-AC treatment on tissue rescue areas as well. Secondly, the number of experimental animals was small, and only adult male rhesus monkeys are included in this study. Further study should recruit female and aging rhesus monkeys to avoid potential influences of age and gender. Finally, histological analysis was only carried out at 30 days after ischemia. It was impossible to make a direct comparison between MRI and histological findings at 7 days after the onset of ischemia.

In conclusion, our findings suggested that reperfusion combined with SI-AC was associated with better preserved neurologic function, remarkable tissue rescue, better white matter integrity, and mitigated histological damage in a NHP model of stroke. All these evidence indicate that adjunctive neuroprotective therapy (including hypothermia) should be further tested at bedside in the era of reperfusion. Given the similarities between large animal models and the human brain, these results may also provide insights into new strategies for other major brain disorders, such as traumatic brain injury and aneurysms [42–45].

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contributions DW performed experiments and wrote the paper. FY, LW, JC, TY, MZ, CW, MS, XH, SJL, YZ, and SLL performed experiments and analyzed results. MH and YD revised the manuscript. DW, YD, and XJ conceived the research and supervised experiments.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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