REMOTE ISCHEMIC CONDITIONING (PRE, PER, AND POST) AS AN EMERGING STRATEGY OF NEUROPROTECTION IN ISCHEMIC STROKE

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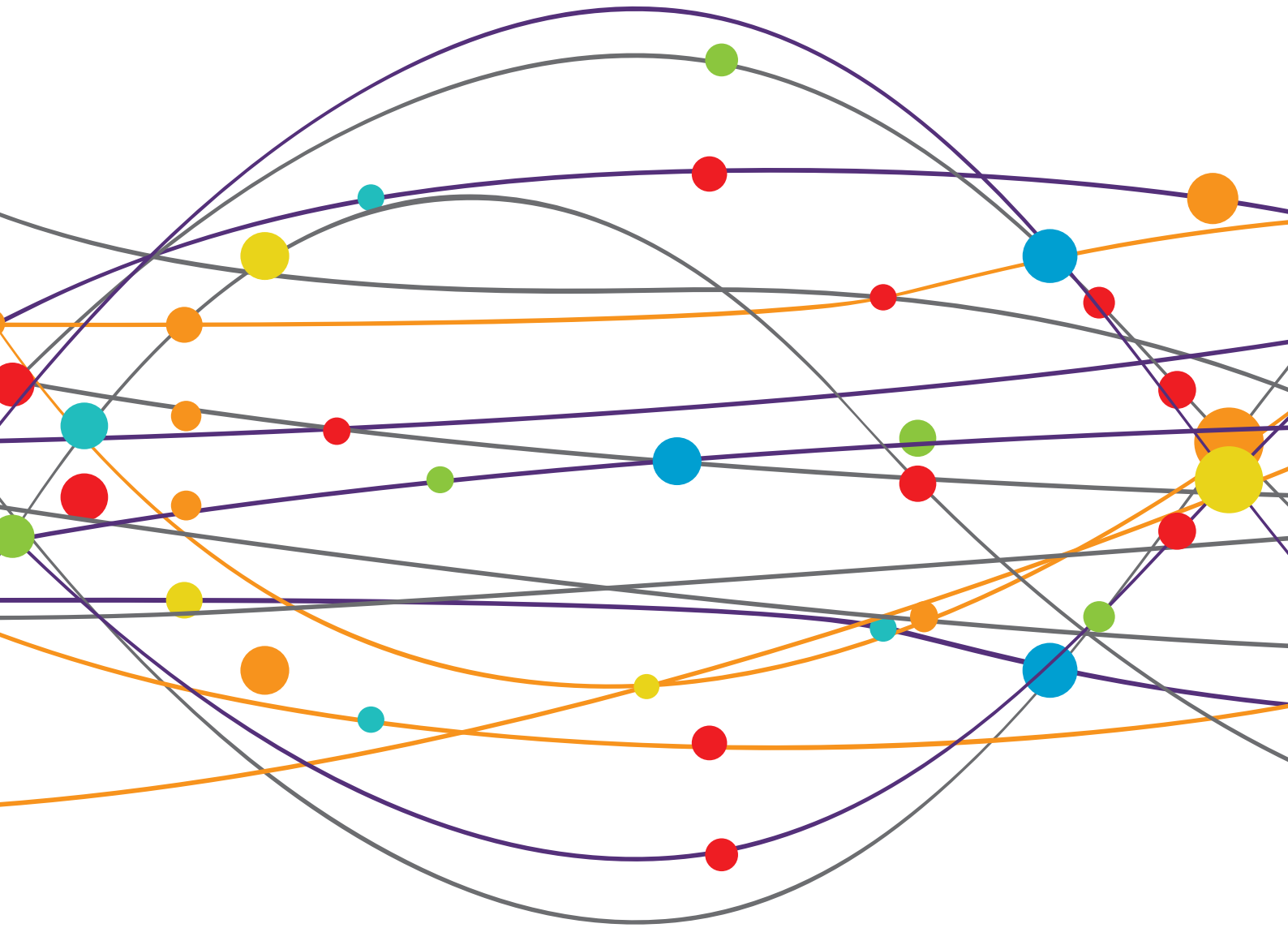

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REMOTE ISCHEMIC CONDITIONING (PRE, PER, AND POST) AS AN EMERGING STRATEGY OF NEUROPROTECTION IN ISCHEMIC STROKE

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EDITORIAL
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Editorial: Remote Ischemic Conditioning (Pre, Per, and Post) as an Emerging Strategy of Neuroprotection in Ischemic Stroke

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Keywords: remote ischemic conditioning, remote ischemia postconditioning, remote ischemia preconditioning, remote ischemia perconditioning, ischemic stroke, neuroprotection

Editorial on the Research Topic

Remote Ischemic Conditioning (Pre, Per, and Post) as an Emerging Strategy of Neuroprotection in Ischemic Stroke

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Stroke is one of the leading causes of death and disability worldwide (1–3). Currently, the only treatments available in the acute phase demonstrating safety and effectiveness are intravenous fibrinolytic treatment and mechanical thrombectomy (4). Unfortunately, many patients cannot benefit from these treatments due to contraindications, time of evolution of the symptoms, or restricted access to mechanical therapies that are currently only offered in specialized centers (5). The efficacy of neuroprotective therapies has great potential, although translation of most neuroprotective trials from the bench to bedside has failed so far (6, 7).

Remote ischemic conditioning (RIC) represents a new paradigm in neuroprotective therapies (8–10), and it has the potential ability to protect the ischemic brain from injury until reperfusion and, later, to protect the brain from reperfusion injury (8, 11). RIC consists of short and controlled cycles of ischemia-reperfusion applied to one limb during the establishment of cerebral ischemia (perconditioning), before (preconditioning), or after (postconditioning) (11). Until now, the underlying mechanisms of RIC are not clear and there are limited data about the clinical translation of RIPerC in ischemic stroke patients (8, 11). Recent trials have only demonstrated the feasibility and safety of this intervention in acute ischemic stroke patients (AIS) (12–16).

In this special issue, we provide new insights into the mechanisms of RIC in ischemic stroke Abbasi-Habashi et al. and Pignataro we compare evidence of the effect of RIC in AIS and myocardial infarction Saccaro et al. Furthermore, we propose new indications or aims for the use of RIC in AIS as preventing further ischemic cerebrovascular events Liu et al. or stroke-associated pneumonia Zhang et al. and improving cognition Poalelungi et al.. Finally, we identify or we propose to identify new subgroups of patients who could benefit from this neuroprotective strategy such as Moyamoya disease Xu et al. and AIS who are not eligible for recanalization therapies Diamanti et al..

Although the exact mechanism by which the protective signal of RIC is transferred from the arms or limbs to the brain remains unclear, preclinical studies suggest that a combination of circulating humoral factors and neuronal signals is involved. RIC could improve the outcomes of AIS treated with reperfusion therapies by reducing reperfusion injury (17). In this issue, Abbasi-Habashi et al. review the putative role of the immune system and circulating mediators of inflammation in these protective processes and the potential role of extracellular vesicles. In this line, Pignataro discusses the role of miRNAs in the activation of endogenous tolerance mechanisms by RIC as transducers of protective messages to the brain and/or as effectors of brain protection.

Myocardial infarction (MI) and AIS have some similarities. Both conditions have an acute onset due to blood vessel occlusion and RIC has been proposed as a strategy to improve reperfusion therapies. Ischemic tolerance and RIC have been first described in MI (11). Clinical trials also started earlier in MI (11) than in AIS patients (9). Saccaro et al. explore similarities and differences of the response of RIC in both conditions. RIC reduces circulating biomarkers of myocardial necrosis, infarct size, and edema although these effects appear to have no effect in the outcomes of MI patients (18). In AIS, RIC is also effective in pre-clinical models (8) but has no significant clinical evidence in the few small studies completed to date (10). The lack of conclusive clinical evidence of RIC efficacy in MI and AIS may be due to heterogenous protocols and different RIC applications. They recommend improving the selection criteria in future RIC clinical trials. Based on pre-clinical studies that demonstrate a greater effect of RIC against reperfusion injury and on its effect in enhancing cerebral collateral circulation, they propose to focus on patients with large vessel occlusion who are candidates for mechanical thrombectomy and can most benefit from the presence of vascular collaterals Saccaro et al.. However, there are still proposals to study the effect of RIC in other subgroups of patients. Diamanti et al. designs the multicenter phase II study TRICS-9 to assess the efficacy of RIC in patients with AIS within 9 h of onset who are not candidates for recanalization therapies.

Repeated RIC post-conditioning (RIPostC) emerges as a promising strategy to improve functional recovery (13). In this special issue, different studies investigate outcomes and indications for RIPostC. Poalelungi et al. in a single center double-blind randomized controlled trial observed that RIPostC during 5 days of hospitalization twice daily might improve disability and cognition at 180 days. Interestingly, Liu et al. in a single-arm open-label phase IIa futility trial (PICNIC-One study) applied RIPostC twice a day for 90 days in 167 acute minor ischemic stroke or moderate-to-high risk transient ischemic attack patients, which seemed to reduce the risk of recurrent stroke. However, only 42% of subjects completed $\geq$50% of 45-min RIC sessions. The compliance of patients to RIPostC for several days or months could be important to guarantee its protective effect. Zhao et al. investigate the factors that influence compliance to long-term RIC. The number of follow-up visits and physiological discomfort associated with RIPostC treatment independently influenced patient compliance. Xu et al. in a small study analyses the effect of RIPostC for 1 year among Moyamoya disease patients. They observe improving cerebral blood flow and slowing arterial progression of the stenotic-occlusive lesions. Finally, Zhang et al. evaluated RIPostC over 6 days in the prevention for stroke-associated pneumonia (SAP) in a "proof of concept" pilot randomized controlled trial. According to these authors the possible anti-inflammatory effect of RIC could prevent SAP. Although proinflammatory cytokines levels at day 5 after admission were significantly lower in the RIPostC group than in the control group, no clinically significant effect was observed, possibly due to the small size of the trial.

RIC is a non-invasive, simple, safe, and cheap neuroprotective strategy with multiple mechanisms of action. Its clinical efficacy in acute ischemic stroke patients remains to be proven. RIPostC seems to be the most effective modality of RIC. Therefore, future trials could focus on patients with large vessel occlusion who are candidates for mechanical thrombectomy. Chronic, daily RIPostC could be an option to reduce stroke recurrence in high-risk patients, to improve disability and cognition after AIS, and to improve cerebral perfusion in Moyamoya disease.

AUTHOR CONTRIBUTIONS

FP wrote the first draft. All authors could review it and did their own contributions.

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Factors That Influence Compliance to Long-Term Remote Ischemic Conditioning Treatment in Patients With Ischemic Stroke

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Objectives: To investigate the treatment compliance of patients with ischemic stroke to remote ischemic conditioning (RIC) and to determine the factors that influence compliance.

Methods: We conducted a retrospective study of patients with ischemic stroke who were treated with RIC. Treatment compliance was determined and analyzed in patients who had received 1 year of RIC training. Factors that influenced patient compliance were also determined using univariate and multivariate regression analyses.

Results: Between March 2017 and February 2018, 91 patients were recruited into this study. The mean (±SD) age was 57.98 ± 10.76 years, and 78 (85.7%) patients were male. The baseline Kolcaba comfort scale of patients with good compliance scores were higher than those with poor compliance. The scores of the four dimensions in the scale and the total score are as follows: physiological dimensions, 15.0 (12.0,17.0) vs 17.0 (13.0,19.0); psychological dimensions, 30.0 (25.0,34.0) vs 31.0 (27.0,35.0); sociological dimensions, 20.0 (18.0,24.0) vs 21.0 (18.0,23.0); environmental dimensions, 19.0 (12.0,24.0) vs 20.0 (17.0,22.0); and total points, 82.0 (69.0,94.0) vs 91.0 (78.0,98.0). the differences between the groups were significant ($p < 0.05$), except for the sociological dimensions. A history of hypertension, number of follow-ups, and the physiological, psychological, and environmental dimensions of the comfort scale were related to patient compliance, out of which the number of follow-ups (Adjusted OR = 2.498, 95% confidence interval (CI) 1.257–4.964) and the physiological discomfort (Adjusted OR = 1.128, 95% CI 1.029–1.236) independently influenced compliance ($p < 0.05$).

Conclusion: In patients with ischemic cerebrovascular disease who were treated with RIC, the number of follow-up visits and physiological discomfort associated with RIC treatment independently influenced patient compliance. Further studies are needed to investigate the RIC protocols and their corresponding nursing models.

Keywords: patient compliance, remote ischemic conditioning, influencing factor, ischemic stroke, secondary prevention

INTRODUCTION

Stroke is the second leading cause of death worldwide and the leading cause of death in countries such as China (1). Furthermore, ischemic stroke is the main subtype of stroke and accounts for 60–80% of all strokes (2). Treatment strategies for acute ischemic stroke (AIS) and its secondary prevention have been advanced significantly in the past decades; however, the prognosis of patients with AIS remains far from satisfactory. Therefore, effective adjuvant therapies for the treatment and secondary prevention of ischemic stroke are needed.

Remote ischemic conditioning (RIC), a non-invasive and easy-to-use method of physical therapy, which encompasses several cycles of ischemia/reperfusion training of an organ (e.g., limbs), confers protection to remote vital organs (3) and has been found to reduce recurrent strokes in patients with symptomatic intracranial artery atherosclerosis (4). It has also been found to improve cognitive function in patients with cerebral small vessel disease (5). Although its mechanism is not fully understood, studies have found that RIC could exert its protective effects immediately after the procedure, and this could last for 3–4 days with a 12-h unprotected interval (6). Therefore, RIC has been recommended for days or months, and previous clinical studies have reported protocols of RIC that range from once daily for 1 week to twice daily for 2 weeks, 6 months, and 1 year (7–9). For patients undergoing RIC for several months or years, compliance to RIC treatment is important to guarantee its protective effects. However, the compliance of patients with AIS to RIC treatment in real-world clinical practice and those factors that influence compliance remain unclear.

In this study, we aimed to investigate the compliance of patients with AIS who underwent repeated RIC for 1 year to RIC treatment and to determine the factors that influence compliance to RIC treatment.

METHODS

Study Design and Participants

This study was based on a prospective randomized controlled trial that investigated RIC in patients with AIS (registered on www.chictr.org.cn/index.aspx with ChiCTR1800014403). Patients with ischemic stroke who participated in the randomized trial between March 2017 and February 2018 and who received RIC were recruited into this study. The inclusion criteria were as follows: (1) age $\geq$18 years; (2) NIH Stroke Scale (NIHSS) score of 0–7; (3) patients who were expected to benefit from non-surgical treatment program; (4) preliminary ultrasound examination excluding extracranial and subclavian artery stenosis with clear intracranial artery stenosis; and (5) patients with stroke that occurred within 30 days. The exclusion criteria were as follows: (1) life expectancy of $<$12 months; (2) acute bleeding diathesis (platelet count $<$100,000/mm^3), heparin received within 48 h resulting in abnormally elevated activated partial thromboplastin time (aPTT) levels above the upper limit of normal, current use of anticoagulant with international normalized ratio (INR) $>$1.7 or prothrombin time $>$15 s, current use of direct thrombin inhibitors or direct factor Xa inhibitors with laboratory tests having increased sensitivity (e.g., aPTT, INR, platelet count, ecarin clotting time, thrombin time, or appropriate factor Xa activity assays); (3) skin diseases or fractures of the upper limbs; (4) pregnancy; (5) incomplete or missing data; and (6) long-term plans to stay abroad during the research period.

The study protocol was approved by the Ethics Committee of Xuanwu Hospital, Capital Medical University (2018009). All participants or their legally authorized representatives provided written informed consent.

Interventions

All patients with AIS were treated according to physician's best judgment: aspirin alone (100–300 mg daily), clopidogrel alone (75 mg daily), or a combination of aspirin and clopidogrel, and antidiabetic or antihypertension when necessary. In addition, all patients received bilateral upper limb RIC, which was performed by an electric auto-control device (Patent No. ZL200820123637.X, China) (4) comprising five cycles of inflation and deflation for 5 min alternately, twice daily for 1 year, with an inflating pressure of 200 mmHg. RIC procedures were performed with the help of an assistant nurse in the hospital or their caregivers after they had been discharged from the hospital.

Data Collection

Data collected included demographic data, medical history, smoking history (smokers who have smoked continuously or cumulatively for 6 months or more in their lifetime and have smoked $>$100 cigarettes in the previous 30 days) and/or alcohol consumption (alcohol intake $\geq$50 ml [50 g] per month), baseline NIHSS score, modified Rankin Scale score, Barthel score, Kolcaba's general comfort questionnaire, baseline blood pressure and blood pressure during the follow-up period, blood lipid- and blood sugar-related indicators, visual analog scale for pain scores, and treatment compliance in patients with 1-year training. Treatment compliance was assessed by calculating the proportion of patients who completed the RIC treatment, which is in line with the regularity of the trial protocol according to the RIC treatment back-end data provided by the device software that collects data via a communication chip, with $\geq$80% indicating good compliance and $<$80% indicating poor compliance.

Statistical Analysis

Measurement data were expressed as mean $\pm$ standard deviation. An independent *t*-test was used to compare independent variables after testing for normal distribution, while Mann-Whitney *U*-test was used for non-normally distributed variables. Count data were described as frequency and percentage, and the chi-square test was used to compare the differences between the two groups. The status of patient compliance and the associated influencing factors were recorded and summarized. After all factors were compared between the groups, factors with *p*-values $<$0.05 were included in the multivariate logistic analysis. Multivariate logistic regression analysis (forward) was performed to detect the factors that influence compliance. All statistical analyses were performed using SPSS software, version 20.0 (SPSS, Inc., Chicago, IL, USA), and a *p*-value of $<$0.05 indicated significance.

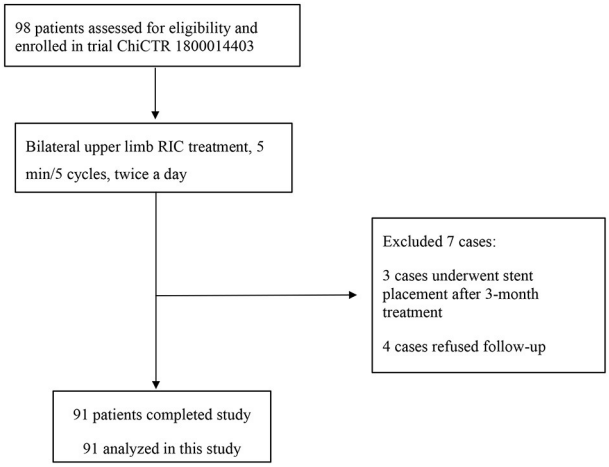

FIGURE 1 | Enrollment flowchart.

RESULTS

Baseline and Demographic Characteristics

A total of 91 patients were included in the final analysis (**Figure 1**). Their baseline and demographic data are summarized in **Table 1**. The mean (±SD) age was 57.98 ± 10.76 years, and 78 (85.7%) patients were male. The Han people were the most predominant ethnic group, accounting for 91.2% (n = 83) of all patients. The educational level of the patients was predominantly junior high school (35.16%) and senior high school (21.98%). Patients whose income was <1,000 RMB accounted for 6.6%, 1,000–3,000 RMB accounted for 22.0%, 3,000–5,000 RMB accounted for 27.5%, 5,000–10,000 RMB accounted for 30.8%, and ≥10,000 RMB accounted for 13.2% of the patients.

Most patients (83.52%) lived in urban areas. The method of medical payment was mainly medical insurance (71.43%). Most participants had chronic diseases, such as hypertension (63.74%), diabetes (36.26%), and hyperlipidemia (32.97%). Approximately 51.6% of the patients had a history of smoking, and 45.1% had a history of alcohol consumption. Patients with severe disease before enrollment into the study had poorer treatment compliance. Until 6 months after enrollment, patients with good compliance during the follow-up period had better blood sugar control. Comparisons of other baseline characteristics are shown in **Table 1**. No significant differences in all baseline characteristics were found between the two study groups ($p < 0.05$), except for the history of hypertension and number of follow-ups. Significant differences in compliance were noted ($\chi^2 = 91.00$, $p < 0.001$).

Total Score of Comfort and Scores in Each Dimension

The baseline comfort of the two groups of patients undergoing RIC treatment were compared, and the results showed that the baseline Kolcaba comfort scale of patients with good compliance scores were higher than those of patients with poor compliance, physiological dimensions, 17.0 (13.0,19.0) vs 15.0 (12.0,17.0); psychological dimensions, 31.0 (27.0,35.0) vs 30.0 (25.0,34.0); sociological dimensions, 21.0 (18.0,23.0) vs 20.0 (18.0,24.0); environmental dimensions, 20.0 (17.0,22.0) vs 19.0 (12.0,24.0); and total points 91.0 (78.0,98.0) vs 82.0 (69.0,94.0) (see **Table 2**). Differences between groups were significant ($p < 0.05$), except for the sociological dimensions.

Factors Influencing RIC Treatment Compliance in Stroke Patients

Multivariate logistic regression analysis was performed to investigate the demographic sociological factors of the patients, pretreatment disease status of the patients, number of follow-ups, and influence of treatment comfort on patient compliance. The variables introduced into the equation were history of hypertension, number of follow-ups, and four dimensions of the comfort scale. The results show that the number of follow-ups [Adjusted OR = 2.498, 95% confidence interval (CI) 1.257–4.964] and physiological discomfort (Adjusted OR = 1.128, 95% CI 1.029–1.236) caused by RIC treatment are independent factors that influence patient compliance (**Table 3**).

DISCUSSION

In this study, we found that in patients with ischemic stroke who received prolonged RIC treatment, the number of follow-ups during the study period was associated with patients' compliance to RIC, and the physiological discomfort related to RIC appears to decrease patients' compliance.

The results of this study showed that the number of follow-ups was positively correlated with patient compliance, and the number of follow-ups was an independent influencing factor of patient compliance. Several studies (10–13) have shown that patients' fear of the potential or actual adverse consequences or disappearance of symptoms can decrease patients' compliance. An effective way of solving the above problems is to communicate with patients to resolve their concerns and convey the rationale and importance of treatment; to elicit and solve specific problems, the above methods can be used to improve treatment compliance. In a long-term treatment study, this can be achieved by increasing the number of follow-ups and by regularly communicating with patients to solve their practical problems.

In this study, the number of follow-up visits was one of the independent factors that influenced patients' compliance to RIC therapy. Therefore, the number of follow-ups should be increased for patients receiving long-term RIC treatment, and at each visit, it is important to make enquiries regarding the patients' doubts and questions, to establish a good relationship with patients and their families, and to provide systematic and continuous health education to correct the patients' wrong attitudes and beliefs. Furthermore, with the rapid development of mobile devices and applications in the field of medicine, appropriate use of these novel techniques may increase communication with patients and this could improve their compliance.

The results of this study indicate that physiological discomfort is an independent influencing factor that reduces patient compliance. Comfort is a subjective sensation that comprises

TABLE 1 | Comparison of baseline data between the two groups.

Item	Poor compliance $n = 35$	Good compliance $n = 56$	Statistics	*p*-value
Age	59.4 ± 9.4	57.2 ± 11.6	−0.892	0.375[a]
Male	30 (85.7)	48 (88.9)	0.013	0.909[b]
Ethnic			4.078	0.253[b]
Han	30 (85.7)	53 (94.6)		
Other	5 (14.3)	3 (5.4)		
Education			5.024	0.413[b]
Primary, below	1 (2.9)	7 (12.5)		
Junior high	15 (42.9)	17 (30.4)		
Senior high	9 (25.7)	11 (19.6)		
Vocational	2 (5.7)	2 (3.6)		
College	6 (17.1)	12 (21.4)		
Bachelor, above	2 (5.7)	7 (12.5)		
Marital status			1.939	0.379[b]
Unmarried	0 (0)	2 (3.6)		
Married	35 (100)	53 (94.6)		
Divorced	0 (0)	1 (1.8)		
Avg. monthly income			1.329	0.856[b]
≤1,000 RMB	3 (8.6)	3 (5.4)		
1,000–3,000	9 (25.7)	11 (19.6)		
3,000–5,000	10 (28.6)	15 (26.8)		
5,000–10,000	9 (25.7)	19 (33.9)		
≥10,000	4 (11.4)	8 (14.3)		
Residence area			1.056	0.304[b]
Rural	4 (11.4)	11 (19.6)		
Urban	31 (88.6)	45 (80.4)		
Payment method			1.213	0.750[b]
Self-pay	3 (8.6)	6 (10.7)		
Public pay	1 (2.9)	4 (7.1)		
Medical insurance	27 (77.1)	38 (67.9)		
Rural medical care	4 (11.4)	8 (14.3)		
Religious			1.115	1.000[b]
Yes	1 (2.9)	0 (0)		
No	34 (97.1)	56 (100)		
Medical history				
Hypertension	28 (80)	30 (53.6)	6.509	0.011[b]
Diabetes	15 (44.1)	18 (33.3)	1.035	0.309[b]
Hyperlipidemia	11 (31.4)	19 (33.9)	0.061	0.805[b]
Atrial fibrillation	0 (0)	2 (3.6)	1.278	0.258[b]
Stroke	8 (22.9)	11 (19.6)	0.135	0.714[b]
Heart disease	6 (17.1)	7 (12.5)	0.379	0.538[b]
Others	4 (11.4)	4 (7.4)	0.420	0.517[b]
Smoking history	16 (45.7)	31 (55.4)	1.088	0.581[b]
Alcohol consumption	16 (47.1)	25 (44.6)	0.050	0.823[b]
BMI	25.8 ± 3.2	26.2 ± 3.8	−0.526	0.600[a]
Baseline NIHSS	2 (0,4.0)	1 (0,3.0)	−1.664	0.096[c]
Baseline mRS	1 (0,3.0)	1 (0,2.0)	−1.554	0.120[c]
Baseline ADL	85.0 (70.0,95.0)	90.0 (70.0,95.0)	−0.610	0.542[c]
Baseline comfort	82.0(69.0,94.0)	91.0(78.0,98.0)	−1.372	0.170[c]
Baseline pain	0 (0, 0)	0 (0, 0)	−0.922	0.356[c]
3-month pain	0 (0, 0)	0 (0, 0)	−1.139	0.255[c]

(Continued)

TABLE 1 | Continued

Item	Poor compliance $n = 35$	Good compliance $n = 56$	Statistics	*p*-value
6-month pain	0 (0, 0)	0 (0, 0)	−1.025	0.306[c]
1-year pain	0 (0, 0)	0 (0, 0)	−1.147	0.246[c]
Baseline glycosylated hemoglobin	6.2 (5.6,7.4)	5.8 (5.4,6.7)	−1.898	0.058[c]
3-month glycosylated hemoglobin	7.5 (6.3,8.1)	6.1 (5.7,7.2)	−1.628	0.104[c]
6-month glycosylated hemoglobin	5.5 (3.8,6.1)	6.2 (5.0,7.2)	−0.671	0.502[c]
1-year glycosylated hemoglobin	7.7 (5.3,8.9)	5.7 (5.4, 6.9)	−1.223	0.221[c]
Times of follow-ups	1.5 (1, 2)	2 (2, 2.75)	−2.782	0.005[c]

[a]Independent t-test; [b]Chi-square test; [c]Mann-Whitney U-test; BMI, Body Mass Index; NIHSS, The National Institutes of Health Stroke Scale; mRS, Modified Rankin Scale; ADL, the Barthel index of activities of daily living.

TABLE 2 | Comparison of the Kolcaba comfort scores in each dimension of the two groups.

Item	Poor compliance $n = 35$	Good compliance $n = 56$	*Z*	*p*-value
Physiological	15.0 (12.0,17.0)	17.0 (13.0,19.0)	−2.691	0.007
Psychological	30.0 (25.0,34.0)	31.0 (27.0,35.0)	−2.082	0.037
Sociological	20.0 (18.0,24.0)	21.0 (18.0,23.0)	−1.610	0.107
Environmental	19.0 (12.0,24.0)	20.0 (17.0,22.0)	−2.295	0.022
Total Score	82.0 (69.0,94.0)	91.0 (78.0,98.0)	−2.604	0.009

The results are expressed as median (inter-quantile range). Mann-Whitney U-test was used to determine the differences between groups.

TABLE 3 | Multivariate logistic regression analysis of patient compliance.

Item	B	SE	*Adjusted P*-value	Adjusted OR	95% CI
Constant	−1.961	0.781	0.012	0.141	
No. of follow-ups	0.916	0.350	0.009	2.498	1.257–4.964
Physiological comfort	0.120	0.047	0.010	1.128	1.029–1.236

B, partial regression coefficient; SE, Standard Error; CI, confidence interval; OR, odds ratio.
The variables introduced into the equation were history of hypertension, number of follow-ups, and four dimensions of the comfort scale.
The model was adjusted for age, sex, ethnicity, education, marital status, average monthly income, residence area, health payment method, religion, smoking history, and drinking history.

both physical and psychological dimensions (14). RIC treatment uses pressure to block blood flow, and this causes pain and numbness, which in turn leads to physiological discomfort. The duration of such discomfort affects the patient's psychological well-being, and this may influence patients' compliance to the RIC treatment, which not only affects the therapeutic effects of RIC but may also lead to drop-out in clinical research (15). Previous studies have shown that complexity, comfort, and duration of treatment programs influence patient compliance (16). Furthermore, the level of comfort had an increasing trend along with continuation of treatment, which indicates that as the test progressed over time, participants gradually adapted to the discomfort at the strongest pressure per cycle, although the discomfort still existed. Therefore, a more comfortable and equally effective RIC treatment protocol can improve the compliance of patients to RIC treatment.

Currently, numerous research teams are actively engaged in clinical transformation research because of the importance of RIC and its broad clinical application prospects (15, 17). However, the clinical transformation of RIC is still in its initial stages. To ensure that the clinical transformation of RIC achieves better results, an evidence-based supportive nursing process is necessary. Therefore, in future studies, researchers should comprehensively consider the patient's efficacy, comfort, compliance, and other factors, while choosing the optimal treatment dose, and improve the RIC nursing process accordingly, which will provide the basis for clinical implementation and scientific research of RIC.

This study has several limitations. First, this was a retrospective study that was based on a randomized controlled trial. This study design has an inherent limitation as we could only use the existing data for analysis. In the future, a prospective research will be necessary to evaluate more factors that may affect patient compliance to RIC treatment. Second, Kolcaba comfort scale is a subjective evaluation tool that may be limited by self-report biases; thus, the findings of this study should be interpreted with caution. In addition, the sample size was small, and some known and unknown factors may have influenced the results. Therefore, larger studies are needed to confirm these results. Finally, our study only focused on patients' factors, and

whether their family and social support influenced compliance to RIC treatment needs further investigation.

In conclusion, in patients with ischemic cerebrovascular disease who were treated with RIC, the number of follow-up visits and the physiological discomfort associated with RIC were independent influencing factors of patient compliance. Further studies are needed to confirm these results and determine appropriate RIC protocols and their corresponding nursing models.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of Xuanwu Hospital, Capital Medical University (2018009). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ and HC: study concept and design, critical revision of the manuscript for important intellectual content, and study supervision. JZ: acquisition, analysis and interpretation of data, and drafting of the manuscript. KF: analysis and interpretation of data. WZ: revision of the manuscript. HY and JM: acquisition of data. All authors read and approved the final manuscript.

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Remote Ischemic Conditioning May Improve Disability and Cognition After Acute Ischemic Stroke: A Pilot Randomized Clinical Trial

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Background and Aim: Remote ischemic conditioning is a procedure purported to reduce the ischemic injury of an organ. This study aimed to explore the efficiency and safety of remote ischemic conditioning in patients with acute ischemic stroke. We hypothesized that remote ischemic conditioning administered from the first day of hospital admission would improve the infarct volume and clinical outcome at 180 days.

Material and Methods: We performed a unicentric double-blind randomized controlled trial. We included all patients consecutively admitted to an Emergency Neurology Department with acute ischemic stroke, ineligible for reperfusion treatment, up to 24 hours from onset. All subjects were assigned to receive secondary stroke prevention treatment along with remote ischemic conditioning on the non-paretic upper limb during the first 5 days of hospitalization, twice daily - a blood pressure cuff placed around the arm was inflated to 20 mmHg above the systolic blood pressure (up to 180 mmHg) in the experimental group and 30 mmHg in the sham group. The primary outcome was the difference in infarct volume (measured on brain CT scan) at 180 days compared to baseline, whereas the secondary outcomes included differences in clinical scores (NIHSS, mRS, IADL, ADL) and cognitive/mood changes (MoCA, PHQ-9) at 180 days compared to baseline.

Results: We enrolled 40 patients; the mean age was 65 years and 60% were men**.** Subjects in the interventional group had slightly better recovery in terms of disability, as demonstrated by the differences in disability scores between admission and 6 months (e.g., the median difference score for Barthel was −10 in the sham group and −17.5 in the interventional group, for ADL −2 in the sham group and −2.5 in the interventional group), as well as cognitive performance (the median difference score for MoCA was −2 in the sham group and −3 in the interventional group), but none of these differences reached statistical significance. The severity of symptoms (median difference score for NIHSS = 5 for both groups) and depression rate (median difference score for PHQ-9 = 0 for both groups) were similar in the two groups. The median difference between baseline infarct volume and final infarct volume at 6 months was slightly larger in the sham group

compared to the interventional group ($p = 0.4$), probably due to an initial larger infarct volume in the former.

Conclusion: Our results suggest that remote ischemic conditioning might improve disability and cognition. The difference between baseline infarct volume and final infarct volume at 180 days was slightly larger in the sham group.

Keywords: remote ischemic conditioning, acute ischemic stroke, neuroprotection, cognition, infarct size, disability

INTRODUCTION

Stroke is one of the leading causes of mortality and morbidity worldwide, with significant global burden and costs (1). It is the first cause of disability and the second cause of cognitive decline (2). Nevertheless, the only approved treatment for acute ischemic stroke (AIS) (which accounts for 87% of all strokes) (1) is reperfusion therapy (intravenous thrombolysis with alteplase and/or mechanical thrombectomy) (3). Unfortunately, in Romania the treatment of AIS remains extremely limited, mainly because patients do not recognize stroke symptoms/signs and arrive late to the hospital, therefore missing the reperfusion therapeutic window. In these cases, there is a great need for neuroprotective interventions in order to improve the outcome of AIS.

Over the last 20 years, many neuroprotective agents and interventions have been studied. Remote ischemic conditioning (RIC) is a new area of interest in stroke and neuroprotection (4). It is a potential non-invasive intervention meant to induce transient and brief periods of ischemia remote from the ischemic injury site. In AIS, single or repeated cycles of transient limb(s) ischemia followed by reperfusion are employed, usually with a blood pressure cuff inflated to a level above the systolic blood pressure for a few minutes, followed by deflation. This stems from the hypothesis that RIC could prevent cerebral damage after AIS by preventing/reducing the ischemia-reperfusion injury (neuroprotection).

Ischemia-reperfusion injury resulting from arterial occlusion in ischemic stroke is characterized by metabolic dysfunction, apoptosis, necrosis, and local inflammatory processes (5). The neuroprotective mechanism of RIC is not clearly understood, but it has been suggested that it is mediated by humoral, neuronal, and inflammatory pathways (6). Remote ischemic conditioning seemingly involves the transfer of a humoral substance from one organ to another. It triggers the release of humoral factors and local autocoids (adenosine, bradykinin, and gene-related peptide) which activate neurogenic transmission (with involvement of muscle afferents and autonomic nervous system) and involve immune pathways by suppressing proinflammatory genes in immune cells. Furthermore, RIC reduces oxidative damage and suppresses the inflammatory responses in the brain. This mechanism can last days after revascularization. More details can be found in excellent detailed reviews about this topic (4, 7, 8).

Ischemic conditioning was introduced in Cardiology in 1986 by Murry et al. who performed short repetitive occlusion/reperfusion of the coronary artery in canine models and observed a significant reduction in infarct size (9). Subsequent studies showed that ischemic conditioning can also be applied remote from the ischemic injury site. Brief episodes of ischemia-reperfusion in a distant organ (e.g., upper or lower limb) were proposed to exert a neuroprotective effect (10). Various studies indicated that limb RIC is neuroprotective in animal models of stroke (11, 12).

In AIS, RIC can be applied prior to the ischemic event (remote ischemic preconditioning), during the ischemic event (remote ischemic perconditioning), or after the vascular event (remote ischemic postconditioning). A mechanical tourniquet or automatic device placed around an arm or leg is meant to perform repetitive cycles of inflation and deflation. Most of the clinical studies chose to perform 3 to 5 cycles of transient limb ischemia, with different duration of mechanical vessel occlusion, ranging from 3 to 5 min. The most frequent site of limb conditioning was a single-arm (13). These protocols were influenced by the Cardiological ischemic conditioning protocols, but currently it is unknown whether these differences modify the efficacy of RIC. The major advantage of remote ischemic perconditioning is the broad therapeutic window- although time-sensitive, it can be carried out even after exceeding the therapeutic window for reperfusion treatment (thrombolysis/thrombectomy).

There is a limited number of clinical trials evaluating the efficacy of RIC in the treatment AIS. The majority of these ongoing trials explore the benefits, feasibility, and risks of applying RIC as soon as possible (even from the ambulance) after AIS onset (14). In particular, they focus on the effect of RIC on clinical outcome scales and final infarct volume.

From all the clinical trials employing RIC in patients with AIS, Purroy F et al. identified only four randomized controlled trials with completed and published data (15). The first study on RIC in AIS was conducted by Hougaard KD et al. in 2014 (14). They tested the effect of RIC vs. sham performed during the prehospital phase (in the ambulance) in AIS patients, in conjunction with thrombolysis. The primary outcome was the penumbra salvage, with a follow-up at 90 days. Overall, a final infarct volume analysis suggested that prehospital RIC might have immediate neuroprotective effects (14). In the RECAST and RECAST-II, England et al. investigated the effect of RIC vs. sham in patients with hyperacute AIS (26 patients recruited within 24 h of stroke onset and 60 patients enrolled within 6 h of stroke onset, respectively) by inducing transient non-paretic arm ischemia through a manual standard blood pressure cuff (16, 17). The primary outcome was feasibility, whereas the second outcome included functional outcomes. The duration of follow-up was 90 days. The conclusion was that RIC applied

twice daily is feasible, well-tolerated, with no serious adverse events, and good adherence in hyperacute stroke (16, 17). Che et al. investigated and demonstrated the feasibility and safety of arm RIC after thrombolysis in 30 patients with AIS (18). The multicenter RESCUE BRAIN trial conducted by Pico et al. inquired whether leg RIC reduces final ischemic volume after AIS. They included 188 patients with confirmed carotid ischemic stroke within 6 hours of symptoms onset and concluded that treatment with RIC during and after AIS reperfusion therapy does not significantly reduce the brain infarct volume growth (19). Considering recent data and evidence, RIC is a simple, inexpensive, well-tolerated intervention, posing minimal risk. Clinical trials have demonstrated the feasibility of delivering RIC at different stages of hospitalization for AIS.

In 2018, Zhao et al. conducted a meta-analysis aiming to assess the benefits and harms of RIC in preventing or treating AIS (20). They included seven randomized controlled studies of RIC vs. sham in subjects with either AIS, chronic cerebral ischemia (i.e., 14 days after symptoms onset)/gradual onset cerebral ischemia, or intracranial/extracranial moderate/severe stenosis/confirmed occlusion, encompassing different protocols for RIC. Out of these, three trials on the effects of RIC on ischemic stroke prevention were included in the analysis, whereas four trials analyzed the effects of RIC on ischemic stroke treatment. The latter category included two studies enrolling patients with cerebral small vessel disease and two studies with AIS subjects. By combining their results, the overall effect (i.e., stroke severity-final infarct volume and clinical scores) was not significantly different between the intervention and sham group in AIS patients (20).

Since clinical trials have employed different RIC protocols in AIS patients, their cumulative results should be interpreted with caution. Temporal inclusion criteria had great variability. Remote ischemic conditioning was initiated during transportation to the hospital or immediately after admission. It was employed isolated or as add-on therapy to revascularization (alteplase or endovascular methods). The procedure was also different among studies, with various repetitions per day or number of days of RIC, possibly responsible for the inconsistent results. Notably, there were significant differences in main endpoints. Some studies focused on final infarct volume, whereas others addressed clinical stroke severity after RIC. Further trials with uniform and standardized protocols might fill in these gaps and provide better knowledge of RIC mechanisms and effects.

Our study aims to explore the efficiency and safety of RIC applied to the non-paretic upper limb in patients with AIS ineligible for reperfusion treatment. We hypothesize that RIC administered from the first day of AIS improves the final infarct volume and clinical outcome at 6 months. Details of the trial design have already been published (21).

MATERIALS AND METHODS

Trial Design

We conducted a unicentric double-blind randomized controlled trial that took place between July 2018 and June 2020. It included all patients consecutively admitted to the Neurology Department of the Emergency Clinical Hospital from Bucharest, Romania, who fulfilled the inclusion criteria. In the original trial design (21), the study was intended to be bicentric, also involving the Neurology Department of Colentina Clinical Hospital from Bucharest, Romania. Nevertheless, since Colentina Clinical Hospital became a COVID-19 support hospital (i.e., a second line hospital that admits patients with comorbidities and SARS-CoV-2 infection in order to support the first line hospitals—infectious disease and pulmonology hospitals) from May 16, 2020, it was excluded from enrollment. The study complied with the Declaration of Helsinki and was approved by the local research ethics committee.

Subjects

We enrolled forty patients aged 50–80 years with AIS who were not eligible for thrombolysis and/or mechanical thrombectomy (e.g., patients who did not fulfill the inclusion criteria required for treatment with intravenous alteplase such as the onset of symptoms < 4.5 h before beginning treatment or patients with one or more exclusion criteria such as ischemic stroke in the previous 3 months, etc.), with National Institutes of Health Stroke Scale (NIHSS) ranging from 5 to 25 points. Exclusion criteria included premorbid dependency [modified Rankin Scale (mRS) < 3], significant comorbidity with any serious diseases, and life expectancy of fewer than 6 months (**Table 1**). Uncontrolled blood pressure (>200/100 mmHg) was also counted as an exclusion criterion. Informed consent was obtained from each patient or legal representative if the patient was unable to consent.

All patients meeting the clinical criteria for stroke underwent brain CT scan at baseline and follow-up at 6-months. The clinical examination and neurological scales were performed at baseline, 3 and 6 months.

All patients continued the standard treatment for secondary prevention of AIS according to the national guidelines.

Randomization and Intervention

The subjects were allocated in a double-masked and randomized fashion 1:1 to either the experimental or control group. The randomization was performed by predefined drawing lots from a large number of sealed opaque envelopes containing the RIC instructions.

A manual tourniquet was placed around the upper limb contralateral to the neurological deficit in the first 24 hours from the onset of symptoms/signs suggestive of AIS. All patients received five consecutive cycles of blood pressure cuff inflation lasting 3 min, each followed by 5 min of reperfusion, twice daily, during the first 5 five days of hospitalization (in the morning and afternoon). In the RIC group, the target of cuff inflation was 20 mmHg above the systolic blood pressure, up to 180 mmHg. Although we included patients with systolic blood pressure up to 200 mmHg, we chose the threshold of 180 mmHg (which would not induce complete restriction of blood flow in these patients) due to safety reasons, to avoid acute limb ischemia. The sham group received cuff inflation up to 30 mmHg, thus inducing sham conditioning. The procedure was performed by one of the authors (A.P.). Patients could stop the RIC treatment at any time.

TABLE 1 | The inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Age: 50–80 years	Reperfusion treatment (intravenous thrombolysis, mechanical thrombectomy)
$5 \leq$ NIHSS ≤ 25	$5 >$ NIHSS > 25
Ischemic stroke confirmed by CT scan	Hemorrhagic stroke on CT scan
$<$ 24 h from onset to treatment	Fluctuating neurological deficit
Signed informed consent	Transient ischemic attack
	Other cerebral lesions: cerebral tumors, arteriovenous malformation
	Uncontrolled blood pressure ($<$ 90/60 or $>$ 200/100 mmHg)
	Difference between systolic blood pressure in the upper arms $>$ 10 mmHg
	Ischemic events in the last 6 months (AIS, myocardial infarction, etc.)
	Premorbid mRS > 3
	Comorbidity with any serious disease and life expectancy of $<$ 6 months.

The patients and the medical staff who processed the data were blinded to the intervention allocation.

All patients underwent head CT scan on days 3–4 and at 180 days in the same hospital, using the same CT scanner (128 slices GE Optimal CT and 128 slices Siemens Somaton CT), unenhanced helical acquisitions, cerebral window, 140 KW(Siemens)/120 KW(GE), collimation 128 × 0.6 mm, 1.5 mm slice thickness (Siemens)/1.25 mm slice thickness (GE). The final ischemic volume was determined by manual contouring on axial CT images slice by slice, using dedicated software (GE version ADV 4.6). The CT scan at 6 months was performed in the same conditions as the first one. The reader of the CT scans was blinded to treatment allocation.

Primary Outcome

Changes in brain lesion were evaluated by infarct volume on head CT at baseline (days 3–4) and 180 days in both groups. We acknowledge that the baseline CT is not a pre-RIC CT, but we wanted to identify a measurable infarct volume in order to calculate the difference at 6 months. We also performed a brain CT at admission in order to exclude hemorrhagic stroke, but we did not include it as a variable in the statistical analysis. We chose CT scan because it is more accessible, faster, and less expensive than MRI scanning.

Secondary Outcome

Changes in clinical stroke severity and disability were assessed by NIHSS, mRS, Barthel Index, Lawton Instrumental Activities of Daily Living (IADL), and Katz Activities of Daily Living (ADL), whereas cognitive performance was evaluated by Montreal Cognitive Assessment (MoCA) and depression occurrence by Patient Health Questionnaire-9 (PHQ-9). All the scales were recorded at baseline, 90 and 180 days in both groups in the same Neurology Department, under similar conditions. We also recorded demographics and vascular risk factors (as defined by the World Health Organization), possible complications related to tolerance and side effects of RIC (local pain or bruising at the cuff side), cerebral complications (cerebral edema, recurrence of stroke, or other vascular events), and systemic complications (bedsores, urinary tract infection, bronchopneumonia, or death rate).

Statistical Analysis

Statistical data were analyzed with SPSS version 20.0 Software. Categorical variables were reported as frequency and analyzed with Chi-square test. Continuous variables were reported as median (minimum, maximum) and analyzed with non-parametric tests (Mann-Whitney U test) provided that neither of them had normal distribution (priorly tested by Kolmogorov-Smirnov test). Hypothesis testing was 2-tailed and statistical significance was defined as $p < 0.05$. Unavailable or unobtainable information (such as MoCA and PHQ-9 in aphasic patients) was recorded as missing values and excluded from the statistical analysis. According to the sample size estimation (for a mean infarct volume difference = 1.814 in the RIC group, 13.416 in the sham group, Alpha = 0.05, Beta = 0.2, Power = 0.8), 60 patients were needed: 30 in the RIC group and 30 in the sham group.

RESULTS

Forty patients were included in our study throughout 24 months. Eighteen patients were randomized into the RIC group and twenty-two were included in the sham group (**Figure 1**). The mean age was 65 years and 60% were men. Because of logistic problems related to the current SARS-CoV2 pandemic (please see above), one of the hospitals designed for enrollment was not available, therefore we could not include all the patients required by sample size estimation.

At hospital admission, the mean values of vital signs were as follows: systolic blood pressure = 154 mmHg, diastolic blood pressure = 85 mmHg, heart rate = 80 bpm, oxygen saturation = 97%, and blood glucose level = 136 mg/dl.

Sixty percent of patients were taking antihypertensive drugs, 35% antiplatelet drugs, 10% oral anticoagulants (7.5% vitamin K antagonists and 2.5% novel oral anticoagulants), and 22.5% statins.

At admission, the mean values of scales for the severity of symptoms, disability, and cognitive function were as follows: NIHSS = 7.7, mRS = 2.9, Barthel = 48, ADL = 4.3, IADL = 3.8,

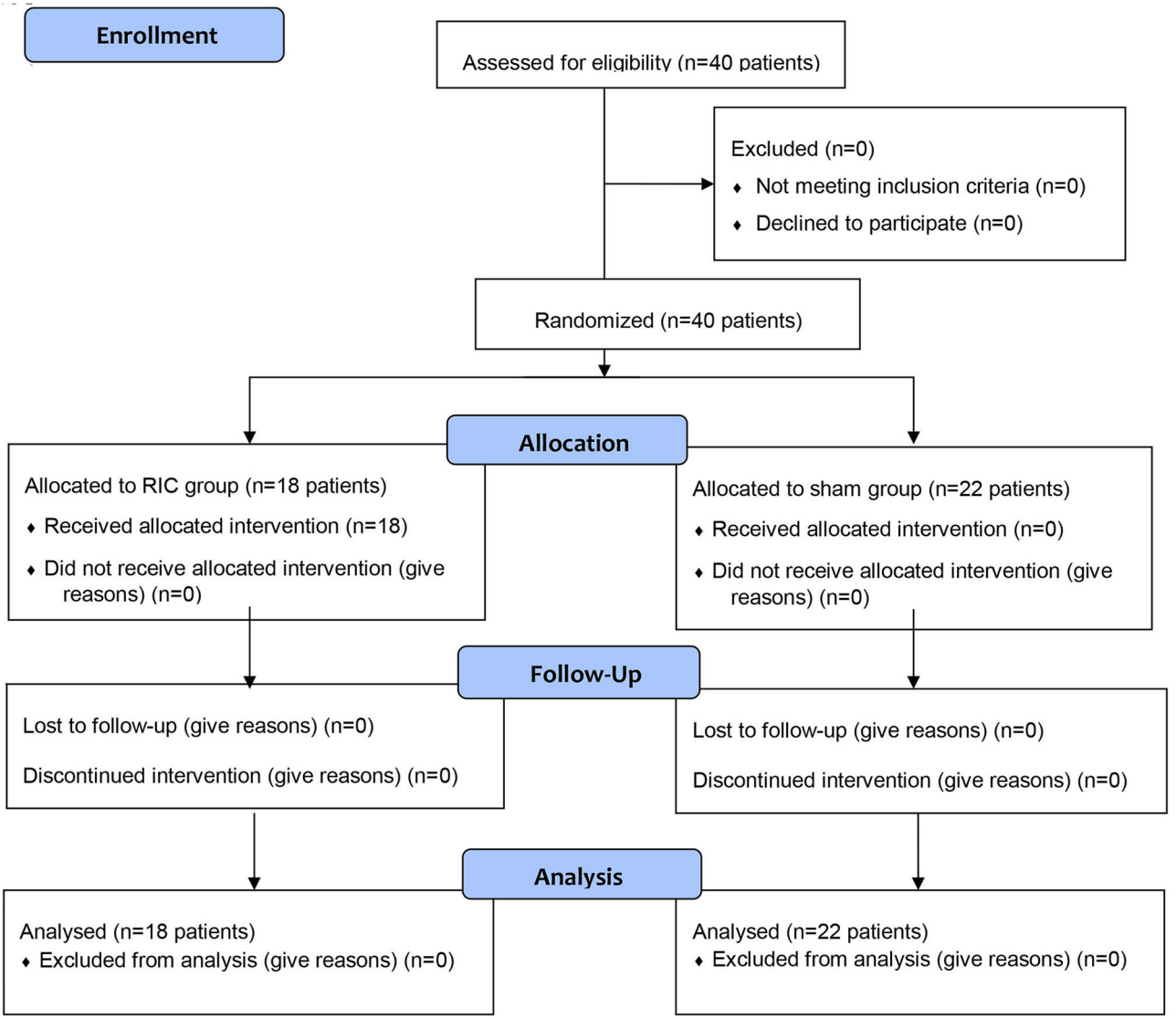

FIGURE 1 | CONSORT flow chart showing the selection process of patients.

MoCA = 15.5, PHQ-9 = 6.2. The average infarct volume on CT was 17.5 cm^3. The most frequent localization of AIS was in the middle cerebral artery territory (75%), followed by basilar artery (12.5%), vertebral artery (5%), and multi-territorial (7.5%). According to the TOAST classification, the most frequent etiology of AIS was large artery atherosclerosis (60%), cardioembolism (13%), small vessel occlusion (15%), and undetermined etiology (13%).

Baseline characteristics were similar in the interventional and sham group (**Table 2**). Concerning medical history/comorbidities, diabetes mellitus was more prevalent in the sham group (36.4 vs. 11.1%, $p = 0.067$) compared to the RIC group. Sedentarism was also more frequent in the sham group (77.3 vs. 44.4%, $p = 0.033$), reaching statistical significance.

Regarding the primary outcome, the mean infarct volume on CT at baseline was 23.19 cm^3 in the sham group and 10.6 cm^3 in the RIC group, whereas the final infarct volume was 10.35 cm^3 in the sham group and 9.38 cm^3 in the RIC group. The median difference of the final infarct volume at 6 months compared to baseline was slightly larger in the sham group, but these results did not reach statistical significance ($p = 0.4$). One of the reasons for a better outcome in terms of stroke volume in the sham group might be the larger infarct volumes at admission compared to the experimental group (**Figure 2**, **Table 3**). Another possible confounder is the baseline CT performed at days 3–4 (after RIC initiation), meaning that RIC could have already affected infarct volume, hence impacting the measure of infarct growth. As expected, larger final infarct volume was associated with increased mRS ($p = 0.001$) (**Figure 3**).

The details related to the secondary outcomes are listed in **Table 3**. The patients in the interventional group had slightly better recovery in terms of disability, as demonstrated by the

TABLE 2 | Baseline characteristics in the two groups.

Characteristics (total $n = 40$)	RIC $n = 18$	Sham $n = 22$	p-value
Age, years	66.78 ± 6.44	64.41 ± 9.02	0.279
Male sex, *N* (%)	11 (61.11%)	13 (59.09%)	0.897
BMI, kg/m^2	27.62 ± 4.13	29.9 ± 4.09	0.471
Abdominal circumference, cm	100.5 ± 12.72	108.5 ± 11.28	0.751
Medical history			
Arterial hypertension, *N* (%)	12 (66.7%)	19 (86.4%)	0.138
Diabetes mellitus, *N* (%)	2 (11.1%)	8 (36.4%)	0.067
Atrial fibrillation, *N* (%)	2 (11.1%)	3 (13.6%)	0.810
Prior stroke, *N* (%)	2 (11.1%)	2 (9.1%)	0.832
Alcohol consumption, *N* (%)	7 (38.9%)	6 (27.3%)	0.435
Smoking, *N* (%)	9 (50%)	10 (45.5%)	0.775
Sedentarism, *N* (%)	8 (44.4%)	17 (77.3%)	0.033
Depression, *N* (%)	5 (27.8%)	8 (36.4%)	0.564
Clinical and laboratory findings at admission			
Systolic BP, mmHg	154.78 ± 35.05	153.05 ± 25.78	0.817
Diastolic BP, mmHg	81.78 ± 17.68	88.05 ± 23.44	0.236
Heart rate, bpm	80.78 ± 15.32	73.82 ± 11.4	0.153
Oxygen saturation, %	97.66 ± 1.41	97.14 ± 2.04	0.555
Temperature, °C	36.42 ± 0.19	36.45 ± 0.22	0.989
Glycemia, mg/dL	125.17 ± 55.32	145.95 ± 52.36	0.128
Total cholesterol, mg/dL	200.28 ± 43.81	206.23 ± 49.45	0.734
Triglycerides, mg/dL	159.94 ± 88.51	165.86 ± 84.25	0.568
Prior therapy			
Antihypertensive drugs, *N* (%)	11 (61.1%)	13 (59.1%)	0.897
Antiplatelet drugs, *N* (%)	6 (33.3%)	8 (36.4%)	0.842
Anticoagulant drugs, *N* (%)	2 (11.1%) (VKA)	2 (9%) (4.5% VKA, 4.5% NOAC)	0.499
Statins, *N* (%)	4 (22.2%)	5 (22.7%)	0.970
Clinical syndrome			
Lacunar, *N* (%)	1 (5.6%)	5 (22.7%)	
Partial anterior circulation, *N* (%)	15 (83.3%)	15 (68.2%)	
Total anterior circulation, *N* (%)	–	–	
Posterior circulation, *N* (%)	3 (16.7%)	4 (18.1%)	
Multi-territorial, *N* (%)	–	3 (13.6%)	
CT findings			
Infarct volume, cm^3	10.6 ± 15.17	23.19 ± 49.02	0.645

differences in disability scores between admission and 6 months (e.g., the median difference score for Barthel was −10 in the sham group and −17.5 in the interventional group, for ADL −2 in the sham group and −2.5 in the interventional one), as well as cognitive performance (the median difference score for MoCA was −2 in the sham group and −3 in the interventional group), but none of these differences reached statistical significance. Nevertheless, the trend was similar in the two groups in terms of clinical stroke severity (median difference score for NIHSS = 5 for both groups) and depression (median difference score for PHQ-9 was 0 for both groups). Cognitive and depression scales could not be performed in 3 patients who were aphasic.

The mortality rate was 4.5% (one patient) in the sham group and 11.1% (two patients) in the RIC group. Recurrent ischemic stroke was 5.6% with one recurrent stroke in the RIC group, which occurred on day 14. There was no intracerebral hemorrhage.

Remote ischemic conditioning was well-tolerated and did not correlate with local (pain or bruising at the cuff side), cerebral (cerebral oedema, recurrence of stroke), or systemic (bedsores, urinary tract infection, bronchopneumonia, or death rate) complications.

DISCUSSION

To our knowledge, this is the first study employing RIC in AIS patients ineligible for reperfusion treatment, which evaluated the final infarct volume on CT scan and the functional outcome scales. We chose to evaluate the final infarct volume on CT scan

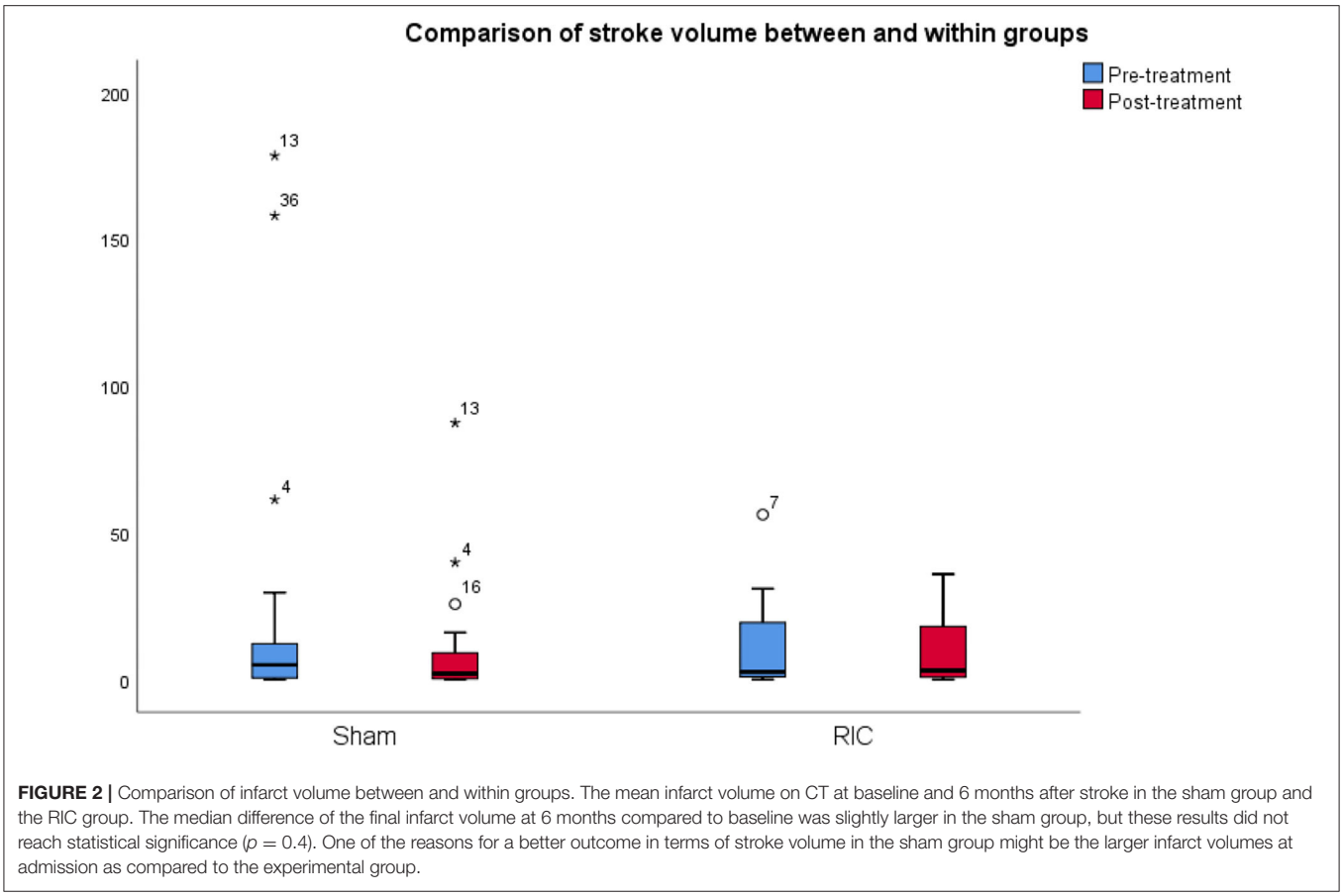

FIGURE 2 | Comparison of infarct volume between and within groups. The mean infarct volume on CT at baseline and 6 months after stroke in the sham group and the RIC group. The median difference of the final infarct volume at 6 months compared to baseline was slightly larger in the sham group, but these results did not reach statistical significance ($p = 0.4$). One of the reasons for a better outcome in terms of stroke volume in the sham group might be the larger infarct volumes at admission as compared to the experimental group.

TABLE 3 | Summary of primary and secondary outcomes in the two groups.

Scale (score difference between 0 and 6 months)	Remote ischemic conditioning						*p*-value (Mann-Whitney *U* test)
	SHAM			RIC			
	Minimum	Maximum	Median	Minimum	Maximum	Median	
Infarct volume	−5.1	156	0.37	−5.4	20.3	0.29	0.4
NIHSS	0	11	5	1	8	5	0.1
mRS	0	4	1	0	4	1	0.3
Barthel	−80	0	−10	−60	100	−17.5	0.9
ADL	−8	0	−2	−6	10	−2.5	0.8
IADL	−8	2	−1	−6	8	−0.5	0.4
MoCA	−19	4	−2	−10	2	−3	0.2
PHQ-9	−12	17	0	−17	7	0	0.5

because it is inexpensive and widely accessible, regardless of the economic status.

Our study indicates that RIC is safe, posing little risk for patients (i.e., no local or systemic events). It is also feasible since all patients finished the cycles of transient limb ischemia and the procedure was well-tolerated and inexpensive. We did not find any serious side effects.

Although RIC has been preliminarily investigated, the optimal protocol has not been found. In previous studies, there were different types of RIC procedures: different sites of limb conditioning (arm, leg, both arms, both legs); different number of cycles and duration of inflation/deflation; different timing of RIC; different frequency of inflation/deflation cycles (once or repeated different times per day) (13, 22, 23). We chose to cover the hyper acute-acute phase of AIS (inclusion within the first 24 h from the onset of symptoms/signs), by applying 5 cycles of inflation lasting 3 min, each followed by 5 min of reperfusion, twice daily,

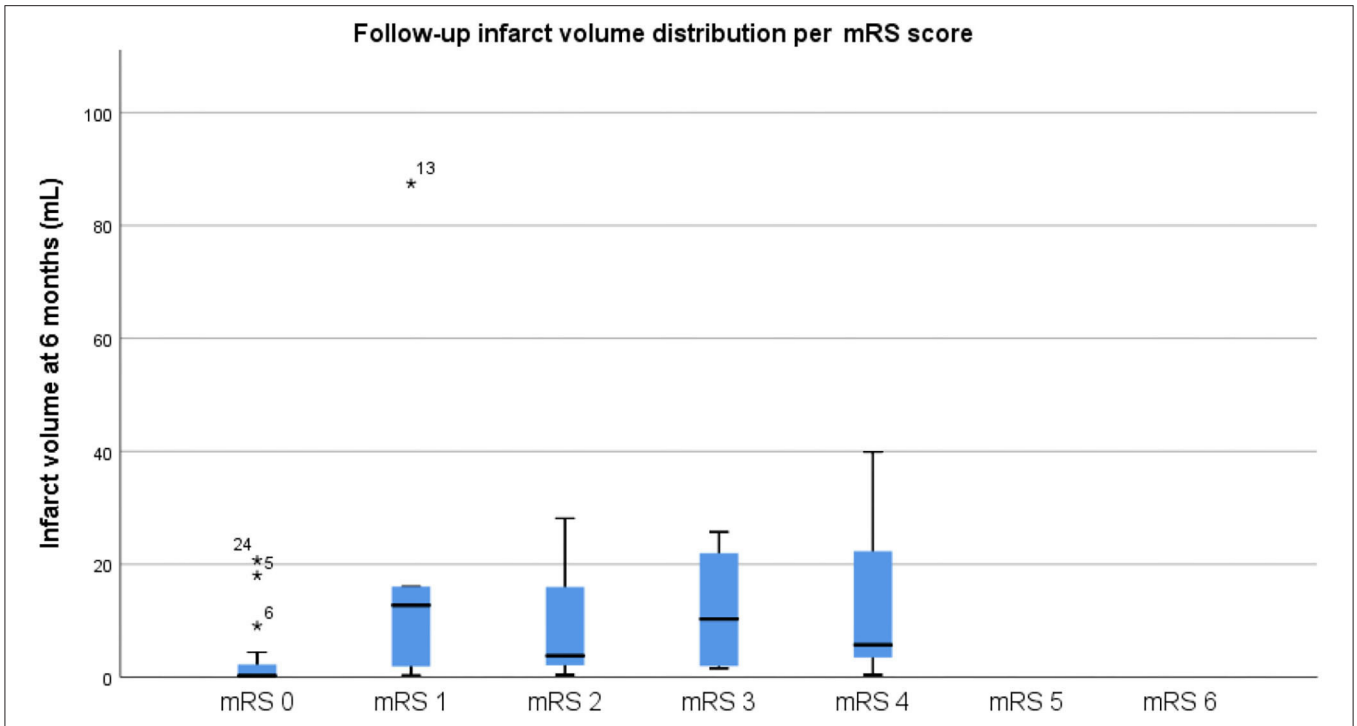

FIGURE 3 | Final infarct volume distribution per mRS score. Please note that a larger final infarct volume was associated with increased mRS ($p = 0.001$), as expected.

during the first 5 five days of hospitalization (in the morning and afternoon).

The neuroprotective effect of RIC supposedly acts in different ways, depending on the time of administration: when applied during the rapid therapeutic time window, RIC might minimize the side effects of sudden reperfusion (i.e., overproduction of reactive oxygen and nitrogen species); during the intermediate therapeutic time window, RIC is likely to enhance survival signaling pathways; during the delayed therapeutic window lasting for the first few days post stroke, RIC might promote synaptogenesis, angiogenesis, and neurogenesis (24). In our trial, RIC was started as soon as the patient arrived at the hospital and lasted for the first 5 days, during the intermediate and delayed therapeutic time window, acting during the post stroke reperfusion.

The primary outcome measure in our study was radiological, showing the difference in brain infarct volume between baseline and 6 months in both the experimental and control group. In the STAIR Group 2016 (Stroke Treatment Academic Industry Roundtable), the final infarct volume on brain imaging has been designated as a biomarker of early efficacy of a new treatment, being linked to the clinical outcome (25, 26). Furthermore, this outcome has been confirmed as a strong independent predictor of functional outcome in patients with ischemic stroke (27). In our study, the difference in infarct volume between baseline and 6 months was slightly larger in the sham group; however, this must be interpreted with caution considering the small number of patients enrolled and the larger infarct volume at baseline (days 3–4) in this group. Moreover, the smaller infarct size in the RIC group at baseline (days 3–4, after initiating RIC) might have been already influenced by RIC.

The clinical outcome measure was investigated as a second target using multiple validated scales: NIHSS, mRS, Barthel Index, IADL, ADL. ADLs refer to the most basic functions of living (eating, bathing, dressing, moving). All these reflect the quality of life of a stroke survivor. Impaired scores are associated with poorer medical health and increasing medical costs. Our results showed improvement in the disability scores at 6 months in the RIC group as compared to the sham group, but none of these findings reached statistical significance. Although the trends appear in favor of RIC, a larger efficacy trial is needed to certify this. In this regard, we draw attention to the larger ongoing phase III trials such as RESIST (NCT03481777), RECAST-3 (ISRCTN63231313), and REMOTE-CAT (NCT03375762) (28–30).

Montreal Cognitive Assessment is one of the most commonly used tests for cognitive status, with high sensitivity for detection of post stroke cognitive impairment. Our study found that MoCA was slightly higher in the RIC group, suggesting that RIC might improve cognitive function after stroke; however, this finding was not statistically significant. These results are consistent with those of a recent study conducted by Feng et al., which included 104 patients and demonstrated that RIC promotes improvement in cognition post stroke (31).

Post stroke depression is a common complication of stroke survivors. It is generally underdiagnosed and undertreated. Nevertheless, in our study, the depression PHQ-9 test was similar

in the two groups (median difference score for PHQ-9 was 0 for both groups).

Our study has both strengths and limitations. The design of the study (i.e., double-blinded, randomized controlled trial) is a valuable asset. All patients had an AIS confirmed on CT scan, with follow-up infarct volume at 6 months as the primary outcome. By contrast, previous studies assessed the brain infarct volume on MRI (6–9). All our patients underwent the whole protocol of RIC, receiving all the cycles of cuff inflation. According to STAIR and STIR recommendations (18, 19), we evaluated the effects of RIC on the long clinical term (NIHSS, mRS) and the final infarct volume at 6 months. One of the most important limitations of the study resides in its unicentric enrollment, with a small sample size that possibly undermines the results. When baseline CT was performed, RIC might have already influenced the infarct size, possibly modifying the measure of infarct growth. We applied RIC manually although an automatic device would be more suitable for better completion of conditioning cycles and to document the treatment compliance. Moreover, we did not record the level of activity and rehabilitation after discharge and until follow-up, which might influence the functionality at 180-days and act as a confounding factor for the outcome. Another possible confounder is the sedentarism which was more frequent in the sham group at baseline.

CONCLUSION AND FUTURE DIRECTIONS

Remote ischemic conditioning is a promising therapy, inexpensive, well-tolerated, supposedly neuroprotective. It might show good results in terms of functionality, and it could be easily implemented as a routine procedure in pre-hospital transport, emergency room, hospitals, or even intensive care units. Further randomized controlled trials with a larger sample size and optimized RIC protocols are required in order to establish and certify the effects of RIC in AIS. Another focal point should be the location of RIC delivering and/or brain lesion, which might influence the outcome of RIC in AIS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Emergency Clinical Hospital Bucharest. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BP is the coordinating investigator and scientific supervisor of the study. AP realized the study design, data collection, and manuscript writing. ET analyzed the CT scans. DT performed the statistical analysis and manuscript writing. DS did the editing assistance. All authors contributed to the article and approved the submitted version.

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REVIEW
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Emerging Role of microRNAs in Stroke Protection Elicited by Remote Postconditioning

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Remote ischemic conditioning (RIC) represents an innovative and attractive neuroprotective approach in brain ischemia. The purpose of this intervention is to activate endogenous tolerance mechanisms by inflicting a subliminal ischemia injury to the limbs, or to another "remote" region, leading to a protective systemic response against ischemic brain injury. Among the multiple candidates that have been proposed as putative mediators of the protective effect generated by the subthreshold peripheral ischemic insult, it has been hypothesized that microRNAs may play a vital role in the infarct-sparing effect of RIC. The effect of miRNAs can be exploited at different levels: (1) as transducers of protective messages to the brain or (2) as effectors of brain protection. The purpose of the present review is to summarize the most recent evidence supporting the involvement of microRNAs in brain protection elicited by remote conditioning, highlighting potential and pitfalls in their exploitation as diagnostic and therapeutic tools. The understanding of these processes could help provide light on the molecular pathways involved in brain protection for the future development of miRNA-based theranostic agents in stroke.

Keywords: microRNA, stroke, remote conditioning, tolerance, preconditioning

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INTRODUCTION

Ischemic conditioning is a neuroprotective approach able to make the brain more resistant to an ischemic insult through the exposure to a subthreshold stimulus. This method provides neuroprotection when the conditioning stimulus is administered either before or after the detrimental ischemia, i.e., preconditioning or postconditioning.

Indeed, ischemic preconditioning is an endogenous defensive process triggered by a subclinical ischemic event that increases tissue resilience or, in other words, organ resistance to a subsequent, typically dangerous, ischemia episode. Non-ischemic conditioning cues can also promote neuroprotection against an ischemic insult, a phenomenon known as "cross-protection"(1). Surprisingly, when the subliminal boost is delivered after the ischemia insult, the neuroprotection achieved, referred to as postconditioning, is comparable to that shown in ischemic preconditioning models. Remarkably, combining preconditioning and postconditioning does not result in greater protection than either treatment alone (2).

We define preconditioning, perconditioning, and postconditioning from a strictly temporal standpoint, depending on whether the conditioning stimulus is delivered before, during, or after the detrimental ischemia (**Figure 1**).

It is now well recognized that stressing preconditioning or postconditioning stimuli elicit multiple endogenous defensive mechanisms in the brain, resulting in a latent protective phenotype. When the lethal ischemic insult is delivered inside this inactive protective phenotype, a partitioned set of reactions are triggered that are strikingly different from the phenotype of the unprimed or non-preconditioned brain, resulting in the so-called ischemia-tolerant phenotype (3).

Surprisingly enough, in the last years we and others produced data supporting the idea that preconditioning and postconditioning exert their effects also when applied to an anatomical site distant from the brain (4–8). In fact, remote ischemic conditioning (RIC) represents an innovative and attractive protective approach in brain ischemia. This method is designed to elicit the initiation of endogenous tolerance processes by providing a not-dangerous ischemic event in a distant tissue, i.e., arms or limbs, leading to a protective systemic response against stroke. Several studies have examined the effectiveness of RIC in reducing the effects of ischemic brain injury, as well as the potential pathophysiological pathways involved (4–8).

Among the multiple candidates that have been proposed as putative mediators of the protective effect generated by the subthreshold peripheral ischemic insult, it has been hypothesized that also in the case of remote conditioning, as it occurs in the case of direct conditioning (9–13), miRNAs may play a vital role in the infarct sparing effect (5, 7).

The effect of miRNAs during RIC can be exploited at different levels: (a) as transducers of protective messages to the brain or (b) as effectors of brain protection.

This review will summarize the most recent evidence supporting the involvement of microRNAs in the protection of the brain caused by remote conditioning, highlighting the potential and pitfalls in their exploitation as diagnostic and therapeutic tools.

microRNAs AS THERAPEUTIC AND DIAGNOSTIC TOOLS

For more than a century, the central view that has dominated molecular biology has been that protein production is mediated by the DNA–RNA–protein axis, which only involves transcription and translation mechanisms to enable decoding of the human genome for production of specific proteins. However, over the recent years, the discovery of non-coding RNAs has radically revolutionized this theory, defining new mechanisms involved in the modulation of protein expression. The human genome project discovered that protein-coding sequences make up only 1.5% of the genome, while introns, regulatory DNA sequences, interspersed elements, and non-coding RNA (ncRNA) molecules make up the remaining 98.5% (14). Without a doubt, the majority of mammalian genomes are translated into ncRNAs, many of which are spliced or processed into smaller products. Short ncRNAs and long ncRNAs are the two types of ncRNAs that have been identified and characterized so far. MicroRNA (miRNA), small interference RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), PIWI-interacting RNA (pi-RNA), transfer RNA (tRNA), circular miRNA (circRNA), ribosomal RNA (rRNA), and other uncharacterized tiny particles are among the short ncRNAs. As of now, miRNAs are characterized as RNA molecules of 18–24 nucleotides in length, transcribed from genes or from intronic regions of other genes, and may promote mRNA degradation or restrict protein translation to control gene expression. More than 60% of mammalian mRNAs are thought to be targeted by at least one miRNA, according to computational predictions (9, 15–19).

Later advances in the topic of miRNAs and their relationship to human diseases have revealed that miRNAs are useful biomarkers and possible disease-modifying agents (20). MiRNA expression profiles exhibit significant changes in response to disease, suggesting that miRNAs are important controllers of disease-related pathways (20).

MiRNAs are considered "downregulators" of gene expression by means of two primary mechanisms: (1) cleavage of mRNA and (2) repression of mRNA translation. MiRNAs interact with mRNA targets through partial sequence complementation, regularly inside the 3′ untranslated region of the mRNA target. In specific, nucleotides 2–7 of the miRNA (beginning from the 5′ end), named the "seed" sequence, are imperative for target binding. The extent of base pairing between miRNA and its target mRNA is currently thought to determine whether the mRNA is degraded or translationally repressed (21, 22).

miRNAs are differentially expressed among tissues, between male and female, during different developmental stages or in response to specific physiological or pathophysiological conditions (17, 23–25). Examples include miR-122, which is preferentially expressed in the liver (24), miR-133a and miR-133b, which are highly enriched in muscle (25), and the miR-302 family members which are specifically expressed in stem cells (26).

microRNAs as Therapeutic Tools

The development of precise and fast assays for miRNA target identification has played a considerable impact in the study of miRNA roles and in the characterization of the biologic activities in which they are implicated. Since the discovery of the potentialities of miRNAs as therapeutic agents, several effective algorithms have been developed for the prediction of miRNA targets (27–32).

There are various extending endeavors to create therapeutics that straightforwardly can target miRNAs, and agreeing to the sort of miRNA, its expression, and its work, diverse approaches are utilized to overexpress or restrain miRNAs (33).

A miRNA mimic is applied to replace the miRNA concentration, eventually reduced in the progression of a pathologic state. This approach was created in 2007 as gain-of-function instrument for particular miRNAs and comprises engineered double-stranded RNAs that are specifically recognized by RNA-induced silencing complex (RISC) (34). These miRNA-like RNA fragments have a 5′ end that gets a sequence with partial complementarity to the 3′UTR of the target genes, in this way imitating the miRNA working mode. This innovation has been further developed by pharmaceutical companies, and a library of miRNA mimics is accessible for

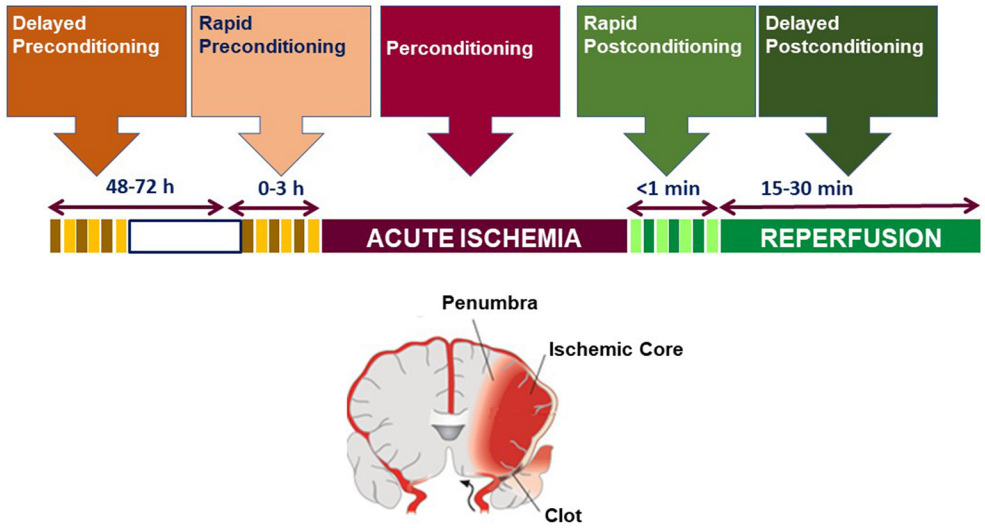

FIGURE 1 | Temporal profile classification of brain conditioning.

all human miRNAs found until presently. In addition, these particles experience modifications in backbone and ribose to advance steadiness *in vivo* and to overcome impediments related to pharmacodynamics. Over the years, other important changes have been made utilizing adeno-associated infection (AAV) vectors and tissue-specific promoters to improve the tissue specificity impact of miRNA mimics (35).

On the other hand, blocking a miRNA may represent a therapeutic option in some pathological conditions characterized by high levels of expression of those miRNAs involved in deleterious pathways. Anti-miRNAs are nucleotide sequences able to bind to the mature miRNA guide strand, causing its inhibition (20). Several methods are used to add modifications with the intent to generate stable and deliverable miRNA inhibitors, creating a class of antagonists known as antagomirs. Among these chemical modifications are included cholesterol conjugation and the use of locked nucleic acid (LNA) (36). LNA modification increases the stability and nuclease resistance of antisense oligonucleotides and, more importantly, improves the efficiency of hybridization to single-stranded RNA.

Chemically modifying the miRNA using either LNAs or the 2′-O-methyl group (OME) can increase stability *in vivo* and is the basis for many antagomir therapies. Antagomirs are the most frequently used approach for therapeutic use of miRNA therapy (37).

An evolution of anti-miRNAs is represented by miRNA sponges, which are able to inhibit multiple miRNAs simultaneously (38). These molecules can contain a seed sequence for an entire miRNA family, however, they could be also used to target multiple miRNAs.

Finally, the usage of miR-Masks is an emerging strategy for altering miRNA activity (39). These masks are single-stranded 2′-O-methyl-modified antisense oligonucleotides that recognize the miRNA target site on the mRNA 3′UTR and hide it.

miRNAs as Diagnostic Tools

Beside their expression within tissues and organs, miRNAs are also present outside the cells (40). Several release mechanisms have been hypothesized including release through microvesicles, resulting from outward budding and plasma membrane separation (41). In particular, some vesicles called exosomes, typified by a specific process of biogenesis, are strongly involved in intercellular communication processes and are characterized by the presence of massive amounts of miRNAs inside them (42). Other miRNA transport systems in biological fluids include high-density lipoproteins (HDL) and apoptotic bodies, generated during the programmed cell death process.

Whatever the origins of miRNA, their presence in the blood and the ability to quantify their levels non-invasively cleared the path for the development of peripheral biomarkers for the diagnosis and prognosis of disorders such as brain ischemia. Undoubtedly, miRNA expression levels within the blood are reproducible and demonstrative of different pathologies (43). The interest in peripheral miRNA and their potential application as a biomarker for rapid diagnosis and prognosis are particularly relevant for ischemic patients (44, 45). In fact, several patient-based studies have already reported the occurrence of significant variations in the circulatory expression of miRNAs during cerebral ischemia, correlatable to the entity of the damage (46).

TRANSLATIONAL RELEVANCE OF miRNAS IN REMOTE CONDITIONING NEUROPROTECTION

Over the years, numerous studies demonstrated that stroke may trigger a re-arrangement of the miRNA profile within the cerebral tissue of animals exposed to brain ischemia (47, 48). The first miRNA expression profiling study in cerebral ischemia was performed in 2008, on the entire brains of rats subjected to

middle cerebral artery occlusion (MCAO) with reperfusion for 24 or 48 h (49). In this study, it has been shown that 106 transcripts were altered 24 h after stroke induction. However, the number of altered miRNAs was reduced to 82 at 48 h, thus indicating that miRNAs were differentially expressed after brain ischemia in a time-dependent manner. In fact, in the 48-h samples, only rno-miR-99a,−181 (a, b, and c),−195,−328,−379, and−539 were found to be exclusively expressed, whereas, in the 24-h samples, 32 miRNAs (rno-miR-16,−17,−20a,−21,−24,−25,−30a-3p,−34a,−92,−124a,−130a,−132,−134,−151*,−210,−215, −324-3p,−322,−329,−342,−361,−374,−382,−383,−422b, −433,−451,−497,−505,−664, let-7d, and let-7f) were found to be exclusively expressed. Notably, rno-miR-206,−214,−223,−290,−292-5p,−298,−327, and−494 were highly upregulated during both ischemia/reperfusion time intervals (49).

These pioneering data were partially confirmed later on in another miRNA profile study carried out in spontaneously hypertensive rats exposed to focal ischemia and reperfusion of different duration (50). In the brain samples obtained from these ischemic animals, it has been found that among the 238 miRNAs examined, 24 miRNAs were upregulated while 22 miRNAs were downregulated at one or more reperfusion time intervals. These data were obtained in the ipsilateral cortex of the ischemic core. Little changes in miRNA expression were detected in the contralateral cortex and in the peri-ischemic cortex. Interestingly, in this study the upregulation of the same five miRNAs identified in the previous work by Jeyaseelan et al., emerged (49), showing the highest upregulation degree at 72 h for miR-206,−214,−223,−290, and−292-5p (49).

Once having established that brain ischemia determines a variation in miRNA expression, the following step was on one side to identify possible miRNAs useful as biomarkers and on another side to identify putative miRNA targets involved in stroke pathophysiology, in order to modulate their expression through miRNAs and to rescue the so-called penumbra region, an area adjacent to the ischemic core, compromised by the ischemic event but not completely damaged.

In this scenario, particular attention was focused to the possibility to restore ionic homeostasis in the penumbra region by using miRNAs to modulate ionic channels and transporters, since the tight correlation between brain damage due to brain ischemia and the disruption of ionic homeostasis is well known (8, 51–53). The plasma membrane sodium/calcium exchanger (NCX1), whose expression is controlled by miR-103 and whose activation ameliorates ischemic damage (51–54), appeared as one of the most promising candidates. Indeed, the capability of AntimiR-103 of inducing a brain-conditioning phenomenon in a rat model of transient brain ischemia has been evidenced (55). The mechanism of action of this LNA anti-miRNA consisted in blocking the detrimental increase of miR-103-1 responsible for the downregulation of NCX1, whose expression is necessary to counteract sodium and calcium imbalance occurring during stroke progression.

Interestingly, more recently, another miRNA, miR-223-5p, emerged as a possible modulator of the K+-dependent Na+/Ca2+ exchanger, NCKX2, a new promising stroke neuroprotective target. In fact, intracerebroventricular infusion of anti-miR-223-5p prevented NCKX2 downregulation occurring after ischemia in rats, thus promoting neuroprotection. Therefore, blocking miR-223-5p by anti-miRNA is a reasonable strategy to reduce the neurodetrimental effect induced by NCKX2 downregulation occurring during brain ischemia (56).

The translatability of miRNAs as biomarkers derives from their physical characteristics. Indeed, as anticipated above, miRNAs are found in a remarkably stable state in human plasma or serum and could be used as biomarkers for a variety of disorders. Chen et al. demonstrated that plasma miRNAs are resistant to RNases as well as other harsh settings such as low/high pH, long-term storage, boiling, and repeated freezing/thawing cycles (57). The levels of miRNA expression in blood have been proven to be repeatable and predictive of illness condition. Although the mechanism of miRNA release into the circulation is unknown, their presence in the bloodstream and relationship with a variety of pathophysiological conditions is now widely established (58) and miRNAs produced by injured or circulating cells are thought to cause enhanced miRNA expression in peripheral biofluids (59).

Many studies demonstrated that this stability is related to the different carriers that mediate miRNA transport, including microvesicles, exosomes, apoptotic bodies, AGO protein complexes to form ribonucleoprotein, and HDL (60, 61). Exosomes, according to current thinking, can regulate the bioactivities of recipient cells by transporting lipids, proteins, and nucleic acids like miRNAs while circulating in the extracellular space, and several studies have shown that exosomes play important roles in immune response, tumor progression, and neurodegenerative disorders.

Taking into account all these considerations and in the attempt to find peripheral markers for stroke, several studies have been conducted to examine expression changes in circulating miRNAs following brain ischemia, at preclinical and clinical levels (10).

Contrasting results have been produced when the expression profiles of miRNAs in the brain were compared to those measured in the blood. In fact, some miRNAs such as miR-290 and miR-494 showed change in their expression in the same direction (upregulation or downregulation) in both tissues at 24 and 48 h, whereas expression levels of some other miRNAs, like miR-150,−195, and−320, exhibited an inverse trend (49). Once again, it should be underlined that brain tissue after ischemic stroke changes over the time, and the per-ischemic region is included in the ischemic core in few hours. miRNA expression is surely influenced by the stage of the disease and the distance from the ischemic core.

Distinct miRNA patterns indicative of the stroke outcome have been found in blood samples of chronic stroke patients within 6–18 months from the stroke onset (62). Interestingly, several miRNAs have shown changes during disease progression. Notably, the number of miRNAs downregulated in all good-outcome stroke samples was usually higher than that of upregulated ones. However, miRNA expression profiles exhibited differential fold change values among the different stroke subtypes; for instance, patients affected by small-artery (SA) stroke show a distinctly different pattern from that

of patients affected by large-artery (LA) stroke. In specific, among the highly upregulated miRNAs measured in samples derived from patients affected by SA stroke, seven miRNAs, miR-130b,−29b,−301a,−339-5p,−532-5p,−634, and 886-5p, changed more than two-fold (62).

Recently, miR-107, miR-128b, and miR-153, three brain-enriched miRNAs, were identified within 24 h from hospital admission in plasma samples of patients affected by brain ischemia, demonstrating that their levels were upregulated after stroke onset and positively correlated with the severity of cerebral ischemic injury (63). Interestingly, no correlation was found between age and smoke with the levels of miRNAs, thus suggesting that this upregulation was mediated only by ischemic insult. In parallel, a further study examined the levels of two atherosclerosis-related miRNAs, miR-185, and−146a, in plasma of ischemic stroke patients in the acute phase, 1–5 days, or subacute phase, 6–30 days (10, 37). MiR-185 was found to be downregulated in both the acute and subacute phases, whereas miR-146a was found to be downregulated in the acute phase but upregulated in the subacute phase. Finally, eight miRNAs were found to be differentially expressed in blood collected 28 h after stroke onset (64). In fact, miR-122, miR-148a, let-7i, miR-19a, miR-320d, and miR-4429 were reduced, while miR-363 and miR-487b were upregulated in the bloodstream of acute ischemic patients. These miRNAs were predicted to be regulators of a number of genes and pathways linked to brain ischemia and involved in immune activation, leukocyte extravasation, and thrombus formation.

In the light of these premises and considering the versatility of miRNAs, the identification of pivotal miRNAs may represent a crucial step in defining new putative theranostic tools in stroke. Therefore, the research in the field has been attracted to the possibility of selecting peculiar miRNAs involved in endogenous neuroprotective phenomena including preconditioning and postconditioning.

The idea that ischemic preconditioning (IP) reprograms the response to ischemic injury and determines an altered expression of genes and proteins is well known and commonly accepted (3). This evidence drives researchers to examine whether cerebral ischemic preconditioning might be related to changes within the expression of miRNAs in brain tissue (9, 12, 13, 48). In specific, the impact of IP on miRNA expression profiles was basically assessed by three experimental works. In the first work, only eight miRNAs out of 360 analyzed were selectively upregulated in the brain of rats 3 h after IP (11). These miRNAs were categorized into the following groups: miR-200 family, including miR-200a,−200b,−200c,−429, and−141, and miR-182 family, including miR-182,−183, and−96. Notably, the increased expression of miR-200b,−200c, and−429 could explain the protective upregulation of HIF1α observed in the brain of rats subjected to IP. Indeed, these miRNAs target prolyl hydroxylase two gene (PHD2), an enzyme involved in HIF1α catabolism (11).

In another study, the effect of IP-only ischemia and post-IP tolerance to ischemia on brain miRNA expression profiles was examined; differences in terms of miRNA expression between male and female ischemic mice were also reported (17). In particular, a large subset of miRNAs was uniquely dysregulated in the IP group, including members of the miR-200 and−182 families, which were overexpressed. In addition, *in silico* prediction analysis allowed the identification of target mRNAs, dysregulated following ischemic tolerance. Notably, the predicted target methyl-CpG-binding protein 2 (MeCP2), which is a global regulator of transcription, appeared of particular interest in the development of the ischemic damage, and further confirmatory analysis demonstrated the relationship between miR-132 and MecCP2. Indeed, the overexpression of MeCP2 observed by immunohistochemical staining in brain tissue during ischemic tolerance was accompanied by the reduced expression of miR-132 (16, 17).

MeCP2 emerged as an important miRNA target also in another study carried out in spontaneously hypertensive rats exposed to ischemic preconditioning (50). Among the 265 miRNA screened, only the expression of 20 miRNAs was exclusively modified by preconditioning. In particular, 11 miRNAs were overexpressed, and nine miRNAs showed a reduced expression (16). Beside MeCP2, other major pathways targeted by stroke-regulated miRNAs and participating in cell regulation, proliferation, and apoptosis comprised MAP-kinase, mTOR, Wnt, and GnRh (50).

Few information is available on the role played by miRNAs in remote ischemic limb post-conditioning (RLIP), and studies in this field are in its infancy as testified by the scarcity of published papers. However, since this neuroprotection strategy presupposes a cross talk between the periphery and the central nervous system, it is possible to hypothesize that miRNAs play a role in this process of cell-to-cell communication.

Indeed, RLIP determines a marked neuroprotection by a not dangerous occlusion of the femoral artery (5). Although this method is being evaluated in clinical trials all over the world, the mechanisms activated by RLIP and implicated in the protection have yet to be fully understood (5, 65–67).

Seeing enormous potential, several research groups have focused their efforts on evidence that a short blockage of a distant artery, such as the femoral artery, can protect the brain against ischemic insults, a process known as RIC (4, 5, 65, 68–71).

In a clinical trial published in 2010, this procedure has led to excellent results in patients affected by myocardial infarction, when performed prior to percutaneous coronary intervention (72, 73).

Independently from tissue or organ affected by the harmful event, the mechanisms underlying the phenomenon of RLIP are classified in three steps:

1. The first events occurring in the remote organ or tissue, generated by the RIC stimulus (71). Indeed, blockage of the arm with a tourniquet or blood pressure cuff can trigger the release of autacoids like adenosine, bradykinin, and calcitonin gene-related peptide, or likely of miRNAs, which protect the target organ or tissue (5, 65).
2. The type of the protection signal that is sent from a distant organ to a target organ. Neural and humoral mechanisms have been postulated as means to transport the peripheral signal from an organ such as a leg to a distant organ such as the brain or heart to explain how this phenomenon works (5, 65).

3. The event that takes place in the target organ and mediates the protective response (69).

Since miRNAs represent good candidates involved in all three phases above described, a putative translational strategy to induce stroke neuroprotection could consist in identifying miRNAs involved in these processes and in modulating these identified miRNAs by using either miRNA mimics or miRNA blockers, anti-miRNAs (9, 18, 19). In this regard, the use of miRNA mimics or anti-miRNAs appears to be a very promising method, given that a single miRNA can regulate the expression of multiple proteins at the same time and that cerebral ischemia is a multifactorial disorder with multiple potential therapeutic targets (9, 16, 18, 19).

Recently, microarray microfluidic analysis of 810 miRNAs in the ischemic rat brain revealed that let-7a-5p, miR-143-3p, miR-451-5p, and miR-485-3p had more significant expression alterations than the others. Their levels did increase significantly 24 h after ischemia induction, but when damaging ischemia was followed by remote ischemic postconditioning treatment, they were practically back to pre-ischemic levels (7). Further, more targeted investigations revealed that only let-7a and miR-143, out of the four miRNAs found and selected, would represent an important tool for stroke intervention. These findings are in line with earlier expression data and functional trials, as well as research on putative miRNA targets (49, 50). Indeed, knocking out the let-7a gene can protect against cerebral ischemia/reperfusion injury by reducing apoptosis and inflammatory reaction markers, as evidenced by a decrease in the number of p-p38 MAPK- and p-JNK-immunoreactive cells, as well as lower levels of TNF-α and IL-6 after treatment with a let-7a inhibitor (74). Let-7a has also been shown to target mitogen-activated protein kinase phosphatase 1 (MKP-1), which inactivates JNK1/2 and p38, suggesting a role for this miRNA during neuroinflammation and apoptosis (74).

Many membrane proteins involved in transduction pathways and solute transporters from different families are identified as probable targets of miR-143-3p (75). As a result, its function appears to be linked to cell responses to external stimuli. Recently, miR-143 has been found to play a role in increasing mitochondrial damage in myocardial ischemia *via* targeting PKCε (75). The activation of PKCε in cardiac myocytes is related to the opening of KATP channels. The latter occurs by preventing the opening of the mitochondrial permeability transition pore (mPTP) (75, 76). Notably, it has been demonstrated that a reduction of focal cerebral ischemic injury induced by delayed remote postconditioning may be achieved also through opening of KATP channels (77). Taken together, these data suggest that reduction of miR-143 expression in the brain after tMCAO plus RLIP might prevent PKCε downregulation, with subsequent opening of KATP channels, thus promoting mPTP closure. Although the action of these two miRNAs occurs at the neuronal level, miRNA-143 expression was also seen in the bodies of astrocytic GFAP-positive cells, validating the idea that astrocytes and neurons show different miRNA expression patterns after ischemia (78). Other investigations have corroborated the distinctive expression of miRNA in neurons and glial cells, and numerous hypotheses have been proposed to explain this phenomenon (78, 79). As a result, after remote postconditioning, astrocytes and neurons express different miRNAs; this could be due to differences not only in their miRNA repertoires but also in their cell-specific roles within the CNS.

It is worth noting that let-7a and miR-143 mimic therapy significantly reduces the neuroprotection afforded by RLIP. As a result, these two miRNAs and the proteins they target appear to be potential RLIP-induced neuroprotection mediators. More research is needed to understand which of the target proteins is involved in remote postconditioning-induced neuroprotection. As a result, altering the levels of expression of these miRNAs and their target proteins appears to be a promising stroke therapeutic strategy.

EXPLOITATION OF miRNAS AS STROKE THERANOSTIC TOOLS: PITFALLS AND CLINICAL PERSPECTIVES

The incessant work of recent years in studying the role of ncRNAs in stroke made important contributions that advance both the so-called miRNA revolution and our knowledge of the possibilities of exploitation of miRNA as theranostic tools in stroke.

A list of miRNAs responsible for the infarct-sparing effect of brain conditioning is now available (7, 9–13, 17). Furthermore, it is now clear that delivery of a class of the miRNA homologs may represent a novel avenue in therapy, and the changes in miRNA plasma concentrations could be used as a biomarkers. However, the preliminary findings raise significant concerns and debates about their applicability in clinical practice.

To begin, it is critical to note that all conclusions are derived from preclinical models. Several interventions that had been shown to enhance outcomes in experimental animal stroke models, however, failed in clinical studies. Systematic assessments of experimental stroke studies have consistently found low-quality scores, unfavorable publication bias, and a lack of data from female, elderly, or comorbid animals, casting doubt on the robustness and predictive utility of single-laboratory preclinical investigations. The new idea of a multicenter preclinical randomized controlled trial (pRCT) is developing as a critical step before transitioning from animal modeling to clinical trial to improve the translation of therapy efficacy from bench to bedside. Notably, we have recently begun a multicenter preclinical study in rats and mice of both sexes to explore the efficacy of RIC in the experimental model of temporary middle cerebral artery (MCA) occlusion (trial registration number PCTE0000177).

An additional and arguably tangential issue that deserves investigation is the assessment that the majority of papers published in this field did not associate the described favorable effects with transport *via* exosomes, a finding that appears to contradict the emerging concept that extracellular vesicles serve as vectors for the RIPC-initiated humoral communication of protective signals to the brain.

The cellular source of the circulating miRNAs released in response to the RIPC stimulation is a third question that will be

difficult to answer: are miRNAs released from skeletal muscle or from a more conventional, blood-borne source?

Fourth is the importance of defining a temporal profile of expression. miRNAs are released in response to a stimulus in a time-related manner. Defining a precise timing of determination after stroke onset is fundamental in the case miRNAs are used as biomarkers, but is also extremely important when miRNA mimics are used as therapeutic tools.

In the vast majority of preclinical studies, MCAO was used to confirm alterations in specific miRNAs in young healthy male rats or mice. Furthermore, the majority of clinical stroke studies did not specify the number of male and female patients who participated in the trials, making it difficult to discuss the importance of gender-specific changes in miRNAs and their contributions to ischemic stroke and/or responses to antagomirs or mimics. In future investigations, men and females animals must be used to confirm the upregulation or downregulation of particular miRNAs. Finally, because most stroke patients have comorbidities and are over the age of 50, animal stroke models with hypertension, hyperlipidemia, and diabetes mellitus, as well as older animals, should be included in confirmation investigations.

Lastly, it will be extremely important to define a delivery system. A successful miRNA therapy necessitates a precise and effective delivery method. To address the intrinsic instability of miRNA in circulation, off-target effects, and improper distribution, a delivery method is required, but it does not need to reduce cell permeability, increase excretion, or accumulate in off-target organs (10). MiRNAs can be injected intravenously or subcutaneously because they are small and water-soluble. Because miRNAs are single-stranded and open-ended, they are either destroyed by systemic nucleases in circulation or eliminated by the kidneys (56).

Accumulation in non-target organs such as liver and spleen, non-specific absorption, excretion, toxicity, and an immune-mediated response are among issues that might arise with various delivery systems (56). Systemic delivery must consider the delivery system's stability in circulation as well as tissue-specific targeting. Some options for systemic delivery have been developed through ongoing research. In this regard, the viral capsid can be modified for tissue-specific delivery, and viral vectors can be used to boost circulatory stability during translocation (56). Adeno-associated viruses have exhibited great tissue specificity and acceptable safety profiles in clinical trials for gene therapy (37). Viral vectors, however, do have pitfalls including immune reactions and viral integration into the host genome (56). Other lipid-based vectors, like liposomes, are protective against nuclease, lysosomal, and endosomal degradation and can be effectively used (40). As natural transporters, exosome delivery systems are an attractive option. These offer specificity by binding in a receptor-mediated fashion, limiting off-target side effects (59). Nanoparticles are another possible strategy and have been used in an attempt to overcome excessive inflammatory reactions (8). An intriguing concept for drug delivery for IR injury is "passive drug targeting" where a colloid-based drug delivery system is utilized. Because the endothelium at these places is weakened, these chemicals collect at sites of inflammation, allowing passive diffusion through the artery (40). Another option for systemic administration is mesenchymal stem cell-derived extracellular vesicles (MSC-EV), which have been investigated *in vivo* and found to be able to transport miRNAs (52). MSC-EV, like the other delivery systems, are currently being studied for clinical usage, with promising results.

CONCLUSIONS

Efforts will be needed in the next decade of miRNA research to enhance and evolve the tools for miRNA analysis and validation, as these technologies will be critical in establishing direct correlations between miRNA-mediated post-transcriptional gene expression and disease (9, 19). Furthermore, it is important to underline the need to speed up all those technical procedures capable of detecting miRNAs in the shortest and simplest possible way, to avoid running into the same problems currently present with the use of CT and MRI to make differentiated diagnosis of ischemic and hemorrhagic stroke.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Remote Ischemic Conditioning in Ischemic Stroke and Myocardial Infarction: Similarities and Differences

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Acute myocardial infarction and ischemic stroke are leading causes of morbidity and mortality worldwide. Although reperfusion therapies have greatly improved the outcomes of patients with these conditions, many patients die or are severely disabled despite complete reperfusion. It is therefore important to identify interventions that can prevent progression to ischemic necrosis and limit ischemia-reperfusion injury. A possible strategy is ischemic conditioning, which consists of inducing ischemia – either in the ischemic organ or in another body site [i.e., remote ischemic conditioning (RIC), e.g., by inflating a cuff around the patient's arm or leg]. The effects of ischemic conditioning have been studied, alone or in combination with revascularization techniques. Based on the timing (before, during, or after ischemia), RIC is classified as pre-, per-/peri-, or post-conditioning, respectively. In this review, we first highlight some pathophysiological and clinical similarities and differences between cardiac and cerebral ischemia. We report evidence that RIC reduces circulating biomarkers of myocardial necrosis, infarct size, and edema, although this effect appears not to translate into a better prognosis. We then review cutting-edge applications of RIC for the treatment of ischemic stroke. We also highlight that, although RIC is a safe procedure that can easily be implemented in hospital and pre-hospital settings, its efficacy in patients with ischemic stroke remains to be proven. We then discuss possible methodological issues of previous studies. We finish by highlighting some perspectives for future research, aimed at increasing the efficacy of ischemic conditioning for improving tissue protection and clinical outcomes, and stratifying myocardial infarction and brain ischemia patients to enhance treatment feasibility.

Keywords: ischemic stroke, remote ischemic conditioning (RIC), neuroprotection, cardiac protection, myocardial infarction

INTRODUCTION

Myocardial infarction (MI) and ischemic stroke are leading causes of morbidity and mortality (1, 2). Both conditions have an acute onset and are due to blood vessel occlusion leading to a certain extent of ischemic necrosis.

MI usually follows thrombotic occlusion of a coronary artery due to a vulnerable plaque rupture. Ischemia-dependent mitochondrial and metabolic alterations lead to systolic function depression and, when persistent, to cardiomyocyte necrosis followed by tissue scarring (3). Similarly, ischemic stroke results from a lack of blood flow to the brain, which reduces oxygen, glucose and nutrient supply, as well as, secondarily, catabolite removal. Blood deprivation is typically caused by large artery atherosclerosis, cardiac embolic events, small vessel occlusion, or stroke of other etiologies (4).

In cardiac, as in brain ischemia, there is a clear major effect of early restoration of blood flow through reperfusion therapies on outcomes. These include pharmacologic (i.e., fibrinolytic therapy) or mechanical interventions, namely primary percutaneous coronary intervention (PPCI) or endovascular thrombectomy. More than 90% of MI patients receive reperfusion therapy against ∼10% of acute ischemic stroke patients (5). Among the factors that account for this difference, the different time windows from symptom onset for beneficial reperfusion treatment should be taken in account. These are usually <12 h (or between 12 and 48 h in some patients with persisting symptoms) for fibrinolytic therapy for ST-segment elevation MI (STEMI), 4.5 h (9 h in some patients with radiological signs of salvageable brain tissue) for thrombolysis of brain ischemia, and <24 h for mechanical thrombectomy in brain ischemia. Furthermore, an arterial occlusive thrombus accessible to catheter-based intervention is found in about 90% of MI patient, but only about half of computed tomography (CT) angiograms performed for acute ischemic stroke (5). Indeed, while there are few contraindications to coronary catheter-based interventions, reperfusion therapies for ischemic stroke are absolutely contraindicated if there is intracranial bleeding or advanced ischemia. Another reason possibly accounting for the difference in the percentage of patients that receive reperfusion therapy between ischemic stroke and MI may be that time to treatment is often longer in the former condition (6). Finally, biomarkers of brain ischemia are missing (while troponins are widely used in cardiovascular medicine), and neurological diagnostic methods [CT or magnetic resonance imaging (MRI)] are expensive, time-consuming, and not routinely performed outside hospital or at the bedside (contrary to an electrocardiogram) (5), although technological advances (such as mobile CT or bedside MRI) may change this (7, 8).

Patients with MI or ischemic stroke who receive successful reperfusion therapies are still exposed to certain risks, as reperfusion itself is an important determinant of end-organ damage. Indeed, ischemia triggers a vicious cycle of cell death, inflammation, and oxidative stress, which is perpetuated by reperfusion and may increase the extent of infarction in otherwise viable brain or cardiac tissue (9, 10), also in association with cerebral edema and blood–brain barrier disruption (11). Reperfusion injury is much more common and more often leads to hemorrhagic transformation in brain infarct than MI (5). Intracranial hemorrhage exposes the patient to life-threatening intracranial hypertension, with the risk of brain herniation (5).

The risk of these detrimental effects is usually counterbalanced by the fact that reperfusion therapies can save the border (or marginal) zone of MI or the ischemic penumbra in ischemic stroke, if administered promptly. The border zone (or penumbra in ischemic stroke) is the salvageable tissue around the ischemic core, in which reduced blood flow causes loss of cell function with normal structural morphology, before irreversible damage, which occurs instead in the ischemic core (12, 13). However, the recanalization rate with thrombolysis in brain ischemia is lower than with endovascular thrombectomy (14). Penumbral salvage becomes more likely with endovascular thrombectomy, which considerably improves clinical outcomes. Despite this, only about half of successful thrombectomies lead to patients' functional independence (15), mainly because the ischemic core is already too large at the time of recanalization. This may partially explain why most stroke patients are still disabled 3 months after treatment (15). As for MI patients, despite the fact that timely PPCI is associated with better outcomes than fibrinolysis, a significant number of patients with reperfused STEMI display the no-reflow phenomenon, which predicts a worse outcome, specifically a greater risk of ventricular wall rupture and arrhythmias, adverse ventricular remodeling with heart failure development, and cardiac death (3).

Reducing the burden of cardiac and cerebral ischemia-related death disability requires the identification of interventions able to "freeze the penumbra," i.e., prevent the growth of the necrotic ischemic core until partial or complete reperfusion, as well as techniques to protect the ischemic tissue from subsequent reperfusion injury (12, 13, 16). However, interventions that aim to improve ischemic stroke and MI prognosis have, so far, shown an inconsistent benefit (17–20). Alternative cytoprotective strategies are being studied (21), but strong evidence on the efficacy of any proposed mechanism is lacking.

An interesting paradigm may be ischemic conditioning (22), first described in 1986 by Murry et al. (23) in the setting of experimental MI. In ischemic conditioning, transient, intermittent ischemia without necrosis is induced either in the organ undergoing spontaneous ischemia (i.e., conventional conditioning), or at a distance from the affected organ [i.e., indirect or remote ischemic conditioning (RIC)]. According to the timing of the intervention (before, during, or after ischemia), remote ischemic pre-, per-/peri-, and post-conditioning, respectively, can be defined. Pre-conditioning has been defined as "an adaptive process of endogenous protection in which small, sublethal doses of a harmful agent protect the organism against a later lethal dose of the same agent" (24). In the settings of acute MI and ischemic stroke, per- or post-ischemic conditioning is more easily realized. This can be achieved by the application of an inflatable cuff around the patient's arm or leg (22). After extensive evaluation in the field of cardiac ischemia, the paradigm of RIC has recently been translated to ischemic stroke (20), and seems to apply to other organs and tissues. Plasma dialysate obtained from animals and humans

treated with RIC has been shown to reduce MI size after ligation of a coronary artery and subsequent reperfusion in isolated heart preparations (25, 26), indicating that the effect may be mediated (at least partially) by humoral substances released from the tissues exposed to intermittent ischemia. In animal models, remote ischemic pre- and post-conditioning have been shown to reduce MI size and biomarkers of myocardial necrosis (27–29). Similarly, in rat models of middle cerebral artery occlusion, brain infarct size was reduced by remote pre-conditioning (30).

This review aims to discuss the existing clinical evidence on RIC in brain and myocardial ischemia. We will first of all synthetically recapitulate potential mechanisms of RIC, then discuss main clinical findings in MI, first, and in ischemic stroke, then, highlight the differences and similarities, as well as future perspectives and therapeutic implications.

PUTATIVE MECHANISMS MEDIATING PRE-, PER-, AND POST-RIC EFFECTS IN PRE-CLINICAL MODELS

The exact mechanisms of this remote organ protection from ischemia are unknown and could differ between pre-, per-, and post-conditioning. While it is beyond the scope of this review to detail all putative mechanisms, we will briefly recapitulate the main ones in the paragraphs that follow and in **Figure 1**. For further details on potential cardioprotective mechanisms, please see Aimo et al. (31), while, for further details on putative neuroprotective mechanisms, please see reviews by Basalay et al. and Chen et al. (26, 32). Of note, more mechanisms may be involved at the same time, and they may be more or less important depending on the setting of pre-, per or post-conditioning. Further investigations of the protective mechanisms of RIC are needed to aid a safe and effective translation to clinical practice. Muscle ischemia may release autocoids (e.g. bradykinin, opioids or adenosine), which might enter the systemic circulation (humoral hypothesis), or locally activate somatic nerve afferents (neural hypothesis). Supporting the neural hypothesis, in several preclinical models of brain infarction peripheral nerve block (either pharmacological or by resection) reduced or abolished RIC neuroprotective effects (30). Further, RIC-dependent vagal nerve activation may have an anti-inflammatory effect mediated by the spleen and liver via the cholinergic anti-inflammatory signaling (24). However, while it is logical to think that protective biomarkers that may be generated by RIC are directly available for the cardiac tissue, it is far from clear whether such molecules can cross the blood-brain barrier and reach the cerebral tissue. Most of the pathways activated by RIC are believed to ultimately affect the mitochondria, preventing for example the formation of the mitochondrial permeability transition pore (MPTP) or leading to the cytoprotective nitrosylation of key mitochondrial proteins, such as those forming complex I and complex IV. This could reduce the generation of mitochondrial reactive oxygen species. Finally, it ought to be noted that RIC has been shown to improve cardiac function, which, in turn, is positively correlated with cerebral blood flux, and could therefore represent yet another mediator of RIC-neuroprotective effects (33).

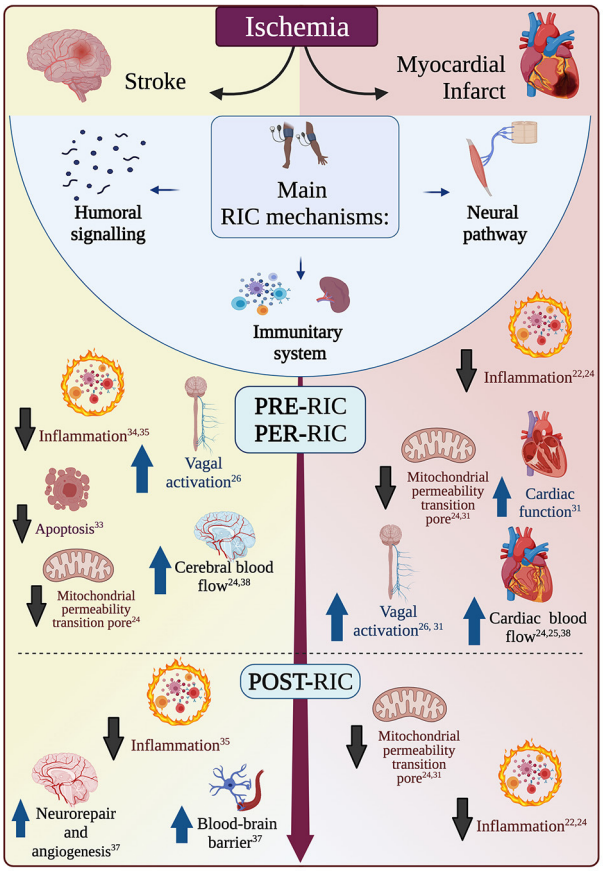

FIGURE 1 | Main mechanisms of remote ischemic conditioning (RIC). RIC can be performed before (pre-RIC), during (per-RIC), or after (post-RIC) an ischemic event (ischemic stroke or myocardial infarction). RIC effects are mostly mediated by humoral signaling, neural pathways, or modulation of systemic immune system. These different pathways generate many effects that have different importance in the setting of myocardial infarction (red, on the right) or ischemic stroke (yellow, on the left), while some effects are likely of similar importance in both conditions (in the white area in the middle). While the same mechanisms may have different importance before, during or after an ischemic event, there is not enough literature to attribute each mechanism to a certain phase only. Upward blue arrows indicated increase and enhancement, while downward black arrows indicate reduction and inhibition.

Putative Mechanisms Mediating Pre- and Per-RIC Effects in Myocardial Infarction

The effects of pre-RIC-induced cardioprotection may be mediated by humoral factors acting through the systemic circulation (e.g., stromal cell-derived factor-1, interleukin-10, microRNA 144, and nitrite, which, for example, may favor vasodilation); by nervous reflexes or neurogenic transmission, through the autonomic fibers activated by, for example, adenosine or bradykinin (26, 31), or, as mentioned, by the effects on circulating immune cells (e.g., inhibition of leucocyte CD11b expression and a reduced number of cardiac macrophages and

neutrophils), reducing inflammation, apoptosis, and oxidative stress (22, 24).

Per-RIC mechanisms are probably similar to pre-RIC ones, comprising humoral and neural autonomic pathways and vagal nerve activation (enhancing the sympathovagal balance), as well as inflammatory modulation (22, 24, 26, 31).

Putative Mechanisms Mediating Post-RIC Effects in Myocardial Infarction

Contrary to pre- and per-RIC mechanisms, post-RIC effects do not seem to be mediated by vagal activation and the aforementioned humoral factors are likely to play a predominant role in reducing inflammation and apoptosis (26, 31).

Putative Mechanisms Mediating Pre- and Per-RIC Effects in Ischemic Stroke

Pre-RIC may have neuro- and cardio- protective effects, reducing the damage of ensuing ischemia. These effects are believed to be mediated mainly by increase of cerebral blood probably mediated by induction of nitric oxide synthase (24) and reduction of inflammation (e.g., inhibition of leucocyte CD11b expression and a reduced number of cardiac macrophages and neutrophils) (22, 24), which is detrimental for ischemic tissue. This anti-inflammatory action may also be mediated by vagal nerve activation triggered by RIC.

In any case, at local levels, pre-RIC-induced neuroprotection has been found to depend on inhibition of inflammatory response and apoptosis in animal models of brain ischemia (34, 35).

Per-RIC effects are likely mediated by an increase of cerebral blood flow (24). Nitric oxide in particular may play an important role in enhancing cerebral blood flow, as it has been shown in animal models of ischemic pre- and per-conditioning (36). As pre-RIC, also per-RIC has been shown in animal models of brain ischemia to reduce ischemia-reperfusion-injury decreasing infarct size, brain edema and neurological deficit scores, through inhibition of pro-inflammatory signals, in particular the TLR4/NF-κ pathway (37).

Putative Mechanisms Mediating Post-RIC Effects in Ischemic Stroke

Post-RIC effects may be, at least in part, mediated by endogenous neuroprotective and neurorepairing responses, such as increased local production of neuronal nitric oxide synthase (38), BDNF or endogenous opioids in the central nervous system, or stimulation of cerebral angiogenesis and inhibition of oxidative stress and inflammatory responses, possibly through vagal nerve activation (39). Speculatively, it might be hypothesized that vagal nerve activation modulates cerebral excitability, and this effect might play a role in improving recovery, but further research is needed on this point. Other potential mechanisms mediating post-RIC neuroprotection involve inhibition of apoptotic signals, alleviation of cerebral edema and enhancement of blood-brain barrier and neurovascular unit integrity (39).

REMOTE ISCHEMIC CONDITIONING IN MYOCARDIAL INFARCTION

Building on the experimental results mentioned in the introduction, several studies have tested RIC in patients with STEMI. In these studies, RIC is delivered together with PPCI. Both peri- and post-ischemic conditioning, generally induced by four 5-min cycles of limb cuff inflation and deflation, were found to reduce the release of creatinine kinase-MB (CK-MB) (41) and high-sensitivity troponin T at different timepoints (42, 43), as well as infarct size (41, 44), microvascular obstruction (44), edema (41), and other markers of myocardial salvage (45). Also, peri-ischemic conditioning (four 5-min cycles of upper arm cuff inflation to 200 mmHg and deflation) associated with thrombolysis for STEMI reduced enzymatic markers of MI (46). In another study of 333 patients with suspected first acute MI undergoing PPCI, peri-ischemic conditioning with four cycles of 5-min inflations and deflations of a blood-pressure cuff had no effect on troponin T release nor infarct size (21). However, peri-ischemic conditioning improved myocardial salvage index, calculated as (area at risk–final infarct size)/area at risk (21). A randomized controlled trial of 151 STEMI patients did not find any additive effect of local ischemic post-conditioning (four cycles of 1-min inflations and 1-min deflations of the angioplasty balloon) to remote ischemic per-conditioning (three cycles of 5-min inflations to 200 mmHg and 5-min deflations of an upper-arm cuff) (47). The latter alone or the two combined had a similar effect in reducing peak CK-MB, the ratio of CK-MB area under the curve to myocardial area at risk, and the ratio of peak CK-MB to the area at risk (47). However, differences in CK-MB area under the curve between control, per-conditioning alone, and per-conditioning with post-conditioning were not statistically significant (47).

While these and other small randomized controlled trials have shown that RIC in addition to reperfusion therapies may blunt the release of myocardial necrosis enzymes and infarct size, or improve myocardial salvage in STEMI patients, results are heterogenous, and different biomarkers gave positive results in different studies. Furthermore, the aforementioned studies do not prove that RIC can improve clinical endpoints, such as mortality or heart failure. Other studies have tried to answer such questions, evaluating the effect of RIC on clinical outcomes in STEMI patients undergoing PPCI. A prospective randomized trial of 696 acute STEMI patients found that combined remote ischemic per-conditioning (three cycles of inflation of an upper-arm cuff for 5 min followed by deflation for 5 min) and post-conditioning (four cycles of 30-s balloon occlusions followed by 30 s of reperfusion) in addition to PPCI slightly reduced the rate of major adverse cardiac events (MACE) and heart failure development at a median of 3.6 years, although the study was not powered for detecting follow-up clinical outcomes (48). However, post-conditioning alone did not decrease MACE compared to controls who received PPCI alone (48). Similarly, two randomized controlled trials showed that remote ischemic per-conditioning in addition to PPCI improved long-term clinical outcomes in patients with STEMI (49, 50). However, these studies had a low statistic power

and were not designed to prospectively detect differences in clinical outcomes between patients receiving RIC and controls (51). Furthermore, none of these trials showed an effect of RIC on MI size reduction (19, 49–51). On the contrary, a large, appropriately powered, international, prospective, randomized controlled trial of 5,401 patients with STEMI who underwent remote ischemic per-conditioning (three cycles of intermittent 5-min lower limb ischemia) did not find any effects on the incidence of MACE at 12 months (51). In this study Hausenloy et al. (51), used the RIC protocol that has been showed to be the most effective in experimental studies (52). Notably, it has been hypothesized that RIC might initiate a form of delayed protection, the clinical benefits of which may not manifest for 2 years or longer (19, 53, 54).

REMOTE ISCHEMIC CONDITIONING IN ACUTE ISCHEMIC STROKE

As in STEMI, pharmacological neuroprotective therapies for ischemic stroke have been disappointing (55). RIC has been shown to be effective in pre-clinical models of acute brain ischemia, both alone and in combination with revascularization therapies (56, 57). In humans, patients with transient ischemic attack (58–60) or peripheral ischemic vascular disease (61) before ischemic stroke have been proposed as possible "natural" models of ischemic pre-conditioning. In both of these populations, subsequent ischemic strokes were attenuated (smaller infarct volumes and lower disability and mortality), compared to ischemic stroke patients without clinical history of transient ischemic attack (58–60) or without peripheral ischemic vascular disease (61). However, these studies have some limitations, such as their retrospective design and the challenges of anamnestic identification of TIA, for instance.

In patients with symptomatic intracranial stenoses, RIC may reduce recurrent stroke, improve cerebral perfusion (62, 63), and decrease ischemic brain injury secondary to carotid artery stenting (64, 65). Nevertheless, evidence of the efficacy of RIC in acute ischemic stroke is lacking.

According to a recent systematic review (20), six studies that applied remote ischemic per-conditioning to acute ischemic stroke patients have been completed and 13 are ongoing. A marked heterogeneity exists in the number of participants, inclusion criteria, remote ischemic per-conditioning protocols, and main endpoints. In most cases, remote ischemic per-conditioning was applied to an unaffected upper limb, most often with an automated device, sometimes manually. Remote ischemic per-conditioning was only initiated in a pre-hospital setting in three trials (20): REMOTE-CAT, RESIST, and a study by Hougaard et al. (66).

The safety of RIC for brain ischemia patients undergoing thrombectomy or thrombolysis has been reported in different contexts by different groups (62, 64, 67, 68), including in octo- and non-agenarians (62), in patients with acute ischemic stroke (68), and in those undergoing thrombectomy (67). In particular, remote ischemic post-conditioning after thrombolysis has been investigated in a small, randomized trial in 30 patients (five 5-min cycles of inflation and deflation on the first day after thrombolysis, and twice each day for 6 consecutive days), which did not highlight any safety issues (64).

Trials evaluating RIC have mostly focused on surrogate markers of efficacy, such as neuroimaging findings (e.g., brain infarct size or tissue at risk for infarction based on cerebral perfusion). Alternatively, some studies have focused on circulating biomarkers. These include putative mediators of protective mechanisms of RIC [e.g., heat shock proteins, which have been associated with ischemic tolerance (69)]; markers of processes known to be detrimental in the course of brain ischemia, such as inflammatory proteins [e.g., C-reactive protein (CRP), serum amyloid protein (SAP), or tissue necrosis factor-α (TNF-α)]; or other possible markers of neuronal degeneration [e.g., S100B or matrix metalloproteinase-9 (70, 71)]. Thus, biomarkers have been used to assess the efficacy of a RIC protocol, either in reproducing the beneficial effects that RIC has shown in animal models (56), or in limiting inflammation and neuronal degeneration.

Most biomarkers did not change in patients with acute brain ischemia undergoing RIC in the RECAST trial (68). However, a significant increase in heat shock protein-27 and reductions in SAP and TNF-α levels were measured in patients undergoing RIC (four cycles of intermittent 5-min upper limb ischemia and reperfusion) compared to controls (68, 72). These results are particularly interesting as SAP levels before RIC displayed a moderate, yet significant correlation with worse clinical outcomes after brain ischemia, and were significantly reduced after RIC compared to before the intervention in intra-subject analyses (72). A decrease in high-sensitivity CRP in stroke patients undergoing ischemic conditioning has also been reported in a recent meta-analysis that included 13 clinical trials, for a total of 794 patients, mainly of Asiatic ethnicity (73).

Pre-hospital manual RIC (four cycles of intermittent 5-min upper limb ischemia and reperfusion) was found to reduce the radiological risk of brain tissue infarction in 443 patients (66). However, no difference in brain infarction volume growth at 24 h after symptom onset was identified in a multicenter study of 188 carotid ischemic patients who were randomized to lower-limb in-hospital remote ischemic per-conditioning (four cycles of 5-min ischemia and reperfusion) after initial MRI in addition to standard therapy or standard therapy alone (74). Other trials that are investigating the effect of RIC on radiological biomarkers, such as brain infarction volume [rtPA-RIC (NCT02886390); PROTECT I (NCT03915782); REVISE-2 (NCT03045055); RICE PAC (NCT03152799); REPOST (75)], are planned or ongoing.

Findings on clinical endpoints of RIC for acute ischemic stroke are even more limited. For example, in the aforementioned trial on 443 ischemic stroke patients by Hougaard et al. (66), neutral results were found: clinical neurological outcomes did not differ significantly between patients undergoing pre-hospital manual RIC and controls. Only four ongoing studies have clinical endpoints as primary outcomes: REMOTE-CAT, SERIC AIS, RESIST, and RICAMIS (20).

On the other hand, a metanalysis by Zhao et al. (73) found that remote ischemic post-conditioning may not only reduce the risk of recurrent stroke, but also the modified Rankin score

TABLE 1 | Summary of the key completed randomized controlled trials (published in English) on RIC with clinical outcomes in patients with acute STEMI or ischemic stroke.

	References	*n*	Conditioning intervention	Clinical findings in the intervention vs. control group
STEMI	Sloth et al. (50)	333	4 × 5-min 200 mmHg, arm (per-RIC)	Reduced rates of MACCE and all-cause mortality at a median of 3.8 years
	Gaspar et al. (49)	258	3 × 5-min 200 mmHg, leg (per-RIC)	Reduced in-hospital HF and lower risks of cardiac mortality and/or hospitalization for HF at a median of 2.1 years
	Stiermaier et al. (48)	696	3 × 5-min 200 mmHg, arm (per-RIC)	Reduced rates of MACE and HF at a median of 3.6 years
	Hausenloy et al. (51)	5,401	4 × 5-min 200 mmHg, arm (per-RIC)	No significant differences in cardiac mortality, hospitalization for HF, or MACCE at 12 months
Acute ischemic stroke	Hougaard et al. (66)	443	4 × 5-min 200 mmHg or 25 mmHg above SBP, arm (per-RIC)	No significant difference in mRS at 90 days
	An et al. (76)	68	5 × 5-min 180 mmHg, both arms (post-RIC)	Favorable recovery (mRS score 0–1) at 90 days in the post-RIC group (adjusted OR 9.85, 95% confidence interval 1.54–63.16; $p = 0.016$)
	Pico et al. (74)	188	4 × 5-min 110 mmHg above SBP, thigh (per-RIC)	No significant difference in mRS at 90 days
	He et al. (77)	49	4 × 5-min 200 mmHg, arm (post-RIC)	No significant difference in mRS nor NIHSS at 90 days

HF, heart failure; MACCE, major adverse cardiac and cerebrovascular events; MACE, major adverse cardiac events; mRS, modified Rankin score; NIHSS, National Institute of Health Stroke Scale; OR, odds ratio; RIC, remote ischemic conditioning; SBP, systolic blood pressure; STEMI, ST-segment elevation myocardial infarction.

(according to two studies) and the National Institutes of Health Stroke Scale score (despite significant heterogeneity in the trials that assessed this variable).

DISCUSSION

This review recapitulates the evidence that RIC reduces circulating biomarkers of myocardial necrosis, infarct size, and edema, although this effect does not appear to translate into better outcomes (19, 51) (**Table 1**). However, concerning ischemic stroke, although RIC is a safe procedure that can easily be implemented in hospital and pre-hospital settings, its clinical efficacy has yet to be proven (20). Furthermore, no biomarkers equivalent to CK-MB or high-sensitivity troponin T (41, 42) exist for ischemic stroke. Thus, only indirect evidence concerning RIC effects in brain ischemia is obtained from existing biomarkers and, as discussed, contradictory results have been reported in existing studies (68, 72).

Further research is needed to better characterize RIC patient responses. Besides those discussed above, putative biomarkers of RIC effects include autocoids (e.g., adenosine, endogenous opioids, or bradykinin), cytokines, and nitrites, but other humoral factors are yet to be better defined (24). The interest in characterizing such biomarkers is two-fold. Firstly, they may clarify the mechanisms underlying RIC. Secondly, they could confirm that a certain protocol is effective in triggering a RIC response, if a defined threshold of a hypothetical biomarker were reached. Defining the exact mechanisms mediating the effects of pre-, per-, or post-RIC will also be crucial to develop drugs or technological devices that may replace RIC. This is important for many reasons. Firstly, because the lack of conclusive evidence on RIC efficacy in terms of clinical outcomes in MI and ischemic stroke may be due to heterogenous protocols and different settings of application of RIC. A pharmacological treatment or an electronic device could allow the design of more standardized protocols, possibly allowing a benefit from RIC to emerge, even in human studies. Secondly, should this benefit be proven in humans, drugs or devices have the undisputable advantage that they can be administered or applied more quickly than the RIC protocol, which typically requires at least 40 min. One interesting perspective, since RIC cardiac protection appears to be at least partially mediated by the activation of vagal fibers (27, 40), is that vagal nerve stimulation may reproduce its effects in MI patients (26). Similarly, as vagus nerve activation has anti-inflammatory (78) and neuroprotective effects reducing cerebral infarct size (24, 79), vagal stimulation may mediate RIC effects in acute stroke (24). However, it is likely that isolated direct transcutaneous vagal stimulation does not trigger all protective processes that are instead activated by RIC protocols inducing muscle ischemia.

Measuring biomarkers mediating the RIC response may also help to stratify patients to identify those who could most benefit from remote ischemic post-conditioning in the days after an ischemic event.

A more stringent selection of included participants in future RIC clinical trials may boost effect size. For example, since RIC is believed to be most effective against cerebral reperfusion injury based on pre-clinical studies (24, 80), stroke clinical trials should focus on patients with a higher likelihood of successful reperfusion (24), who are thus exposed to the risk of reperfusion injury. Besides reperfusion, other risk factors include hypertension, cerebral vascular dysregulation, and late recanalization (81, 82).

Another interesting criterion of stratification of stroke patients may be the presence of collateral brain vessels, which can limit the extension of ischemic penumbra or border zone (83). Importantly, recent pre-clinical studies have found that RIC

enhanced cerebral collateral circulation (84, 85). For this reason, the effects of RIC may be most evident in patients who can most benefit from the presence of vascular collaterals, such as those undergoing large vessel occlusion, who are also candidates for mechanical thrombectomy. For such reasons, testing RIC in this specific population may yield new, interesting results (20).

A possible cause of the discrepancy between results of pre-clinical and clinical studies on RIC is a difference in protocols. In fact, pre-clinical studies mostly employed RIC of the hindlimb, while upper limb RIC is usually performed in patients (24). It cannot be excluded that the higher proportion of muscle tissue undergoing RIC in the hindlimb may explain the greater efficacy of RIC in pre-clinical studies, especially if we consider that human patients might be elderly individuals with muscle deconditioning and comorbidities. Thus, leg RIC might be systematically used in future studies, as has previously been studied (41, 49, 74). Alternatively, a higher number of ischemia/reperfusion cycles and/or longer duration of the stimulus repetition might be more effective. Furthermore, post-conditioning long-term RIC protocols in patients are complicated by problems of compliance. Technological devices can also be used to document compliance and to ensure adherence to the protocol. Smart devices to monitor real-time post-conditioning, long-term RIC protocols, in association with smartphone applications, are currently being tested (86).

Finally, in both MI and acute ischemic stroke, it is crucial to apply RIC as soon as possible, even in the pre-hospital setting, to freeze the penumbra, or the border zone, thus possibly extending the time window for reperfusion therapies (20, 74). To make this possible, patients' early triage and stratification with pre-hospital scales is warranted (20). Importantly, it is possible to apply RIC in pre-hospital emergency settings, not only in an ambulance (21, 44), but also during air medical transportation (87). However, challenges to pre-hospital RIC administration include the need for dedicated personnel, if using a manual cuff, and the fact that average pre-hospital transport times may not be long enough to administer the full cycles. For instance, in one of the studies discussed above, transportation time was too short to administer four RIC cycles in 18% of patients (66). Once again, an automatic device can relieve both manpower and cognitive resources in the emergency setting, as it can be left in place once a pre-programmed protocol has been set. Interestingly, RIC can also be given in the emergency department, and even in the catheterization laboratory (88).

CONCLUSIONS

RIC is "non-invasive, simple, safe, and cheap" (89), can be used alone or in combination with existing reperfusion therapies, and can be initiated in pre-hospital settings, but its clinical efficacy in MI and acute ischemic stroke patients remains to be proven (20, 47, 49–51, 66, 74). Further research is needed regarding the optimal time window and protocol for RIC application, its mechanisms and biomarkers, and the populations that could most benefit from this strategy in both cardiology and neurology settings.

AUTHOR CONTRIBUTIONS

LFS: design and writing. AA, ME, and FP: design and critical revision. All authors contributed to the article and approved the submitted version.

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STUDY PROTOCOL
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Multi-Center Randomized Phase II Clinical Trial on Remote Ischemic Conditioning in Acute Ischemic Stroke Within 9 Hours of Onset in Patients Ineligible to Recanalization Therapies (TRICS-9): Study Design and Protocol

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Aim: To assess the efficacy of remote ischemic conditioning (RIC) in patients with ischemic stroke within 9 h of onset, that are not candidates for recanalization therapies.

Sample Size Estimates: A sample size of 80 patients (40 in each arm) should yield 80% power to detect a 20% difference in early neurological improvement at 72 h at $p = 0.05$, two sided.

Methods and Design: TRICS-9 is a phase II, multicenter, controlled, block randomized, open-label, interventional clinical trial. Patients recruited in Italian academic hospitals will be randomized 1:1 to either RIC plus standard medical therapy or standard medical therapy alone. After randomization, RIC will be applied manually by four alternating cycles of inflation/deflation 5 min each, using a blood pressure cuff around the non-paretic arm.

Study Outcomes: The primary efficacy outcome is early neurological improvement, defined as the percent change in the National Institute of Health Stroke Scale (NIHSS) at 72 h in each arm. Secondary outcomes include early neurologic improvement at 24 and 48 h, disability at 3 months, rate of symptomatic intracerebral hemorrhage, feasibility (proportion of patients completing RIC), tolerability after RIC and at 72 h, blood levels of HIF-1α, and HSP27 at 24 h and 72 h.

Discussion/Conclusion: RIC in combination with recanalization therapies appears to add no clinical benefit to patients, but whether it is beneficial to those that are not candidates for recanalization therapies is still to be demonstrated. TRICS-9 has been developed to elucidate this issue.

Clinical Trial Registration: ClinicalTrials.gov, identifier: NCT04400981.

Keywords: acute stroke, ischemic stroke, remote ischemic conditioning, RIC, phase II, clinical trial, TRICS-9

INTRODUCTION AND RATIONALE

Remote ischemic conditioning (RIC) relies on a transient ischemia being applied to a certain body site with the aim of increasing ischemic tolerance in distant organs by endogenous protective mechanisms (1–3). Although these mechanisms remain elusive, evidence supports the role of both humoral and neuronal factors, such as the release of adenosine, bradykinin, and nitric oxide in the blood, and the activation of neuronal p-AKT and several miRNAs (4). Recent observations have suggested that HSP27 and HIF-1α are mediators of remote ischemic conditioning and hence potential biomarkers (5–12). Many experimental studies on animal models of ischemic stroke have demonstrated that RIC is neuroprotective against cerebral ischemia and that benefit is obtained in stroke models with or without reperfusion up to 6 h after stroke induction (13–19). Despite numerous preclinical studies, clinical evidence is limited. Clinical studies showed that remote ischemic conditioning, applied by transient limb ischemia using a blood pressure cuff, is well-tolerated, feasible, and safe (20, 21) and that long-term treatment decreases the recurrence risk of stroke due to symptomatic intracranial arterial stenosis (22). Two clinical trials on RIC in combination with intravenous thrombolysis or mechanical thrombectomy gave neutral results (23, 24); however, no studies have investigated whether RIC is beneficial to patients with acute ischemic stroke who are not candidates for recanalization therapies. These patients represent a significant proportion of those with acute ischemic stroke and although criteria for patient eligibility are continuously evolving, the proportion of ischemic strokes potentially eligible for endovascular thrombectomy or intravenous thrombolysis is limited (25, 26).

The Italian Stroke Organization (ISO) Basic Science Network, a nationwide network that promotes translational research on acute ischemic stroke, has launched a multicenter translational research program on RIC. This program provides for a multi-center pre-clinical study of experimental ischemic stroke (27) and a multi-center randomized phase II clinical trial on remote ischemic conditioning in patients with acute stroke within 9 h of onset who are not candidates for recanalization therapies (TRICS-9).

The TRICS-9 trial is designed to investigate the efficacy of RIC in a carefully selected population of ischemic stroke patients whose neurological improvement could be attributed to no acute treatments but RIC, to evaluate the potential role of RIC as acute therapy for patients that are not a candidate for recanalization therapies and who would otherwise receive secondary prevention only.

The study protocol of the clinical trial is described hereafter.

METHODS AND ANALYSIS

Design

The study is a phase II, multicenter, controlled, block randomized, open-label, interventional clinical trial comparing RIC plus standard medical therapy to standard medical therapy alone, in patients with acute ischemic stroke within 9 h of onset that are not eligible to intravenous thrombolysis and/or mechanical thrombectomy. The study has a prospective randomized open blinded end-point (PROBE) (28) design.

Selection/Treatment of Subjects

Patient enrollments (**Figure 1**) will be carried out in three ISO-associated academic hospitals provided with Comprehensive Stroke Centers of the Italian Hub-and-Spoke network for stroke. Patients will be eligible for inclusion in the trial if they have evidence of acute ischemic stroke due to either small or large vessel occlusions of the anterior circulation within 9 h of symptom onset, are 18 years of age or older, have a score on National Institutes of Health Stroke Scale (NIHSS; scores range from 0 to 42, with higher scores indicating a more severe deficit) equal to or higher than 5 and lower than 25, a pre-stroke score of 0 or 1 on the modified Rankin scale (which ranges from 0 to 6, with a score of 0 indicating no disability and higher scores indicating more severe disability), and patients or they surrogate provided informed consent. Strokes with unknown time of onset will also be considered for enrollment. For patients with stroke symptoms on awakening, the midpoint of sleep (i.e., the time between going to sleep and waking up with symptoms) will be considered as time of stroke onset and patients will undergo randomization if the estimated time of onset is $\leq$9 h (29); for patients with neurologic deficits (e.g., aphasia, anarthria, altered mental status) that keep them from reporting the time of onset, enrollment will be possible whenever the time elapsed since the patient was last known to be well is $\leq$9 h. Information regarding the time of going to sleep or the last time the patient was seen well will be obtained by the patient or anyone who last had contact with the patient before stroke onset. Patients eligible for either or both intravenous thrombolysis and mechanical thrombectomy will be excluded from the trial. Other exclusion criteria will be evidence of intracranial hemorrhage, vascular malformation, intracranial masses on brain CT or MRI, rapidly improving neurological symptoms at the time of first evaluation, transient ischemic attack with the resolution of symptoms at the time of first evaluation, amputation of the upper non-paretic arm, ulcers, phlebitis, or bad skin condition in the upper limbs, and history of peripheral artery disease or sickle cell disease. All inclusion and exclusion criteria are summarized in **Table 1**.

Randomization

Participants will be randomly assigned to either control or experimental groups with a 1:1 allocation as per computer-generated block randomization schedule stratified by site. A specific "Randomization Procedure" document will be prepared, signed, and stored within a sealed envelope before the first randomization, The allocation sequence will be created by an individual separated from trial investigators and analysts. Treatment allocation will be disclosed after the patient has been recruited.

Interventional Method

The experimental intervention will be performed in the Emergency Department immediately after randomization.

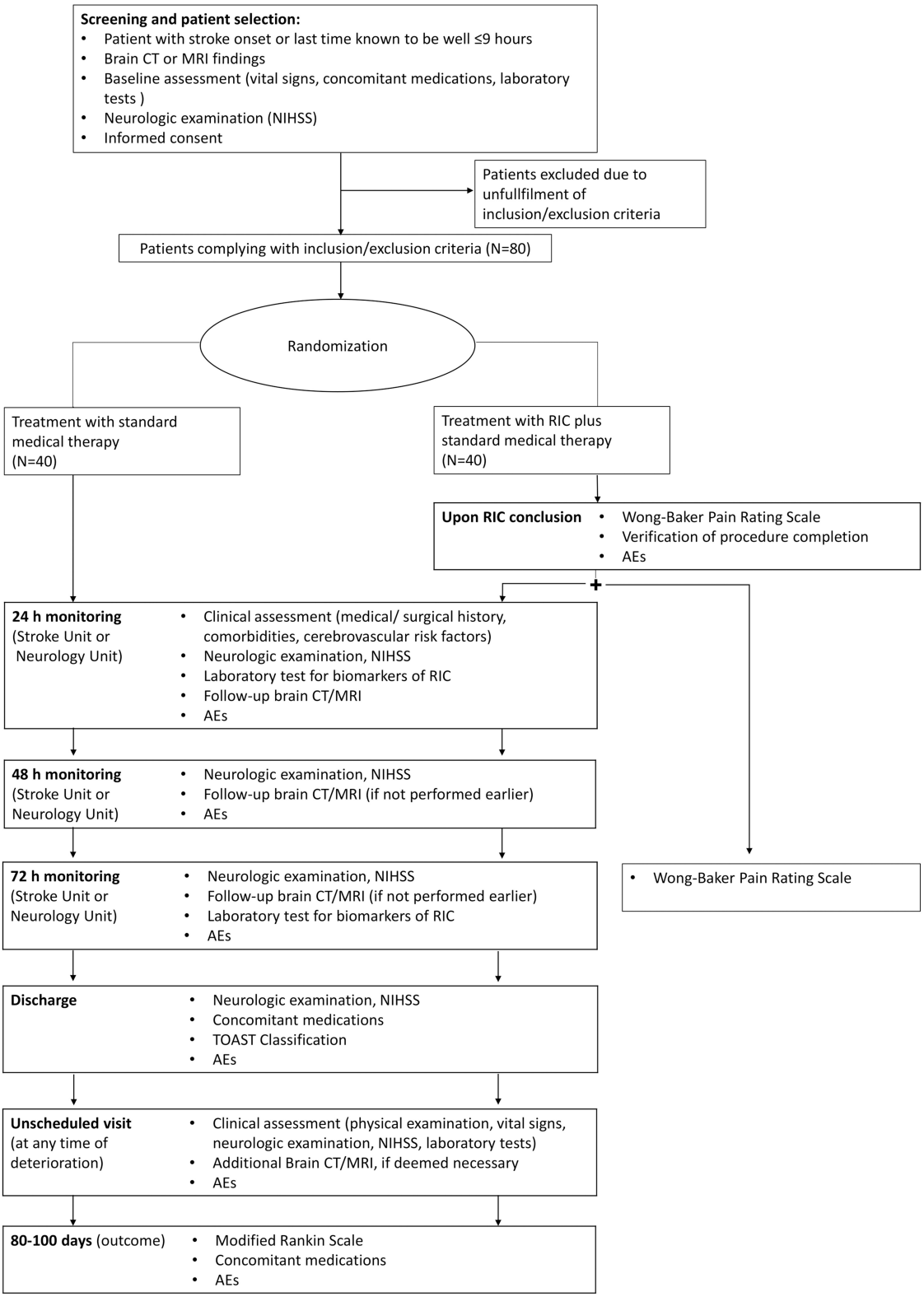

FIGURE 1 | Flowchart of TRICS-9.

TABLE 1 | Inclusion and exclusion criteria.

Inclusion criteria
• Clinical diagnosis and/or diagnosis on neuroimaging of anterior circulation acute ischemic stroke (due to either large or small vessel occlusion) within 9 h of symptom onset
• Age ≥ 18 years
• NIHSS ≥5 and <25
• Stroke with Unknown Time of Onset: this will include wake up strokes with estimated time of onset ≤ 9 h of enrollment or patients unable to report symptom onset but that were last known to be well ≤ 9 h of enrollment
• Modified Rankin scale ≤2 prior to stroke onset
• Written informed consent obtained from patient or legally responsible person
Exclusion criteria
• Candidates for intravenous thrombolysis and/or mechanical thrombectomy according to AHA/ASA guidelines
• Brain CT or MRI detecting intracranial hemorrhage, vascular malformation, intracranial masses, or any medical condition unrelated to stroke that could explain symptoms
• Rapidly improving neurological symptoms at the time of first evaluation
• Transient ischemic attack
• Amputation of the upper non-paretic arm
• Ulcers or a bad skin condition in the upper limbs
• History of peripheral artery disease, sickle cell disease, or upper limb phlebitis
• Pregnancy
• Ongoing participation in any interventional study
• Terminal illness or life expectancy ≤6 months
• Patients unable to give informed consent

CT, computed tomography; MRI, magnetic resonance imaging; NIHSS, National Institutes of Health Stroke Scale.

A standard blood pressure cuff will be placed around the non-paretic arm. Patients randomized to RIC will undergo remote ischemic conditioning by four cycles of intermittent, manually induced, upper limb ischemia alternating 5 min of inflation (20 mmHg above the systolic blood pressure) to 5 min of deflation. Patients in the control group will have the blood pressure cuff placed around the non-paretic arm for 40 min but will undergo no inflations. All patients will receive usual care, at the discretion of the investigator and according to available guidelines and standards of care.

Outcomes

Admission NIHSS will be assessed before randomization, whereas NIHSS at 72 h (NIHSS72) will be obtained by a neurologist blinded to treatment allocation.

The primary efficacy outcome will be early neurological improvement at 72 h, defined as percent change in NIHSS score [(Admission NIHSS—NIHSS72) × 100/Admission NIHSS]. This definition has been adopted in a recent study that re-assessed the primary outcome of the NINDS part 1 trial on intravenous thrombolysis, wherein percent change in NIHSS score resulted to be more sensitive in demonstrating early clinical benefit and more predictive of long-term neurological outcome (30, 31) than the absolute changes in NIHSS score.

Six secondary outcomes will be assessed: (1) NIHSS percent change at 24 h and 48 h: NIHSS will be assessed 24 h (NIHSS24) and 48 h after randomization (NIHSS48) and 2% changes will be independently calculated: [(Admission NIHSS—NIHSS24) × 100/Admission NIHSS] and [(Admission NIHSS—NIHSS48) × 100/Admission NIHSS]; (2) disability at 3 months, as assessed by the modified Rankin scale (mRS); (3) symptomatic intracerebral hemorrhage per the SITS-MOST definition: a local or remote Type 2 parenchymal hemorrhage on imaging 22–36 h after treatment or earlier if the imaging scan was performed due to clinical deterioration combined with a neurological deterioration of 4 NIHSS points from baseline or from the lowest NIHSS score between baseline and 24 h or leading to death within 24 h. A grading of Type 2 parenchymal hemorrhage for intracranial hemorrhage indicates a coagulum exceeding 30% of the infarct with substantial space occupation; (4) feasibility will be estimated by the proportion of patients randomized to a treatment group that complete the experimental intervention; (5) tolerability will be assessed by the Wong-Baker Faces Pain Rating Scale following the procedure and after 72 h; (6) an exploratory analysis on blood levels of HIF-1α and HSP27 24 h and 72 h after remote ischemic conditioning will be also carried out. Whole blood HIF-1alpha mRNA levels will be assessed at 24 and 72 h by quantitative reverse transcription polymerase chain reaction (HIF1a F, TCATCCAAG- GAGCCTTAACC; HIF-1a R, AAGCGACATAGTAGGGGCAC, Takara Bio, CA, USA). HIF-1alpha mRNA levels will be expressed as a ratio to the housekeeping gene GAPDH and the difference between HIF-1alpha mRNA levels at 24 h and 42 h will be defined as HIFDiff72 (HIF72 - HIF24). Plasma levels of HSP27 will be quantified using a colorimetric enzyme immunoassay (ELISA) kit (Enzo Life Sciences, Roma, Italy). The difference between plasma levels at 24 and 72 h will be expressed as HSPDiff72 (HSP72 - HSP24). An increase of HIF-1alpha and HSP27 levels at 72 h compared to 24 h is a biomarker of the peripheral vascular effect of RIC.

Sample Size Estimates

In the NINDS trial, the NIHSS percent change in the control group was 15% +/− 35% at 24 h. The median of the NIHSS percent change in absence of treatment is expected to slightly increase, and the standard deviation to slightly decrease, at 72 h, due to stabilization of the neurological deficit. An estimated total sample size of 80 patients (40 patients in each arm) should yield 80% power to detect a clinically significant difference of 20% (40% in treatment vs. 20% in control arm) in the median percent change in NIHSS at 72 h, considering a standard deviation of 30%, at the two-sided statistical significance threshold of $p = 0.05$, when using a Wilcoxon-Mann-Whitney test. The primary null hypothesis is that there is no or negligible difference in clinical benefit between remote ischemic conditioning plus standard medical therapy and standard medical therapy alone.

Data Monitoring Boards

An independent Data and Safety and Monitoring Board (DSMB) will be constituted by an experienced neurologist (E.Be.) of the Istituto di Ricerche Farmacologiche Mario Negri, a statistician (E.Bi.) of the Istituto di Ricerche Farmacologiche Mario Negri and a lay member (E.C.) of the Italian stroke patients' association A.L.I.C.E. Onlus (Associazione per la Lotta all'Ictus Cerebrale). The DSMB will provide expertise by periodical review and evaluation of the accumulated study data for participant safety, study conduct, and progress; it will monitor any serious adverse

events, guarantee patient's dignity and fundamental rights with regard to treatments and care, and provide recommendations on continuation, modification, or termination of the trial.

Data Analysis

The baseline patient characteristics of each treatment group will be described as means and standard deviations, medians, and quartiles, for numerical variables, and as frequencies and percentages per class for qualitative variables. Between-group unbalances among baseline characteristics will be considered for adjustment whenever deemed important for the outcome, in which case an adjusted secondary logistic analysis will be performed. The primary analysis of treatment effect on early neurological improvement will be performed using a Wilcoxon-Mann-Whitney test. Secondary analysis of the primary outcome will be performed using a mixed linear regression including treatment, centers, and any unbalanced baseline characteristics. Secondary outcomes will be analyzed using a Wilcoxon-Mann-Whitney test to evaluate treatment effect on early neurological improvement at 24 h or 48 h, and using a logistic mixed model to assess the effect of RIC on secondary variables (i.e., Wong-Baker face pain rating scale, modified Rankin scale, and occurrence of symptomatic intracerebral hemorrhage). The mRS scores will be dichotomized as a good functional outcome (mRS 0–2) and poor functional outcome (mRS 3–6). Wong-Baker Face Pain Rating Scale scores will be dichotomized around medians. Whole blood levels of HIF-1α mRNA and plasma levels of HSP27 in each arm will be represented by histograms. We expect no missing data for the primary outcome since patients will still be hospitalized. In case of missing results, a multiple imputation procedure will be used. Analyses will be conducted with an intention-to-treat approach (ITT). For the primary outcome, patients with neurological worsening will be given a percent change of 0; those deceased within 72 h will be given the last percent change available and a percent change of 0 will be assigned in case admission NIHSS is the only available data. A population of interest is composed of patients that completed the experimental intervention: a specific section will be devoted to this group, where main analyses will be repeated. The cut-off for statistical significance will be set at 0.05, two-tailed. Analyses will be carried out in blind using Stata/IC v. 15 or higher (Statacorp). A detailed Statistical Analysis Plan (SAP) will be written within 1 month after enrollment of the first patients.

Allocation Concealment

This is an open label experimental study. Treatment allocation will be disclosed after patient recruitment, i.e., after inclusion/exclusion criteria have been recorded. Only outcome assessors (at 72 h and 3 months) and data analysts will be blinded after assignment to interventions.

Study Organization and Funding

TRICS-9 is funded by a grant from the Italian Ministry of Health - PRIN 2017CY3J3W. This funding source had no role in study design and will have no role during its execution, analyses, interpretation of the data, reporting of the study, or decision to submit results.

DISCUSSION

The primary objective of the TRICS-9 trial is to assess the efficacy of RIC in patients with acute ischemic stroke that are not candidates for intravenous thrombolysis or mechanical thrombectomy due to ineligibility or contraindication, and that present to hospital within 9 h of symptom onset.

Previous clinical studies on the efficacy of RIC in acute ischemic stroke have provided neutral results (21, 22). A possible limitation of previous clinical trials was the inclusion of patients that received either or both intravenous thrombolysis and mechanical thrombectomy, since their efficacy may have concealed the effect of RIC. If RIC in combination with recanalization therapies appears to add no clinical benefit, whether it is beneficial to patients who are not candidates for recanalization therapies is still to be demonstrated. These patients represent a significant portion of those with acute ischemic stroke; indeed, despite progressive widening of eligibility criteria, the proportion of patients treated with recanalization therapies is limited (25, 26). TRICS-9 will involve patients that are not candidates for recanalization therapies due to ineligibility or contraindication and that may still benefit the neuroprotective action of RIC. Among these, common examples are (1) patients with distal occlusions with contraindications to intravenous thrombolysis because of ongoing anticoagulant treatment, elevate blood pressure despite the administration of blood pressure lowering medications, recent surgery or trauma or previous intracranial hemorrhage; (2) patients with lacunar strokes with contraindications to intravenous thrombolysis due to stroke onset beyond 4.5 h and insufficient mismatch ratio on perfusion imaging; (3) patients with large vessel occlusion with early infarct signs on brain imaging and insufficient mismatch ratio to be a candidate for thrombectomy, but who may still exhibit some salvageable brain tissue on perfusion studies. We decided to exclude mild strokes (NIHSS <5) due to the propensity they show toward spontaneous favorable neurologic outcomes, and those with very severe strokes (NIHSS ≥ 25). The choice to select such a population has two advantages: (1) it offers RIC to patients that otherwise would not be offered any acute treatment and would receive only secondary prevention, and (2) it tests the efficacy of RIC in acute stroke patients without the confounding factor represented by recanalization therapies that might mask the benefit of RIC.

The 9-h time window has been inspired by recent clinical trials on recanalization therapies in acute ischemic stroke, such as EXTEND and WAKE-UP trials, that demonstrated potentially salvageable tissue up to 7–10 h from symptom onset, even in patients without large vessel occlusion (29, 32). The neuroprotective effect of RIC is presumably advantageous in presence of salvageable brain tissue, whose survival can last many hours after stroke onset depending on multiple factors. Many preclinical studies indicate that RIC helps the survival of salvageable brain tissue by reducing apoptosis,

downregulating inflammatory response, and improving collateral circulation in the ischemic penumbra (33). Furthermore, two pre-clinical studies on a rat model of experimental ischemic stroke demonstrated that RIC is beneficial up to 6 h after ischemic stroke due to permanent large vessel occlusion (18, 19). Based on this evidence, we hypothesize that the application of RIC may be beneficial up to several hours after stroke onset in presence of salvageable brain tissue.

In a previous clinical trial (23), wherein penumbral salvage was the primary outcome and both infarct growth and final infarct size were secondary outcomes, no between-group differences were found in patients treated with RIC plus standard therapy and in those that received standard therapy alone. However, radiological findings represent surrogate outcomes allowing only indirect measurement of treatment efficacy and the lack of demonstration of a positive radiologic effect does not exclude a clinical benefit with certainty. Taking a different approach, in TRICS-9 a clinical outcome has been chosen to obtain a direct measure of RIC efficacy.

The primary outcome will be early neurological improvement, defined as NIHSS percent change at 72 h, following the definition adopted in a recent study that re-assessed the NINDS part 1 trial on intravenous thrombolysis in acute ischemic stroke (30, 31). This primary outcome has many advantages: (I) it accounts for the wide variation in admission scores across patients, (II) it is sensitive enough to allow a higher study power than that achievable using absolute changes in the NIHSS score, even with a relatively small sample size, (III) it is sufficiently hard to test a large effect size of immediate clinical interest, and (IV) the study power associated with a continuous outcome variable is expected to be higher than that achieved using a dichotomous outcome variable.

In a previous study (23), RIC was applied by emergency medical services to patients with suspected stroke during transfer to hospital. Although application shortly after symptom onset is reasonable in view of a prompt activation of neuroprotective mechanisms, many stroke mimics were initially recruited. In our trial, patient selection will be carried out in the emergency department only after neurologic examination and radiologic investigations, to prevent the inclusion of stroke mimics.

RIC has been investigated in many studies; however, the underlying mechanisms remain elusive and no reliable biomarkers are available. Despite the observation of HIF-1α and HSP27 to be associated with RIC, there is no solid evidence of their role as mediators. HSP27 is neuroprotective against cerebral infarction when purified from human lymphocytes and injected in mice models (5, 6), as well as when overexpressed in mice models of ischemic stroke (7). A significant increase in serum total HSP27 and phosphorylated HSP27 (pHSP27) was detected in stroke patients 4 days after RIC compared to controls (16). HIF-1α is involved in neuroprotection against ischemic brain injury (8–10), and RIC promotes HIF-1a activation in the peripheral blood (11, 12). These observations suggest HSP27 and HIF1α to be good candidates as biomarkers. Hence, an exploratory analysis on blood levels of HSP27 and HIF1-α will be performed to assess their potential role as biomarkers of neuroprotection following RIC.

This trial has limitations. First, the expected effect size of RIC in TRICS-9 was inspired by evidence from experimental studies on *in vivo* models of ischemic stroke (13, 15) that demonstrated an absolute difference of about 50% in the infarct size of rats with transient middle cerebral artery occlusion treated with RIC as compared to those untreated. However, the expected effect magnitude of RIC in TRIC-9 has been voluntarily set at 20% to reduce the risk of an overambitious outcome, resulting in smaller effect size than those observed in other experimental studies. If TRICS-9 will provide neutral results, it will not exclude that RIC may determine a milder clinical effect than that considered in this trial, in which case further dedicated studies will be necessary: in any case, it will provide valuable information for a potential phase III study. Second, the sample size is relatively small; however, the study design is adequately powered to detect an early neurological improvement, and both the characteristics of the highly selected study population and recruitment rate have been taken into account. Third, RIC will be carried out manually rather than automatically, which may implicate heterogeneity in manoeuver execution; however, the manoeuver is not supposed to be significantly influenced by the operator, since the interventional procedure is intentionally simple and will be performed by dedicated and instructed personnel. Fourth, the choice of a highly selected patient population could reduce the probability of recruitment; however, to maximize the probability of recruitment, only Comprehensive Stroke Centers, wherein a high influx of patients is expected, will be involved in the study.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico ASST Monza. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SB conceived the study and initiated the study design. SD contributed to study design and helped with its implementation. MT provided expertise in clinical trial design. CF is the grant holder. All authors contributed to the refinement of the study protocol and approved the final manuscript.

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REVIEW
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Immune Modulation as a Key Mechanism for the Protective Effects of Remote Ischemic Conditioning After Stroke

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Remote ischemic conditioning (RIC), which involves a series of short cycles of ischemia in an organ remote to the brain (typically the limbs), has been shown to protect the ischemic penumbra after stroke and reduce ischemia/reperfusion (IR) injury. Although the exact mechanism by which this protective signal is transferred from the remote site to the brain remains unclear, preclinical studies suggest that the mechanisms of RIC involve a combination of circulating humoral factors and neuronal signals. An improved understanding of these mechanisms will facilitate translation to more effective treatment strategies in clinical settings. In this review, we will discuss potential protective mechanisms in the brain and cerebral vasculature associated with RIC. We will discuss a putative role of the immune system and circulating mediators of inflammation in these protective processes, including the expression of pro-and anti-inflammatory genes in peripheral immune cells that may influence the outcome. We will also review the potential role of extracellular vesicles (EVs), biological vectors capable of delivering cell-specific cargo such as proteins and miRNAs to cells, in modulating the protective effects of RIC in the brain and vasculature.

Keywords: cerebral ischemia, collateral circulation, remote ischemic conditioning (RIC), inflammatory response, extracellular vesicles (EVs), microRNAs

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INTRODUCTION

The incidence, mortality, and prevalence of neurological disorders are increasing worldwide, primarily because of the growing elderly population (1). Stroke is one of the most common neurovascular conditions with a prevalence of 101.5 million people worldwide (2021 Heart Disease and Stroke Statistical Update) (1). Of these strokes, 76% were classified as ischemic stroke (∼77.2 million), ∼ 20% as intracerebral hemorrhage (∼20.7 million), and about 8% as subarachnoid hemorrhage (8.4 million) (1). Acute ischemic stroke (AIS) occurs when a major artery that supplies oxygen and nutrients to the brain becomes obstructed, leading to the formation of two injury zones: The ischemic core and the "penumbra." The infarct core is severely hypoperfused, such that neurons undergo rapid and irreversible necrotic cell death (2). In response to ischemia and cell death in the core, inflammatory signals are released into the peripheral circulation, attracting immune cells to the damaged area and exacerbating the inflammatory response. The core of the ischemic region is surrounded by a relatively hypoperfused zone called the penumbra, which

defines the tissue at risk for further infarction (3). Because cell death in this penumbral region occurs gradually, it is possible to rescue this peri-infarct area in the hyper-acute phase of AIS prior to cell death and infarct expansion.

One important factor contributing to the viability of penumbral tissue is the existence of strong collateral circulation that reduces ischemia in the penumbra, reducing injury, and improving the clinical outcomes (4). Pial collateral vessels—also called leptomeningeal collaterals–are auxiliary vascular networks on the brain surface that connect the distal part of major branches of the anterior and posterior cerebral artery (ACA, PCA) with the distal branches of the middle cerebral artery (MCA). These vascular anastomoses provide oxygen and essential nutrients *via* retrograde blood flow to the deprived ischemic tissue when the primary artery is blocked (5). Currently approved treatments for AIS, such as thrombolysis through recombinant tissue plasminogen activator (rtPA) administration or recanalization *via* mechanical endovascular treatment (EVT, i.e., mechanical thrombectomy), work in a time dependent manner and have a limited therapeutic window (6). While good collateral blood flow can extend this window, rapid restoration of flow to the brain remains the best treatment for acute stroke. However, this is restricted to ∼10–20 percent of stroke sufferers who can make it to a primary stroke treatment center in time. Treatments that can improve collateral blood flow may extend the window for recanalization therapy and improve outcome for stroke patients (5).

Even after flow is restored in an occluded cerebral vessel, cellular injury can be exacerbated by reperfusion injury (7). Recanalization of the occluded artery can lead to damage to the integrity of the capillary endothelium, known as ischemia/reperfusion (IR) injury. Restored flow can increase BBB permeability when a high blood volume re-enters the already collapsed vasculature. Following this reperfusion, activated endothelial cells (ECs) produce reactive oxygen species (ROS), which further triggers the influx of inflammatory cells to the ischemic site (8). Increased leukocyte stimulation, trafficking and release of proinflammatory chemoattractant substances amplifies local inflammation. Elevated expression of adhesion molecules on ECs can further potentiate interactions between circulating blood cells and ECs, particularly neutrophil-endothelial interactions that can lead to neutrophil aggregation in the capillary bed (9). Reducing IR injury is key to improving outcome after recanalization therapy. So far, several approaches have been attempted to inhibit leukocytes aggregation and attenuate IR injury, but none have proven effective in clinic (10). Additional therapies are urgently needed to protect brain tissue from the ischemic and post-reperfusion damage. One such approach may be remote ischemic conditioning (RIC) (11, 12). RIC has shown to be a clinically safe and straightforward intervention which helps to attenuate the detrimental effects of ischemia. Multiple molecular signaling pathways contribute to the protective effects of RIC against reperfusion injury, with key signaling pathways converging on transcription factors that regulate cell survival and apoptosis (13). Of these signaling cascades, the reperfusion injury salvage kinase (RISK) and the survivor activating factor enhancement (SAFE) pathways are well-characterized. Below, we will review RIC for stroke treatment, its established mechanisms, and discuss RIC induced modulation of inflammatory immune cells and their gene expression profiles.

RIC: CONCEPT AND ORIGIN

Remote Ischemic Conditioning (RIC) is a therapy that involves brief, intermittent episodes of sublethal ischemia and reperfusion that is applied to a peripheral tissue, organ or a vascular territory. This peripheral signal is then transmitted to the distal target organ (e.g., brain or heart) to relay protection against prolonged ischemia and subsequent IR injury (14, 15).

In 1986, ischemic preconditioning was described by Murry et al. in relation to cardiac ischemia (16). A preconditioning (PC) intervention was directly applied to the dog heart *via* four cycles (each for 5 min) of alternative occlusion/reflow of the left anterior descending (LAD) coronary artery prior to initiation of 40 min cardiac ischemia (16). Their results showed that PC was associated with a considerable reduction in myocardial infarction size. However, in another animal cohort, the same PC protocol preceding 3 h of sustained coronary occlusion failed to salvage the heart tissue injury, suggesting that PC has a protective time window and it may only delay the cellular death up to a few hours and then dissipates (16). Thereafter, additional investigations advanced the theory of "two time windows for protection" based upon these results (17–19). The early phase of protection occurs immediately, within minutes after the PC application, and lasts for ∼3 h. It is thought that the early phase is mainly caused by rapid alterations in protein kinase signaling pathways that converge on the mitochondria to stop the apoptotic pathways (20, 21). The late phase starts 18–24 h after PC and lasts for ∼4 days. The protection during the late period is probably due to *de novo* synthesis of proteins that are involved in inflammation, ischemia and vascular dynamics (12, 22, 23), and the suppression of genes involved in IR injury.

In 1993 the conditioning concept was extended to remote ischemic conditioning (RIC), in which ischemia is induced to an organ far from the target organ, often using a blood pressure cuff, offering a safe and feasible approach (24).

RIC MODALITIES

The remote application of RIC provides a safe, non-invasive and clinically applicable method, often involving intermittent cycles of inflation and deflation of a blood pressure cuff around the upper arm in humans and upper hind limb in preclinical studies in rodents (25). RIC has been used in three temporal windows during or after cerebral ischemia: (1) remote ischemic preconditioning (RIPreC) is applied prior to the injurious ischemia. While less practical as a therapeutic approach, because the stroke event is not always predictable, RIPreC can be used as a preventive measure for post-operative ischemic complications in known hospital settings. For example, prior administration of RIPreC to patients undergoing endovascular procedure can potentially reduce the high risk of ischemic or haemorrhagic

stroke insult after surgical treatments for several clinical settings including intracranial aneurysms and carotid endarterectomy (26–29); (2) remote ischemic per-conditioning (RIPerC), which is applied during the ischemic event (prior to any recanalization); and (3) remote ischemic post-conditioning (RIPostC), which is applied after the ischemic event (i.e., following recanalization) or during reperfusion. The latter two conditioning paradigms have promise for translation, as they are non-invasive and can be administered pre-hospital (i.e., in an ambulance while transferring stroke victims to the emergency center) or following recanalization therapy (14).

RIC EFFICACY

Several parameters might affect the overall efficacy of RIC in reducing the infarct size following the focal ischemic stroke, including sex, age, animal species and different models of focal ischemia (30). A recent meta-analysis and systematic review has shown that there is no significant difference in RIC beneficial effects between reperfusion (e.g., intraluminal and embolism models) and permanent (e.g., cauterization, use of a permanent clip, permanent distal MCA ligation, permanent intraluminal suture) models of focal brain ischemia (30). However, RIC was shown to be more efficacious in male rodents relative to their female counterparts (30). As expected, older animals show significantly larger ischemic damage when compared with younger adult group due to several factors, namely, rarefaction of cerebral collaterals, decreased arteriole dimeter, higher tortuosity in cerebral vessel (31–33). All these factors affect the aged animals' ability to compensate for the poor blood flow circulation. On the other hand, the cellular and biochemical alterations associated with aging process, such as higher expression levels of pro-inflammatory cytokines and exacerbated oxidative stress, will reduce the cell survival rate in aged population and increases the neuronal cell injury and death (34, 35). Consequently, aged stroke groups may benefit less from the neuroprotective effects of RIC and may show more limited functional recovery in both pre-clinical and clinical settings (36).

RIC: UNDERLYING MECHANISMS OF ACTION

Extensive research has been conducted in the preclinical and clinical settings to investigate the underlying mechanisms of RIC. Still, the primary molecular pathways are somewhat equivocal, possibly due to the contribution of several complex and overlapping signaling pathways. Although much of the research to date focuses on the protective role of RIC on cardiomyocytes in the heart, there is a growing focus on brain ischemia, and the underlying mechanisms of cardio- and neuro protection likely overlap. Multiple hypotheses have been proposed on how the protective signal is transferred from the periphery to the target organ. Generally, three pathways are thought to play a role in RIC protection (**Figure 1**).

Neurogenic Pathway

Modulation of autonomic nervous system has been shown to play a key role in RIC-induced distant organ protection in both experimental and clinical studies (37–39). In a rat model of cerebral ischemia investigating the neurogenic mechanism led to the neuroprotective effects of RIPreC, pharmacological inhibition of autonomic ganglia with hexamethonium (a ganglionic blocker) reversed the reduction in cerebral infarct size in animals undergoing RIPreC, thereby indicating the potential role of neural pathways in relaying the protective signals generated by conditioning stimulus (37).

Humoral Pathway

Following the conditioning stimulus, blood-borne molecules are released into the circulation and then travel from the remote site toward the target organ to exert their protective functions. The humoral nature of the conditioning signal is supported by several lines of evidence: First, every brief ischemic cycle is followed by a brief reperfusion cycle. This allows the factors secreted during the ischemic conditioning to flow in the bloodstream toward the target organ; Second, cross-individual blood transfer from a conditioned subject to an unconditioned control can confer the protection against injury in preclinical models (40). Recently, Pickard et al. reported that there is an interdependence between the neural and humoral pathways in mediation of cardioprotection following RIC (41). In other words, the secretion of circulatory factors may rely on the prior firing of the vagal nerves and stimulation of autonomous nervous system (41), and the humoral release of some factors may lead to the activation of sensory afferent nerves. For example, the release of autacoids at the site of remote ischemia may initiate neuronal and humoral signal transduction, and contribute to the protective effects of RIC (41). Prominent autacoids such as adenosine, bradykinin, catecholamines, opioids, and prostaglandins are secreted locally in the conditioned limb. Some autacoids can stimulate the afferent neural pathways, while others, such as nitric oxide and endothelin (ET), are mainly characterized by vasoactive effects on the blood vessels (14).

Immune-Mediated Pathway

Neuroinflammation involves the activation and release of proinflammatory mediators from the brain resident immune cells (microglia and astrocytes) as well as the peripherally derived immune cells, such as neutrophils, monocytes, and T cells (42–44). Evidence suggests that RIC can inhibit not only the activation of microglia and astrocytes following an acute ischemic stroke but also the recruitment of circulating peripheral immune cells into the ischemic brain (45). Several studies have shown that RIC can reduce the infiltration of leukocytes in the brain, and therefore alleviate the inflammatory status in the brain. Considering the integral presence of leukocytes during cerebral ischemia, modulation of leukocyte gene expression by RIC is probably inevitable (46); however, limited studies have focused on the regulatory effects of RIC on leukocyte gene transcription (46). In the setting of cerebral ischemia, emerging evidence

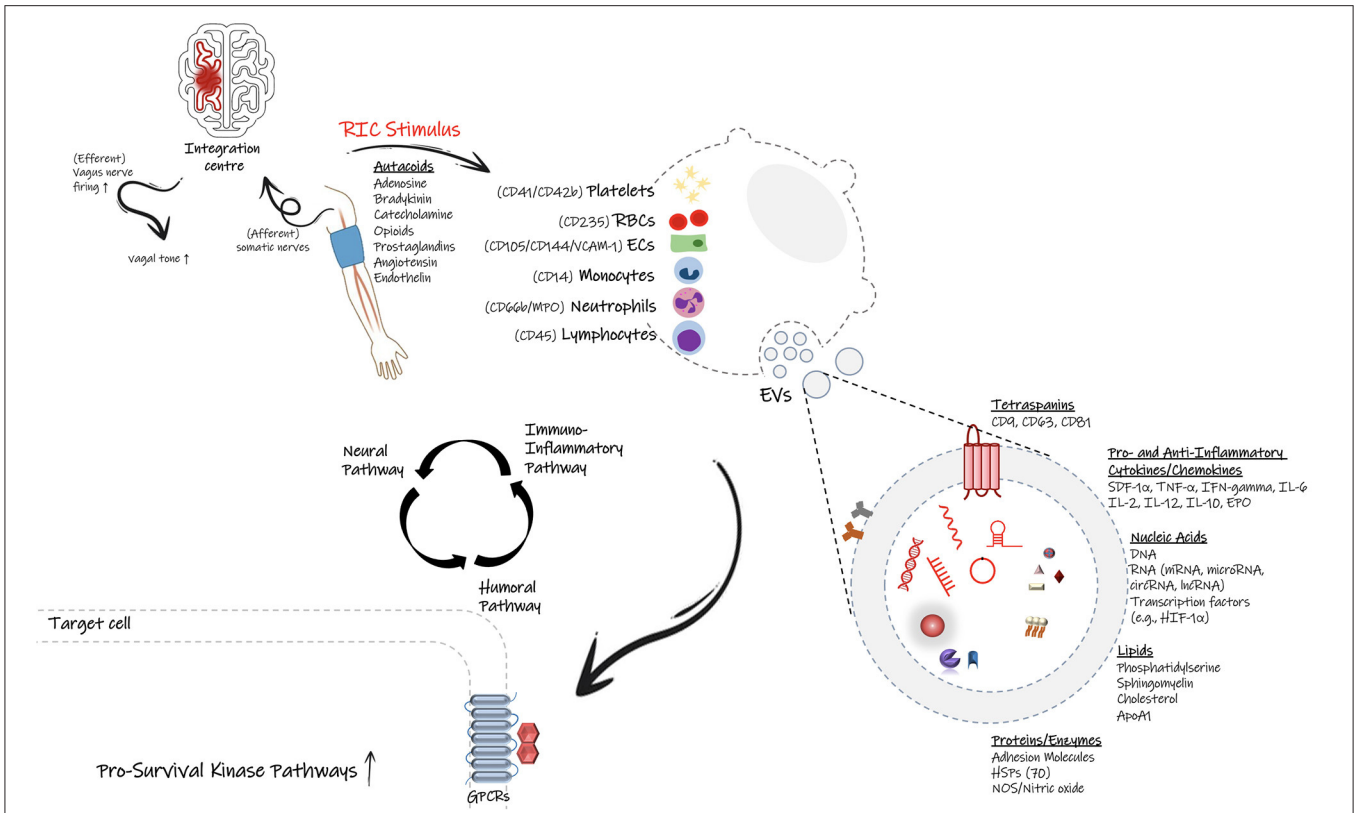

FIGURE 1 | Proposed signal transmission pathways during remote ischemic conditioning (RIC). Following application of RIC protocol on the upper arm, the initiated protective signal appears to involve a complex and overlapping activation of neural pathways (autonomic nervous system), humoral mediators, and the peripheral immune system. It is proposed that small endogenous particles called extracellular vesicles (EVs) can facilitate the transfer of protective effects of RIC through the flow stream. Many cell types including endothelial cell, immune cells as well as platelets can generate EVs with signature surface markers and cargo defined based on the parent cell and the physio/pathophysiological conditions. EVs can carry cytokines, chemokines, genetic material and many more biological substrates, which allow them to inter-connect distant cells, tissues or organs and affect the target cells' transcriptional profiles and likely their function and phenotype. Based on the stimulus, they can deliver either proinflammatory or anti-inflammatory factors, therefore modulate the immune response and the fate of recipient cells.

suggests systemic immune cell responses change during RIC-mediated neuroprotection, which will be further discussed later in this review.

RIC: COLLATERAL BLOOD FLOW ENHANCEMENT

In addition to direct effects on target organs, RIC has direct effects on improving blood flow in vulnerable tissue. Preclinical stroke studies in mice suggest that RIPerC is effective alone and in combination with i.v. r-tPA in enhancing penumbral flow in young male mice, ovariectomized female mice, and 12-month old male mice (14, 47–49). Remote ischemia has also been associated with increased cerebral blood flow in humans (50–54).

The collateral circulation is a key determinant of infarct progression in AIS. Good collateral flow is associated with reduced infarct expansion and better stroke outcome (55–64). However, a progressive constriction of collateral arterioles over time after ischemic onset may contribute to infarct growth (36, 65, 66). RIC may improve collateral flow by preventing narrowing of key collateral vessels, and is associated with improved collateral flow and reduced infarct in preclinical studies (4, 65, 67–69). Thus, preventing collateral failure is thus critical to improve outcome in stroke patients, and RIC may improve collateral flow. However, the exact mechanisms of enhanced collateral flow due to RIC are not defined.

RIC may increase cerebral blood flow (CBF) either through formation of new vascular branches (angiogenesis and/or arteriogenesis) or strengthening of the existing vasculature. Some of the major signaling mediators of CBF enhancement are discussed below.

The eNOS/NO/Nitrite System

Nitric oxide (NO) is a key regulator of vascular tone and blood flow in the brain (70). NO is primarily generated *via* enzymatic function of three types of nitric oxide synthase (NOS), namely endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (70). Following an ischemic insult, nNOS is activated soon after elevation of intracellular Ca2+ levels and produces NO to regulate cerebral vascular tone and blood flow (71). Afterwards, NO derived from eNOS in vascular endothelium contributes to flow-mediated vasodilation

(71). Available evidence suggests that NO released during a brief period of ischemia (produced by nNOS and eNOS) may play a neuroprotective role against prolonged focal ischemia, mainly through formation or strengthening of collateral vessels in order to maintain the cerebral microcirculation, as well as preventing platelet aggregation. In addition, NO is the main driver of blood flow through the collateral circulation toward the site of injury (71). By contrast, large amount of NO produced by iNOS is associated with neurotoxic effects, such as lipid peroxidation, reaction with superoxide (O2●-) to form peroxynitrite (OONO–), and protein nitrosylation. Nevertheless, iNOS is only expressed when it is induced by proinflammatory factors (70).

In the field of stroke research, several studies have demonstrated RIC neuroprotection has been associated with collateral flow enhancement. For instance, in a rat model of chronic cerebral hypoperfusion (CCH, bilateral common carotid arteries were ligated), RIC treatment (3 cycles of 8-min occlusion/release of bilateral hindlimbs, for 28 days) significantly augmented cerebral perfusion measured by laser speckle contrast imaging (LSCI) at day 14 after CCH onset, compared to non-treated group (72). The results were associated with an increased number of vessels in hippocampus, and accordingly a better learning capacity and spatial memory ability in RIC-treated rats. Mechanistically, based on western blot data RIC caused neuroprotection through preservation of eNOS activity (i.e., by promoting neovascularization in hippocampus); however, a NOS inhibitor (L-NAME) abolished all the RIC protective effects (72). As mentioned earlier, NO is primarily derived from eNOS; moreover, Nitrite can also be a prominent source of NO, which circulates in the bloodstream with RBC/hemoglobin and it is reduced to NO, especially in response to ischemic insult to mediate vasodilation (73). In a mouse bilateral CCAO model, Hess and coworkers noticed a dramatic increase in plasma nitrite after 2 weeks of daily RIPostC treatment, 4 cycles of 5 min occlusion/reopening of both hindlimbs, started 7 days after bilateral CCAO, and rise in nitrite level was correlated with augmented CBF (47). Altogether, literature suggests that NO and nitrite are two signaling molecules that play a key role in RIC mechanism of neuroprotection, in particular *via* strengthening the collateral vessels and maintenance of cerebral microcirculation (47, 72).

Notch Signaling Pathway

There is also evidence that stimulation of Notch1 signaling pathway by RIC can mediate neuroprotection. Preclinical experiments in rats with focal cerebral ischemia demonstrated a higher rate of arteriogenesis in brain sections from RIC (RIPerC + RIPostC)-treated rats, as indicated by increased arterial diameter and more proliferative (BrdU+) smooth muscle cells in peri-ischemic core when compared to non-treated MCAO rats (74). Additionally, RIC improved local CBF on the cortical surface supplied *via* leptomeningeal collateral anastomoses. Increased arteriogenesis induced by RIC was correlated with activation of Notch 1 signaling, as the expression of Notch receptor and its intracellular domain (NICD) was significantly elevated in ischemic arteries by RIC (74). Therefore, RIC-induced arteriogenesis and increased cerebral perfusion through enhanced collateral branches can be somewhat attributed to the activation of Notch signaling pathway (74).

VEGF/VEGF Receptor Signaling Pathway

Vascular endothelial growth factor (VEGF) signaling is another possible mechanism underlying the effect of RIC on cerebral blood flow. VEGF is known to modulate vascular tone after binding to its corresponding tyrosine kinase receptor on the vascular endothelium thereby promoting the release of vasodilatory compounds such as prostacyclin and NO (75, 76). Elevated levels of NO in response to VEGF binding to VEGF receptor type 2 (VEGFR2), can induce angiogenesis and regulate the endothelial function and migration (76). Some studies have identified that VEGF mRNA and protein expression level is up-regulated following RIC treatment (77, 78). In a mouse model of spinal cord ischemia, ischemic preconditioning applied to the abdominal aorta (3 × 5 min of alternative clamping and reperfusion) resulted in high VEGF protein levels in plasma, with a resultant neuroprotective effect (78). Although early increase in VEGF/VEGFR expression can cause BBB permeability and exacerbate the ischemic injury, later up-regulation at the border of ischemic core can increase the number of capillaries (neovascularization) and restore the cerebral microvascular circulation after stroke (79).

RIC: REGULATION OF CELL SURVIVAL AND APOPTOSIS SIGNALING

RIC increases tolerance and viability of brain tissue during cerebral ischemia by activating signaling that supports survival and inhibits apoptosis, and by reducing inflammation. Several protective signaling pathways and pro-survival kinases and mediators have been shown to be involved in RIC-induced protection (**Figure 2**). The two most widely studied pathways are (1) the reperfusion injury salvage kinase (RISK) pathway, with its major signaling *via* Akt and Erk1/2, and (2) the survivor activating factor enhancement (SAFE) pathway, with its major signaling *via* Janus Kinase (JAK) and signal transducer and activator of transcription 3 (STAT-3) (15, 80–83). These signaling pathways can be triggered by a variety of factors, including SDF-1α, MIF, HIF-1α, heat shock proteins (HSPs), nitric oxide (NO), mammalian target of rapamycin, MMPs, adenosine, bradykinin, erythropoietin (EPO), endocannabinoids, and tumor necrosis factor-α (TNF-α) (15, 80, 84), and are discussed in greater detail below. Furthermore, AMPK signaling pathway is increasingly recognized as a potential mediator of cell survival following RIC, and is discussed below (85–88).

Risk Pathway

The RISK pathway is a possible protective signaling cascade through which RIC may exert its protective effects against reperfusion injury, *via* the activation of pro-survival kinases in two parallel signaling cascades—phosphoinositide-3 kinase (PI3K)/Akt and MEK1/2-ERK1/2 (80, 81, 83, 89). The RISK pathway was described by Schulman et al. when examining the

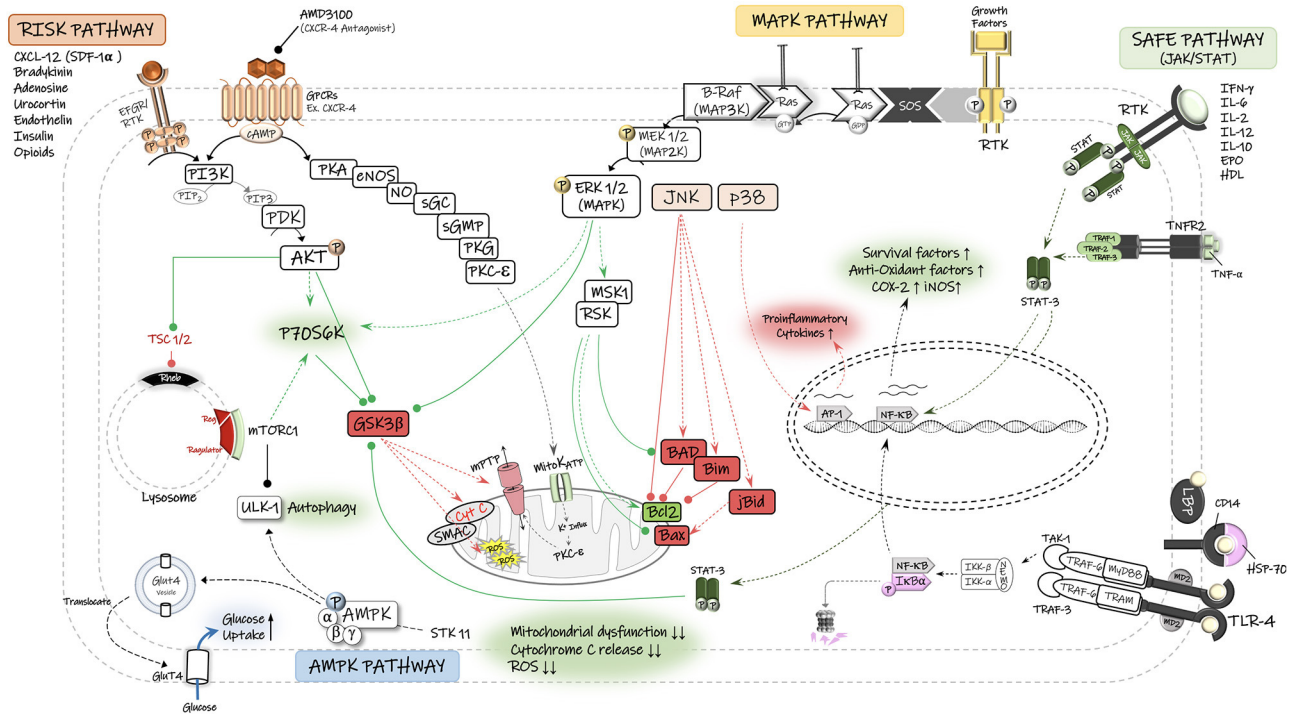

FIGURE 2 | Schematic diagram of the potential signaling pathways involved in neuroprotective effects of remote ischemic conditioning (RIC). The reperfusion injury salvage kinase (RISK) pathway and its major pro-survival kinases, including PI3-K, phosphatidylinositol 3-kinase; PDK, phosphoinositide-dependent protein kinase; AKT/PKB, protein kinase B. The survivor activating factor enhancement (SAFE) pathway and its major signaling components, including STAT, signal transducer and activator of transcription, JAK, Janus kinase. The Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) cascade is activated through ligand binding to surface receptor. MAPK signal transduction results in activation of mitogen and stress activated kinase 1 (MSK1) and pp90 ribosomal S6 kinase (RSK), thereby leading to inactivation of the pro-apoptotic protein Bad and up-regulation of anti-apoptotic proteins of the Bcl-2 family. There is also an interplay between MAPK and RISK pathways that together with SAFE pathway converge on the mitochondrial permeability transition pore (mPTP) and prevent its opening and the consequent release of cytochrome C (Cyto C) and reactive oxygen species (ROS) into the cytosol. Under stress (Ischemia/hypoxia) conditions and low energy levels (↑AMP:ATP), the cell energy sensor adenosine monophosphate-activated protein kinase (AMPK) gets phosphorylated and activated by its upstream serine/threonine kinase 11 (STK11) and enhances the translocation of glucose transporter GLUT4 to cell membrane. AMPK-dependent autophagy contributes to functional recovery and neuroprotection. Autophagy is also regulated by the mammalian target of rapamycin (mTOR) complex-1. TNF-α, tumor necrosis factors-alpha; TNFR2, tumor necrosis factors receptor type-2; RTK, receptor tyrosine kinase; mitoKATP, mitochondrial ATP-sensitive potassium channel; GSK3β, glycogen synthase kinase 3 β; GPCR, G protein-coupled receptor; P70S6K, p70 ribosomal S6 protein kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; cGMP, cyclic guanosine monophosphate; NO, nitric oxide; Ulk, autophagy-initiating kinase; TSC, tuberous sclerosis complex; Rheb GTPase, direct mTORC1 activator; AP-1, activator protein-1; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; TLR-4, toll-like receptor-4.

cardioprotective potential for the growth factor urocortin on both isolated and *in vivo* models of acute myocardial infarction in adult rats (89). Urocortin is a peptide which belongs to the corticotropin-releasing factor (CRF) family and can regulate the mitogen activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway (89, 90). A significant reduction in the myocardial infarct size following the urocortin administration at reperfusion has been reported, which was associated with a notably higher levels of phosphorylated (ERK1/2) MAP kinase. This protection was not altered by the inhibition of other subfamilies of MAP kinases, p38 MAPK and Jun N-terminal kinase (JNK), suggesting that ERK1/2 (MAPK1) is responsible for the cell survival, while p38 and JNK are part of the death pathway in the ischemic setting (89). In a rat model of neonatal hypoxia-ischemia (HI), RIPostC reduced cerebral infarct size in an opioid-mediated activation of PI3K/Akt/Bax signaling pathway, as pharmacological inhibition of PI3K (*via* wortmannin) or opioid receptor (*via* naloxone) decreased the phospho-Akt expression levels and abrogated the infarction reduction and improved neurological outcomes achieved by RIPostC treatment. The serine/threonine kinase Akt (also known as protein kinase B) acts as an effector protein of PI3K pathway and it phosphorylates several downstream targets, including GSK-3β, Bad, and Bax (91). Akt-induced phosphorylation of Bax at Ser-184 reduces its half-life and inactivates its insertion into mitochondrial membranes, therefore blocking Bax-mediated proapoptotic pathways (91).

Activation of the RISK pathway following the ischemic conditioning, and phosphorylation of downstream effectors is believed to cause tissue protection *via* preventing mitochondrial permeability transition pore (mPTP) and inhibiting the release of cytochrome C into the cytosol and thereby activation of caspases and apoptosis will not be initiated (80).

Safe Pathway

Another signaling pathway involved in RIC-induced protection is the survivor activating factor enhancement (SAFE) pathway, with the participation of key proteins Janus Kinase 2 (JAK2) and the signal transducer and activator of transcription (STAT-3 and−5) (81–83). As demonstrated by Heusch and colleagues, RIPreC application in patients undergoing coronary artery bypass surgery increased the phosphorylated form of STAT-5 (pSTAT-5) in myocardial biopsies (92). In agreement, treatment of isolated rat and mice hearts (93) with either Tryphostin AG490 (JAK Inhibitor) or PPI (Src kinase blocker), which both inhibit the phosphorylation of STAT5a *via* the upstream kinases (JAK and Src kinase), abolished preconditioning-mediated cardioprotection. Moreover, preconditioning protection cannot be achieved in STAT5a-deficient (knock-out) mice (93) and genetic depletion of functional STAT-3 in mice cardiomyocytes prevented preconditioning-induced protection against the ischemic injury (94). In a pig myocardial IR injury model, Heusch et al. demonstrated that RIPostC activates the mitochondrial STAT-3 in the heart that preserves the function of mitochondria in cardiomyocytes and confers cardioprotection against IR injury, and pharmacological inhibition of STAT-3 abolished the effects (95).

There is evidence that SAFE and RISK pathways can confer tissue protection independently from each other. Lecour et al. reported that "pharmacologic" preconditioning of rat hearts (subjected to 30-min regional IR injury) with a low dose of TNF-α injection confers the same cardioprotective properties as "ischemic" preconditioning (96). The activation of TNF-α receptors triggers phosphorylation of STAT-3 by either JAKs or MAPKs and initiation of cell survival pathways (96). In this study, despite blocking different components of RISK pathway, including PI3K (*via* wortmannin), MAPK-Erk1/2 (*via* PD-98059), and mTOR (*via* rapamycin), STAT-3 expression did not change and cardioprotection achieved by TNF-α preconditioning was not abrogated, indicating the independent function of SAFE pathway (96).

Conversely, Tamareille et al. indicated that there is a crosstalk between the RISK and SAFE pathways in RIPerC alone, local IPostC (with conditioning and ischemia in the same target organ) alone, and combined RIPerC + IPostC in rat myocardial IR injury model (83). They confirmed this interaction since cardioprotective effects against reperfusion injury were fully abrogated *via* the pharmacological inhibition of either RISK (with wortmannin, an inhibitor of PI3 K/Akt signaling pathway, and with U0126, an inhibitor of MEK1/2) or SAFE (with AG490, an inhibitor of JAK/STAT pathway) (83). In other words, inhibitors of RISK abrogated the phosphorylation of STAT-3, and inhibitor of SAFE (AG490) blocked the phosphorylation of survival kinases from RISK (Akt, ERK1/2, and GSK-3β) pathway (83).

Emerging evidence indicates that these pro-survival signaling pathways (SAFE and RISK) potentially converge on the mitochondrial permeability transition pore (mPTP), high-conductance channel proteins located in the mitochondrial inner membrane important in cell death signaling (97, 98). In case of excessive calcium entry or high ROS exposure under ischemia/hypoxia conditions, the opening of mPTP allows the release cytochrome C into the cytosol and that can lead to the cell death (99). Therefore, inhibition of mPTP opening supports cell survival under pathologic conditions (99). The cytoprotection received through the phosphorylation of key prosurvival kinases of RISK (Akt, Erk1/2, GSK-3β) and SAFE (STAT3) pathways is dependent on the inhibition of mPTP, suggesting a key protective role for this pathway in molecular signaling induced by RIC (97).

AMPK Pathway

Compelling evidence has suggested that AMPK signaling can contribute to RIC-mediated neuroprotection against the cerebral IR injury (88). AMPK (5'-AMP-activated protein kinase) is a member of the serine/threonine (Ser/Thr) kinases and an early energy sensor that responds to stressful stimuli such as ischemia/hypoxia and energy deprivation (100). Under low energy conditions, higher activation of AMPK signaling pathway contributes to elevated glucose uptake and utilization in neurons. We have recently shown that RIPerC-mediated neuroprotection and collateral flow enhancement in a rat model of focal ischemia is associated with an increase in pAMPK/eNOS activity (86). AMPK is considered to be a direct activator of eNOS/NO system. Hence, the improved cerebral blood flow in RIPerC-treated rats can be attributed to AMPK-mediated eNOS activation and NO production, resulting in vascular relaxation and flow increase (86, 101).

There is also evidence that AMPK reduces the ischemic injury by triggering autophagy (catabolic) pathways in several organs, including heart (102–105) and kidney (106). However, the extent to which AMPK-induced autophagy plays a protective or destructive role in conditions of cerebral ischemia is unclear (107, 108). In general, autophagy serves as a prosurvival/cytoprotective mechanism during metabolic stresses and protects the cell through degradation of damaged organelles and aggregated proteins into basic biomolecules, which are then recycled for energy regeneration (107). Up-regulated autophagy processes through AMPK-related signaling have been associated with suppressed neuronal apoptosis and alleviated cerebral ischemic damage (88). In a mouse model of cerebral ischemia, RIPostC, applied *via* 3 cycles of 10-min occlusion/reopening of bilateral femoral arteries at the time of reperfusion following 2 h MCAO, was associated with improved neurological outcome as well as a smaller infarct size (88). However, neuroprotective effects of RIPostC were abolished when mice were given the autophagy inhibitor 3-methyladenine (3-MA) prior to RIPostC treatment and partially abolished when mice received compound C, an AMPK inhibitor, indicating RIPostC mediated neuroprotection *via* activation of AMPK-dependent autophagy (88). In addition, anti-apoptotic properties of RIPostC were abrogated by 3-MA treatment, as indicated by up-regulation of apoptotic agents like Bax and caspase-3, and downregulated anti-apoptotic Bcl2 (88). Liu et al. demonstrated that metformin-treated mice had reduced brain injury after 90-min MCAO (109). Metformin is a glucose-lowering medication for type 2 diabetes (110) that can protect against the inflammation and endothelial dysfunction associated with the cerebral ischemia reperfusion injury through the activation of AMPK signaling pathway (109). Metformin

alleviates cerebral I/R injury through activation of AMPK-dependent anti-inflammatory mechanisms including AMPK-induced suppression of NF-κB pathway, reduced expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α) and adhesion molecules (ICAM-1), reduced neutrophil infiltration, and reduced endothelial injury and BBB permeability (109).

RIC: HUMORAL MEDIATORS

The alternative hypothesis to the "signal transfer through the activation of neural pathway" is that the protective signal generated locally in the remote site (like a limb) may have humoral (blood-borne) nature. Several blood-borne mediators have been identified as important for protection *via* RIC. These factors can travel *via* the circulation toward target tissue, wherein they can modulate inflammation and cell death.

SDF-1α

The chemokine stromal-derived factor-1 alpha (SDF-1α, also termed as CXCL12) is one of the most studied humoral factors involved in cerebral and cardiac ischemic conditioning (84, 111–113). It is classified as an atypical cytokine that binds to the G-protein-coupled CXCR4 receptor, which has an abundant expression on the endothelial cells (114). SDF-1α binding to CXCR4 activates several down-stream signaling pathways (115) including the Gi-protein/Src/PI3K-Akt-NF-κB and the PKC pathways that activate the Ras/Raf/MAPK axis (115). Activated MAPK p42/44 (Erk1/2) will in turn translocate into the nucleus and mediates transcriptional activation (115) (**Figure 2**). Activation of JAK proteins and recruitment of STAT transcriptional factors may also be associated with the activation of SDF-1α-CXCR4 axis in some cell types (116). SDF-1α stimulation can lead to the activation of nuclear factor (NF)-κB, well-known for its role in inflammation (115). Following translocation into the nucleus, NF-κB binds to specific DNA sequences in the promoter region of critical mediators of immune and inflammatory responses and regulates their gene expression levels (117). Activated NF-κB also enhances the expression of anti-apoptotic target genes (118, 119) and the expression of target genes that encode for antioxidant proteins, reducing necrotic cell death (119).

Of particular relevance to the ischemic conditioning-induced tolerance is the finding that preconditioning treatment with SDF-1α could induce the activation of antiapoptotic pathways and protect cardiac myocytes from hypoxia/reoxygenation damage (112). Hu et al. reported that infusion of SDF-1α into the left ventricular cavity of mice prior to 30 min of LAD coronary artery occlusion enhanced cell survival equivalent to ischemic preconditioning (112). Ischemic PC, which was applied *via* 6 cycles of 4-min occlusion/reopening of coronary artery, was associated with a 4.5-fold increase in SDF-1α mRNA transcript level and a significant reduction in the myocardial infarct size. Incubation of isolated mice cardiomyocytes with SDF-1α significantly increased the phosphorylation of Erk and Akt within 5 min; conversely, JNK and p38 phosphorylation sharply declined (112). Notably, pretreatment of cultured myocytes with specific antagonist of CXCR4 (AMD3100) prior to SDF-1α exposure prevented the protective effects of SDF-1α on myocyte survival, suggesting SDF-1α-CXCR4 binding mediates anti-apoptotic mechanisms of preconditioning (112).

MIF

Macrophage migration inhibitory factor (MIF) is another putative candidate for the "remote signal" in the conditioning paradigm (120–122). MIF is a pleiotropic chemokine-like inflammatory cytokine that acts as a key regulator of innate and adaptive immunity states such as neuroinflammation and the stress response (123). In response to proinflammatory stimuli, MIF is released from cytosolic pools of almost all types of immune cells into the circulation rather than being rapidly upregulated at the transcriptional levels (123). MIF can engage several different receptors as well as intracellular binding partners, and thereby exert varied biological functions (123). MIF can not only bind to its cognate cytokine receptor (CD74), but also it can be a non-cognate ligand for the CXCR2 and CXCR4. CXCR2 is dominantly found on the surface of neutrophils and monocytes/macrophages, while CXCR4 is ubiquitously distributed on many cell types (123, 124). There is evidence that MIF binds to CXCR2 and 4 with high affinity and provokes the recruitment of leukocytes to the site of inflammation (125, 126). Of note, MIF binding to CD74 can initiate the formation of a functional complex comprising CD74, proteoglycan CD44 and Src kinases, which then leads to the sustained activation of the Erk1/2 MAPK pathway (127), giving rise to different regulatory effects on expression of downstream transcription factors including Elk-1, AP-1, and cMyc (127). MIF can also interact with intracellular binding proteins, such as JUN-activation domain-binding protein 1 (JAB1), through which it can block the activity of JNK pathway and its target transcription factor AP-1 (128). Recent studies implicate that MIF may play a mediatory role in conditioning-induced protection (120, 121). In an animal study of ischemic heart disease, ischemic PC (3 cycles of 5-min myocardial ischemia/reperfusion) mediated cardioprotection against the prolonged 60 min ischemia/3 h reperfusion injury in wild-type (WT) mice (120). However, ischemic PC had no protective effects in mice with MIF-knock out (MIFKO), suggesting that MIF released from the preconditioned myocardium mediates the PC protection. Notably, PC lowered the density of infiltrated inflammatory cells [by 35% in CD45+ cells (leukocytes) and 63% in CD68+ cells (macrophages)] in the WT, but not in MIFKO hearts (120). Western blotting data revealed a noticeable increase in phosphorylation and activation of RISK and AMPK signaling components in PC-treated WT hearts. PC significantly increased p-Erk1/2, p-Akt, p-p70S6K, and p-GSK3β (proteins involved in RISK pathway), AMPK phosphorylation, cell-surface GLUT-4 translocation and glucose uptake. MIF deficiency abrogated all these effects of PC, indicating that MIF is exerting its role through activation of RISK and AMPK pathways (120).

ApoA1

Apolipoprotein (ApoA1) is the main structural constituent of high-density lipoproteins (HDL). The plasma levels of HDL

cholesterol is inversely correlated with the risk of cardiovascular diseases (CVDs) (129), meaning that patients with low HDL levels are more prone to CVD incidence, particularly to the formation of atherosclerotic plaques. Clinical strategies to increase HDL levels can lower the risk of CVDs (129, 130). In addition to anti-atherogenic properties of HDL and ApoA1, both have proven to be protective by modulating anti-inflammatory, anti-oxidative, and antiapoptotic pathways. Moreover, compelling evidence suggests that ApoA1 may act as a humoral mediator of RIC through induction of pro-survival signaling pathways. The plasma levels of ApoA1 have been shown to be upregulated in response to RIC in animals and humans (131, 132). Kalakech et al. suggested that ApoA1 may be involved in the protection conferred by RIC, based on data showing i.v. administration of ApoA1 prior to prolonged myocardial ischemia (MI, 40-min occlusion of coronary artery in rats) could mimic cardioprotection achieved by RIPreC (81). ApoA1-treated rats *in vivo* showed a significant reduction in myocardial infarct size along with an increase in phosphorylation and activation of RISK and SAFE signaling, including Erk1/2, Akt, and GSK3β. However, pretreatment with either Wortmannin (a PI3K/Akt pathway inhibitor) or U0126 (MEK1/2-ERK1/2 pathway inhibitor) prior to ApoA1 administration markedly abolished the cardioprotective effects of ApoA1 (81) (**Figure 2**). Furthermore, acute injection of ApoA1 exhibited anti-inflammatory properties, including lower infiltration of leukocytes to the infarcted area, downregulated adhesion molecules (e.g., ICAM-1) and hence lower leukocyte-EC interaction and adhesion, lower expression of pro-inflammatory cytokines (e.g., TNF-α and IL-6). Taken together, experimental and clinical data suggests that ApoA1 may contribute to the RIC protection during MI through activation of anti-apoptotic proteins and modulation of inflammatory response.

TNF-α

Tumor necrosis factor-alpha (TNF-α) has been identified as an essential contributor to the induction of ischemic tolerance. TNF-α is small (17 kDa) inflammatory cytokine produced by macrophages, monocytes, neutrophils, mast cells, T and B lymphocytes upon stimulation (e.g., ischemic injury) during acute phase of inflammation (133).

Intriguingly, the function of TNF-α in the cerebral ischemia and ischemic conditioning is controversial. Pathophysiological levels can not only compromise the integrity of BBB and exacerbate the inflamed brain injury, but also can activate the pro-apoptotic factors and caspases and cause cell death. On the other hand, genetic deletion of TNF receptors in mice prior to focal stroke has shown to increase neuronal cell death because of higher oxidative stress and suppressed microglial reactivity, implicating TNF-α as a neuroprotectant in ischemic brain (134).

TNF-α can exert pleiotropic effects by signaling through two types of TNF receptors, either TNFR1 or TNFR2. TNF-α binding elicits complex signaling cascades that varies according to receptor subtype. Given the neurotoxic and neuroprotective signaling elicited by TNFR1 and TNFR2, respectively, the ratio of TNFR1:TNFR2 may be a key determinant in TNF-α overall effects (135). There are two bioactive forms of TNF: transmembrane TNF (tmTNF) and soluble TNF (solTNF) (133, 136). While tmTNF can activate both TNFR1 and TNFR2, solTNF can only signal through TNFR1 that is widely expressed on almost all cells (133, 136). TNFR1 activation triggers the recruitment of TNFR1-associated death domain protein (TRADD), which in turn can initiate two different signaling cascades regulating both cell survival and apoptosis (133, 136, 137). TNF-α can confer resistance to cell death through formation of protein complex I, where TRADD recruits TNFR-associated factor 2 (TRAF2) and leads to the stimulation and activation of NF-κB transcription factor. Translocation of NF-κB into the nucleus can promote the transcription of protective genes, including antioxidant enzyme Mn-superoxide dismutase (Mn-SOD) and calcium chelator calbindin (135). Therefore, TNF-α can modulate reduction of reperfusion injury by binding to TNF receptors and triggering the upregulation of antioxidant activity through NF-κB-dependent dismutase Mn-SOD synthesis. Alternatively, TRADD can induce programmed cell death *via* formation of complex II through interaction of Death Domain (DD) sequence of TNFR1 with Fas-associated death domain protein (FADD) and caspase 8 (138). Unlike TNFR1, TNFR2 does not contain DD and its expression is confined to regulatory T cells (Tregs), endothelial cells and some subset of cells in CNS. TNFR2 directly engages TRAF2 and activates pro-survival (PKB/Akt) and NF-κB pathways (139).

Several preclinical and clinical studies have provided evidence supporting the neuroprotective role of TNF-α and its upregulation following the conditioning stimulus (140–142). Therefore, mild elevation of TNF-α during ischemic conditioning can induce protective properties by neutralizing the oxidative insult and enhancing cellular defense mechanisms against severe ischemic attack (137, 143).

RIC: IMMUNE-MEDIATED NEUROPROTECTION

Preclinical and clinical evidence suggests that RIC confers neuroprotection in the setting of AIS (144). A growing number of studies are suggesting part of this effect might be due to differential responsiveness of peripheral circulating immune cells following the conditioning stimulus (140, 145). The immune response to ischemic conditioning is itself composed of molecular, cellular, and systemic mediators that may play a role in conditioning tolerance (145). The conditioning stimulus can prime the brain in advance by mobilizing both innate and adaptive immune responses so that by the time severe IR injury happens, the brain enters the "resolution of inflammation" or "recovery phase." Thus, immunomodulation may contribute to conditioning-induced protection in brain and heart (140, 145–147) (**Figure 3**).

Several studies have demonstrated that molecular and cellular profile of inflammation change following the conditioning stimulus (140). In a rat model of focal ischemia, Liu et al. reported that RIPreC-mediated neuroprotection was associated with altered immune cell populations and cytokine profiles (140). In this study, a shift in the phenotype of splenic

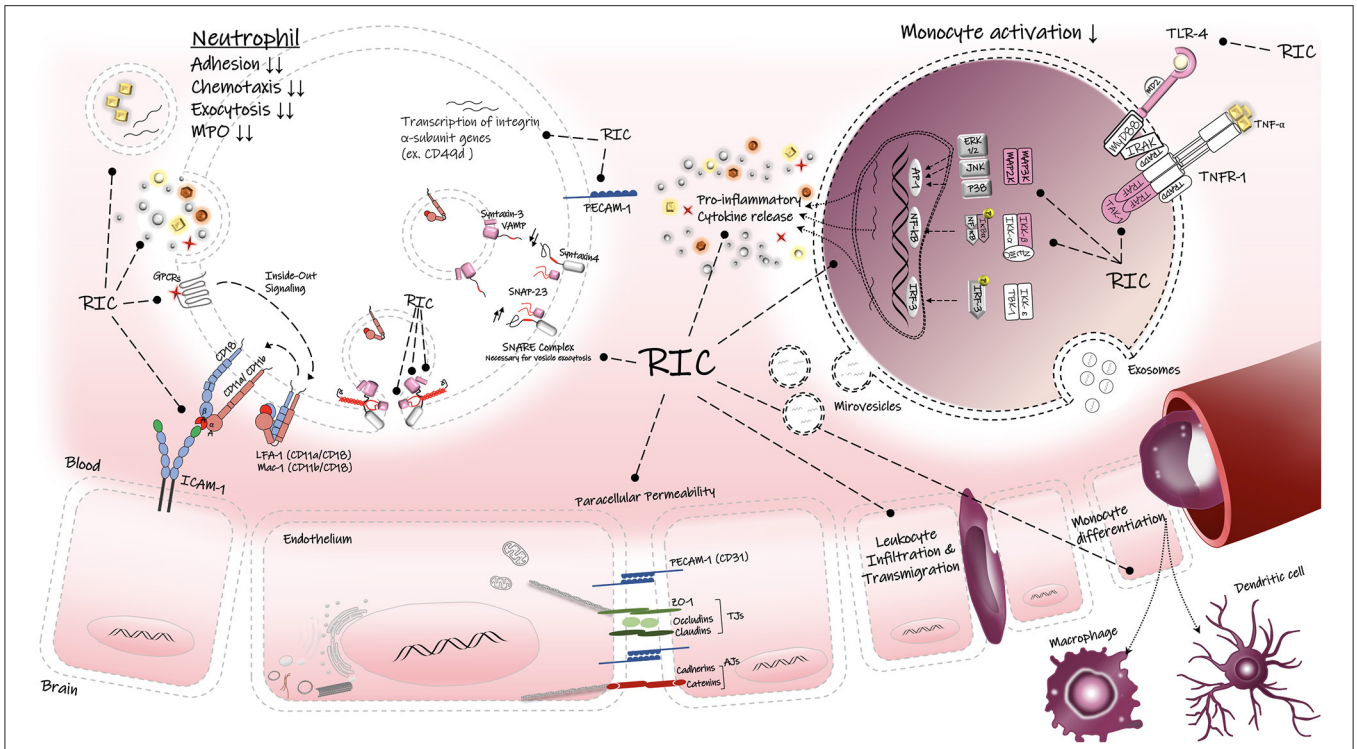

FIGURE 3 | Regulatory effects of RIC on inflammatory responses. RIC can decrease the peripheral immune activation, infiltration and transmigration into the brain parenchyma. RIC can suppress the TLR-4/NF-κB associated proinflammatory cytokine release in the immune cells. LFA-1, lymphocyte function-associated antigen-1; Mac-1, macrophage-1 antigen; ICAM-1, intercellular adhesion molecule-1; PECAM, platelet endothelial cell adhesion molecule-1; Z0-1, zonula occludens-1; AJs, adherens junctions; TJs, tight junctions, TNF-α, tumor necrosis factors-alpha; TNFR1, tumor necrosis factors receptor type-1; TLR-4, toll-like receptor-4; SNAP-23, synaptosomal-associatedprotein-23; VAMP, vesicle-associated membrane protein; GPCRs, G protein-coupled receptor; TAK1, transforming factor-β-activated kinase 1; Iκβα inhibitor kappa beta kinase.

monocytes toward less or non-inflammatory (non-classical) monocytes ($CD43^+/CD172a^+$) was observed in the RIPreC-treated rats (140). Monocytes with inflammatory phenotype (classical monocytes) can infiltrate the brain and lead to highly inflammatory type of cell death *via* their potent ability to secrete inflammatory mediators and free radicals and to differentiate into macrophages and dendritic cells (148). Thus, RIPreC-mediated change in favor of non-inflammatory monocytes prior to focal stroke has been beneficial against ischemic attack (140). In addition, the expression levels of proinflammatory cytokines such as TNF-α and IL-6 were significantly elevated by RIPreC, suggesting that conditioning induced manipulation of the immune response may be a key mechanism of protection (140) (**Figure 3**).

Beside the importance of peripheral immune cells in conditioning effect, the brain's resident immune cells, microglia and astrocytes, are also considered to be cellular mediators of conditioning stimulus, as they contribute to resolution of neuroinflammation by promoting immunosuppression (145). These cells can release anti-inflammatory cytokines (e.g., TGFβ and IL-10) to inhibit the inflammatory response. In addition, astrocytes and neuronal cells arrange the "repair and regeneration" phase by producing growth factors such as insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) that promote neuronal sprouting and angiogenesis (145).

Microglia exhibit a high level of TLR4 expression on their cell surface which helps them to initiate the innate immune response. While hyperactivation of these receptors are known to aggravate the inflammatory status, partial activation during a brief ischemic conditioning may confer neuroprotection by priming the brain against severe and prolonged ischemia (149). Pradillo et al. reported that prior exposure to ischemic preconditioning (IPC, 6-min occlusion of bilateral common carotid arteries) can induce immunological tolerance and therefore protect against the permanent MCAO in wildtype (WT) mice with normal TLR4 expression (149), as shown by a better neurologic outcome and reduced infarct size. However, genetic deletion of TLR4 receptors abolished the protective properties of IPC, indicating the importance of TLR4 for activation of innate immunity and induction of ischemic tolerance by IPC (149). They further observed IPC upregulated the protein levels of TNF-α, iNOS, and COX-2, p65 subunit of NF-κβ transcription factor and downregulated inhibitory kappa B alpha (IκBα). These molecular proteins have been suggested to mediate the ischemic tolerance by IPC (42, 149). Likewise, all the results were reversed in TLR4-deficient mice. Taken together, TLR4 signaling pathway mediates the IPC-induced neuroprotection *via* activation of transcription

factor NF-κβ and therefore upregulation of TNF-α, iNOS, and COX-2 (149, 150).

Considering the fact that peripheral and resident immune cell activation can dramatically change the inflammatory status during and after the cerebral ischemia, RIC's ability to modify the immune response and thereby improve the stroke outcome poses this novel treatment as a potential therapeutic adjunct to already approved stroke therapies.

RIC: TRANSCRIPTIONAL ALTERATIONS IN CIRCULATING LEUKOCYTES

Gene expression in circulating peripheral immune cells rapidly changes after AIS, and several studies have utilized gene expression profiling to investigate transcriptomic alterations in these cells (151–153). Genes differentially expressed after stroke in humans including neutrophils and monocytes (151, 152), suggesting neutrophils and monocytes play key roles in the genomic responses of circulating blood cells to AIS (151, 152, 154). Alterations in genomic patterns happens at an early stage (<3 h) after stroke onset, and these rapid changes can be used to make an early diagnosis of AIS in humans (151). Notably, neutrophils are the first immune cells to arrive at ischemic brain tissue and are key contributors to BBB permeability, cerebral edema and brain injury (154). Therefore, therapeutic approaches that target the deleterious aspects of neutrophil activation, including neutrophil-mediated BBB disruption, neutrophil transmigration and infiltration, and their interaction with the neurovascular unit (NVU), may be helpful to reduce brain edema and therefore improve the stroke outcome (155).

The significance of transcriptional gene screening of blood cells lies in its potential to identify and validate specific genes as molecular biomarkers in ischemic stroke diagnosis and prognosis (152, 156). A refined gene expression signature would allow a readily available clinical evaluation by blood test, especially when brain imaging facilities are limited (153). Unraveling the differential expression of transcriptomic profile in the whole blood as well as the isolated immune cell populations would not only help to understand the underlying mechanisms during the stroke pathology, but also aid in the development of novel treatments for stroke. Due to RIC's early potential in reducing brain ischemic infarct caused by severe AIS, evaluation of transcriptome in peripheral blood cells following RIC and its comparison with stroke-related changes in gene expression may provide key mechanistic insight into neuroprotection.

The first study of genome expression in human leukocytes following RIPreC was reported by Konstantinov et al. who found that conditioning stimulus achieved by transient forearm ischemia (3 cycles of 5 min I/R) in healthy individuals significantly downregulated the expression of proinflammatory genes in leukocytes (46). These suppressed genes are known to be responsible for the inflammatory responses, including genes involved in TLR4-signaling, proinflammatory cytokine release (TNF-α), leukocyte chemotaxis and extravasation (PI3KCA), leukocyte adhesion (e.g., integrins, ADAM 8,10, PECAM), and exocytosis and secretory granule release (SNAP-23) (46). In another study by the same research group in 2010, these alterations in human leukocyte gene expression showed strong correlation with functional responses of neutrophils, in particular a significant reduction in neutrophil adhesion and phagocytosis ability (40) (**Figure 3**).

RIPreC was also associated with a 3-fold reduction in synaptosome-associated protein (SNAP-23) in leukocytes, a protein known to mediate exocytosis in mast cells and neutrophils. Lack of SNAP-23 prevents the formation of ternary complex with other SNARE proteins and therefore inhibits the fusion of granules in these cells (46). It is well-established that neutrophils mainly contribute to the inflammatory responses through secretion of specific cytoplasmic granules containing cytotoxic species and proteolytic enzymes; therefore, RIPreC-induced downregulation of SNAP-23 gene may partially explain the mechanisms that underlie the protection (46, 157). In addition, decreased levels of platelet endothelial cell adhesion molecule (PECAM1 or CD31) gene expression after RIPreC may be responsible for the observed reduction in the chemotactic ability of neutrophils (46, 157). PECAM1 is known to stabilize and preserve the integrity of BBB, and it is normally expressed on endothelial cells, platelets, neutrophils, monocytes and specific members of leukocytes. However, in neuroinflammation, PECAM1 mediates paracellular diapedesis across the vascular wall and its blockage abolishes the leukocyte migration (158). Therefore, lower expression of PECAM1 mRNA in RIPreC group compared to the controls may reduce the neutrophil transmigration in the brain (46).

RIPreC also suppresses the expression of the CCR2 gene that encodes for C-C chemokine receptor type 2, an essential protein needed for monocyte migration, infiltration and macrophage trafficking. This result is aligned with a significant reduction in the number of tightly adherent leukocytes and reduced leukocyte accumulation at the inflammatory sites in CCR2-deficient mice, suggesting that CCR2 modulation by RIC may reduce leukocyte adhesion (159). Taken together, RIC modulatory effects on immune responsive cells results in attenuation of inflammatory responses. This modulation includes reduction of excessive release of proinflammatory mediators during AIS, and enhancing the release of anti-inflammatory cytokines such as IL-6 and IL-10 (46).

RIC: ROLE OF EVs IN TRANSFERRING THE PROTECTIVE SIGNAL

Extracellular vesicles (EVs) are submicron-sized membrane-derived particles that are generated from different cell types under physiological and pathological conditions (160). Their contents include lipids, proteins, and genetic materials (i.e., microRNAs and circRNAs). EVs function *via* transferring their cargo, especially miRNAs, to neighboring target cells, or can act over long distances as an intercellular messenger (160). Fundamental biological processes in the target cells (e.g., proliferation, apoptosis, survival, and differentiation) can be modulated by EVs (160). EVs are classified into three main

groups based on size distribution, chemical composition, and route of biogenesis: exosomes (30–150 nm), microvesicles or microparticles (MVs or MPs, 150–1,000 nm), and apoptotic bodies (500–5,000 nm) (160). However, since there is no strict size distribution for these sub types, and because different physiological or pathophysiological situations may affect their size and surface protein expression, it is recommended by the International Society for Extracellular Vesicles (ISEV) to use the general term "Extracellular Vesicles" while referring to the three subsets (161). EVs inherit their composition and physicochemical properties from their parent cells (162). Other than general EV markers, like tetraspanins CD9, CD63, and CD81, EVs carry signature markers of their cells of origin (e.g., common surface markers in humans are CD146+ for endothelial-derived EVs, CD41+ for platelet-EVs, CD45+ for leukocyte-EVs, and CD235+ for erythrocyte-EVs) (163). The ubiquitous nature and abundant presence of EVs in most body fluids and their ability to reflect cellular and molecular alterations under pathological states qualify them as promising and powerful tool in biomarker studies (163). For instance, platelet-derived microvesicles (PMVs) are known to play a key role in the pathogenesis of acute atherothrombotic events, such as thrombosis, recurrent ischemia, stroke, and vascular inflammation (164). Notably, there is a correlation between an increase in microvesicles released from platelets and endothelial barrier dysfunction (164, 165). Under normal physiological conditions, the majority of the circulating EV population is derived from circulating platelets and platelet precursors in the bone marrow (163–165). However, EV number, origin, and composition can change in pathology (164).

PMVs are important mediators of vascular homeostasis, inflammation, and angiogenesis (165). Accordingly, PMVs can contribute to the vascular homeostasis by maintaining the balance between their procoagulant and anticoagulant properties, depending on the composition of their surface markers or molecular contents. While the expression of phosphatidylserine (PS) and tissue factor (TF) on these vesicles can trigger the activation of coagulation cascades (166), the presence of glycoprotein 1b and annexin V is necessary for activation of protein C and its co-factor protein S, which are best characterized for their roles in anticoagulation pathways (167). PMVs can also play immunomodulatory role in modulating inflammation (165). PMVs exert pro-inflammatory actions mainly *via* provoking monocytes and neutrophils, thereby inducing them to release inflammatory mediators, including IL-1β, TNF-α, MCP-1, and MMP-9 (168). Notably, PMVs can boost the immune response by promoting leukocyte-endothelial interactions (169), *via* PMV uptake by activated neutrophils (polymorphonuclear cells, PMNs) and endothelial cells (ECs). Activation of neutrophils and ECs by PMV uptake was confirmed by an increased surface expression of CD11b and adhesion molecules (ICAM-1 and P-selectin), respectively (169).

Endothelial-derived microvesicles (EMVs) are also important players during various aspects of inflammation (170). They are believed to be secreted from activated ECs as an early response to any alterations in vascular homeostasis. In particular, EMVs contribute to fundamental processes affecting vascular endothelial cell fate, such as apoptosis, cell survival and proliferation, and homeostasis (170). A recent study investigated the content and vascular effects of endothelial-derived microvesicles (EMVs) during inflammation (171). It was shown that the protein levels of c-Src kinase inside the isolated EMVs from mouse was elevated (171) and led to dissociation of endothelial adherens junctions and increased vascular permeability (171). Additionally, src kinase hyperactivity caused higher adhesion and interaction between neutrophils and ECs, as was shown by elevated expression of adhesion molecules (ICAM-1 and VCAM-1) and integrins (CD11b) on the endothelium and neutrophils, respectively (171). In a rat model of focal brain injury and CNS inflammation (induced by IL-1β microinjection into the striatal region), Couch and colleagues demonstrated that the number of circulating CD31-positive EVs (i.e., endothelial cell origin) significantly increased in the acute phase of brain injury compared to age-matched controls (172). Proteomic analysis revealed that circulating EVs in the bloodstream after stroke contain upregulated proinflammatory proteins and can activate peripheral immune cells to induce an inflammatory response (172).

Numerous studies have been conducted to identify the conveyor of RIC protective signals from the remote site to the target organ. EVs may be a potential carrier of this signal (40, 173–177). EVs can transmit cargo (e.g., lipids, proteins, and nucleic acids) from the donor cells to nearby or far-away target cells to modify biological processes in them (178). In this regard, Shan et al. investigated whether transfusion of isolated PMVs from RIPreC-treated rats (donor) to rats who underwent transient MCAO (recipient) can confer protection (179). Their findings revealed a significant increase in PMV (CD41+ and annexin V+) levels in the PMV-treated recipient mice compared to control mice, with a resultant reduced infarct size and better neurologic outcome, indicating that PMVs may be a carrier of the RIPreC protective signal (179). Likewise, Li et al. reported neuroprotection conferred by RIPreC (three cycles of 10-min occlusion/reopening of mouse hindlimb) against permanent MCAO in mice was associated with increased levels of exosomes (<100 nm in diameter) in plasma (180). Interestingly, the transfer of purified exosomes from RIPreC-treated mice (donor) to non-treated stroked mice (recipient) reduced infarct volume and improved neurologic outcome in the recipient mice, indicating RIPreC protective signal may be conveyed through exosomes. Furthermore, RIPreC treatment upregulated the HIF-1α in the purified exosomes compared to the control group (180). Notably, sublethal hypoxic or ischemic conditioning also upregulates transcription factor HIF-1α, which in turn translocates into the nucleus and dimerizes with HIF-1β (181). After dimerization, HIF-1α binds to the hypoxia response elements on specific target genes, such as VEGF and erythropoietin (EPO), thereby counteracts the cell apoptosis. The neuroprotective properties of VEGF and EPO in ischemic brain have been linked to their ability to induce angiogenesis and neurogenesis, respectively (182, 183).

Similarly, whether RIC cardioprotection is transferable from the RIC-treated subject's plasma to naïve untreated subjects, and if this is mediated by circulating EVs, has been examined

in both rodents and humans (184–186). Notably, these studies suggest RIC increased the release of EVs from the heart, RIC-induced protective signal is conveyed in part to the target organ *via* EVs, and this protection is transferable intra and across species. Using an *ex-vivo* langendorff-perfused rat heart method, Giricz et al. assigned the isolated hearts to two "donor" and "recipient" groups (173). A group of donor hearts were preconditioned *via* 3 alternate cycles of 5 min ischemia/5 min reperfusion prior to 30 min of global ischemia. Western blots against the EV marker (HSP60) revealed higher EV levels in coronary perfusates collected from preconditioned donor hearts compared to untreated control hearts, (173) as well as a smaller infarct, suggesting an EV-mediated transmission of RIC protective effects. Infarct size was significantly decreased in naïve hearts that received the coronary perfusate from the preconditioned donors, while no reduction of infarct size was noted in the hearts recipient of EV-depleted coronary perfusate (173). Likewise, RIPreC-induced cardioprotection has been associated with increased EV concentration and differential expression of specific microRNAs in plasma from patients who underwent coronary bypass surgery (187). Isolated EVs from RIC-treated patients added to cultured rat cardiomyoblasts *in vitro* conferred the same protection against hypoxia, indicating RIC protection is mediated *via* circulating EVs, and it is transferable across species (187). These studies also confirmed the presence of a well-recognized endothelial surface marker (i.e., CD146) on the isolated EVs, suggesting endothelial cells as a likely cellular source of RIC-induced EV release (187).

ROLE OF MICRORNAS IN TRANSFERRING THE RIC PROTECTIVE SIGNALS

MicroRNAs (miRNAs or miRs) are a key regulator of many fundamental cellular and molecular processes, such as cell growth, differentiation and apoptosis (188). MiRNAs are small single-stranded non-coding nucleic acids (∼22 nucleotides long), which function through base-pairing with a complementary region in mRNA transcript and repress their translation into functional proteins (188).

The emerging role of miRNAs in stroke pathogenesis has recently been the focus of investigations in this field (189). MiRNA and target mRNA expression levels can change rapidly after the cerebral ischemia (190–193). Besides, numerous studies demonstrated that RIC alters the miRNA profile and thereby the expression and translation of genes and proteins. This again can reprogram the transcriptional response to the ischemic event (176, 194–196). Therefore, identifying the miRNAs and miRNA targets involved in stroke pathophysiology appears to be a promising candidate as either diagnosis or therapeutic tool.

Evidence from animal studies of cerebral ischemia suggests that miRNA (non-coding genes) genes may have a higher sensitivity to preconditioning (PC) stimulus than protein-coding mRNAs (191, 197, 198); since differential expression was observed in more than 20% of miRNAs, while <5% of coding mRNAs changed following PC application (197, 198). MiRNA profiling analysis by Dharap et al., in the cerebral cortex of preconditioned mice after a 10 min MCA occlusion reported a rapid change in the expression of 51 miRNAs following PC. Bioinformatics and pathway analysis suggested MAP Kinase and mTOR signaling are the main downstream signaling pathways of up-regulated miRNAs (26 out of 51), and Wnt and GnRH signaling pathways are the main targets of down-regulated miRNAs (25 out of 51) (197). Of these 51 differentially expressed miRNAs, the most up-regulated and down-regulated miRNAs 24h after PC were miR-21 (13-fold) and miR-466c (27-fold), respectively. Notably, miR-21 is anti-apoptotic factor that attenuates the expression of certain pro-apoptotic genes, including programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), tropomyosin1 in neurons (199–203).

miRNAs play an important role in apoptotic signaling pathways through regulation of many pro-apoptotic genes (204–207). For a detailed review on apoptotic factors regulated by many different miRNAs, see Jang and Lee (204). For instance, Wu et al. demonstrated that upregulation of miR-21 (measured by rt-PCR Quantitative Kit) in human keloid fibroblasts were associated with a host of cellular events, which all led to the inhibition of cell apoptosis, including lower ROS, increased ratio of Bcl-2/BAX, decreased cytochrome C release into the cytosol, lower activity of caspase-3 and 9. All these events are critical components involved in the mitochondrial-mediated apoptotic pathway (206). In a rat embolic MCA occlusion model, Buller et al. demonstrated that elevated miR-21 level after focal ischemia attenuated the expression of Fas ligand (FasL) *via* complementary base-pairing with FasL transcript and blocking its translation into FasL protein ligands (208). Thus, miR-21 attenuates the neuronal cell apoptosis in the ischemic brain area by targeting critical cell death-inducing factors (200, 202, 203). FasL belongs to the TNF family, and its binding to one of the apoptosis signaling receptors FasR (apoptosis antigen-1, APO-1) initiates a cascade of events that leads to the activation of caspases and eventually causes neuronal cell death (209).

An important consideration is the potential use of miRNAs in maintaining the ionic balance in the ischemic region of the brain (176). During brain ischemia, there is a disruption in sodium and calcium balance due to downregulation of necessary ion channels and transporters by regulatory effects of some miRNAs (210, 211). To this end, blocking the expression of these miRNAs can be a therapeutic strategy to interfere with their detrimental behaviour (212). Anti-miRNAs evolution represents an efficient approach to inhibit and alter the action of miRNAs. Interestingly, in a rat model of transient cerebral ischemia, miR-103-1 was shown to downregulate the expression of Na/Ca exchanger (NCX1), a plasma membrane transporter which regulates the ionic homeostasis in ischemic brain. Notably, anti-miR-103-1 could significantly upregulate the expression of NCX1 mRNA and proteins levels in the brain cortex and striatum of ischemic rats, inducing a strong neuroprotective effect (∼60% reduction in infarct volume) (211).

Aside from the regulatory role of miRNAs in the underlying mechanisms of conditioning-induced neuroprotection, miRNAs have also shown to be involved in the cardioprotective effects of conditioning (184). For example, Lassen et al. demonstrated that beneficial effects of RIC are delivered through EVs and

their miRNA content. The transferability of EV-mediated RIC cardioprotection from the RIC-treated patients to *in vitro* cultured murine myoblasts was also demonstrated (184). In this study, the three miRNAs were most upregulated in association with cardioprotection after the RIC treatment were miR-144-3p, miR-451a, and miR-16-5p. These miRNAs demonstrated a two-fold upregulation, and each was linked to fibroblast growth factor 2 (FGF2) mRNA. Pathway analysis and gene ontology analyses suggested that all three differentially expressed miRNAs are associated with the mTOR signaling pathway and mediate the protein turnover, stress response, and apoptosis (184). Additionally, IR injury in the mouse myocardium reduces miR-144 expression levels, and this was reversed in mice receiving either RIPreC or systemic injection of miR-144 into the tail vein 30 min prior to global ischemia. These manipulations resulted in a marked reduction in infarct size and improved functional recovery of the heart. However, these beneficial effects were abolished after systemic injection of antagomir-144 (specific antisense oligonucleotide against miR-144), indicating the significance of miR-144 in RIPreC -induced cardioprotection. Moreover, miR-144 elevation led to downregulation of mTOR. Of note, mTOR signaling is an inhibitor of autophagy, which is a vital regulator of cell survival and a natural homeostatic mechanism of cell to remove the unnecessary or damaged components. Improved functional recovery after RIPreC may involve suppression of mTOR signaling and improved cardiomyocyte survival through increased autophagy (213).

Characterization and identification of EVs provides a "snapshot" of the environment of their origin cell at any given time. Additionally, they have a ubiquitous nature with high abundance in most body fluids. These features, along with their capacity as a vehicle for intercellular communications, position them as an ideal diagnostic and/or therapeutic target in many pathological states, including AIS. Moreover, these natural lipid mediators can be modulated for the delivery of specific agents or drugs to the target cells or organs, exhibiting superior properties relative to synthetic nanoparticles, including natural targeting ability, biocompatibility and safety. Thus, by identifying key EV-based mechanisms of RIC, new avenues of therapy to improve outcome after AIS can be developed.

AUTHOR CONTRIBUTIONS

SA-H researched, wrote, and designed figures for the manuscript. IW and GJ designed, co-wrote, and edited the manuscript with SA-H. All authors contributed to the article and approved the submitted version.

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Preventing Ischemic Cerebrovascular Events in High-Risk Patients With Non-disabling Ischemic Cerebrovascular Events Using Remote Ischemic Conditioning: A Single-Arm Study

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Background: Secondary stroke prevention after a high-risk, non-disabling ischemic cerebrovascular event needs to be enhanced. The study was conducted to investigate whether remote ischemic conditioning (RIC) is effective in preventing recurrent ischemic events within 3 months.

Methods: This was a four-center, single-arm, open-label Phase IIa futility trial (PICNIC-One Study). Adult patients (≥18 years of age) who had an acute minor ischemic stroke (AMIS) with a National Institutes of Health Stroke Scale score ≤ 3 or a transient ischemic attack (TIA) with moderate-to-high risk of stroke recurrence (ABCD score ≥ 4) within 14 days of symptom onset were recruited. Patients received RIC as adjunctive therapy to routine secondary stroke prevention regimen. RIC consisted of five cycles of 5-min inflation (200 mmHg) and 5-min deflation of cuffs (45 min) on bilateral upper limbs twice a day for 90 days.

Results: A total of 285 patients met the study criteria, of which 167 provided signed informed consent and were enrolled. Data from 162 were analyzed with five subjects excluded. Recurrent AIS/TIA occurred in 6/162 (3.7%) patients within 3 months, with no

occurrence of hemorrhagic stroke. The top three adverse events were upper limb pain (44/162, 27.2%), petechia (26/162, 16.0%), and heart palpitation (5/162, 3.1%). About 68 (42.0%) subjects completed ≥ 50% of 45-min RIC sessions.

Conclusions: RIC is a safe add-on procedure and it has a potential benefit in reducing recurrent cerebrovascular events in patients with high-risk, non-disabling ischemic cerebrovascular events as the risk of stroke/TIA events is lower than expected; however, its compliance needs to be improved. Our study provides critical preliminary data to plan a large sample size, randomized controlled clinical study to systematically investigate the safety and efficacy of RIC in this population.

Keywords: remote ischemic conditioning, stroke, transient ischemic attack, secondary prevention, acute minor ischemic stroke, transient ischemic attack

INTRODUCTION

Stroke is the most common cause of mortality and disability in China (1). High-risk non-disabling ischemic cerebrovascular events consist of acute minor ischemic stroke (AMIS) and moderate-to-high risk transient ischemic attack (TIA). The most commonly used definition of AMIS is a National Institutes of Health Stroke Scale (NIHSS) score < 4 at the time of the event (2).

In particular, a large-scale clinical trial among Chinese patients with AMIS/TIA (CHANCE study) has indicated that dual antiplatelet therapy (clopidogrel and aspirin) reduced the risk of recurrent stroke, as compared to single antiplatelet therapy (3). Subsequently, the latest American and Chinese guidelines for the secondary prevention of stroke has also recommended the combination of aspirin and clopidogrel within 24 h of a high-risk non-disabling ischemic cerebrovascular event, which is continued for 21 days (4, 5). However, according to the CHANCE study results, 9.4% of patients would still have another ischemic stroke/TIA within the next 3 months, despite receiving the recommended dual-antiplatelet therapy (3). Moreover, only fewer than two-thirds of stroke patients were reported to arrive at hospitals within 24 h to receive dual-antiplatelet therapy in China (6). Furthermore, there have been no published data demonstrating whether guidelines have changed real-world clinical practice in China up to our knowledge.

Remote ischemic conditioning (RIC) involves repetitive and brief cuff inflation around the limb to the pressures level above systolic blood pressure and subsequent deflation to induce repetitive ischemia and reperfusion, which has been demonstrated to protect distant organs, such as the heart, kidney, or brain (7–9). Our prior small sample-size, clinical studies have indicated that long-term, regular (twice a day for at least 90 consecutive days), bilateral upper-limb RIC was effective in reducing stroke recurrence in patients with symptomatic intracranial artery stenosis (10, 11). One recent clinical trial in China found repeated remote ischemic post-conditioning during hospitalization combined with intravenous thrombolysis significantly facilitate functional recovery at day 90 in patients with acute ischemic stroke (12). Similarly, another study in UK showed that with 4 cycles of RIC within 24 h of onset of stroke symptoms can lead to a significant decrease in day 90 NIHSS score (13).

Therefore, we hypothesized that adjunctive, twice-daily RIC for 3 months can further reduce cerebrovascular events in patients with AMIS/TIA. To that end, we performed a single-arm, open-label, multi-center, Phase IIa futility study.

MATERIALS AND METHODS

A team of researchers from Xuanwu Hospital of the Capital Medical University (China) and the Medical University of South Carolina (USA) designed this study. A detailed rationale and study design of the Preventing Ischemic Cerebrovascular events in patients with acute Non-disabling Ischemic Cerebrovascular events using RIC (PICINIC-One) study was published previously (14). This study was conducted at four sites in China: Xuanwu Hospital of Capital Medical University, Shengli Oilfield Center Hospital, the First Affiliated Hospital of Hainan Medical University, and Taoyuan People's Hospital.

The study was approved by the Institutional Review Board (IRB) of each study site: Xuanwu Hospital Capital, Shengli Oilfield Center Hospital, Taoyuan People's Hospital, and the First Affiliated Hospital of Hainan Medical. Informed consent was obtained from all included participants prior to the study. The study was registered on clinicaltrial.gov (NCT03004820). All procedures in this study were performed in accordance with the ethical standards of the institutional and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Study Population

We recruited adult (≥18 years) patients of either sex, who had an AMIS with NIHSS score ≤ 3 or a TIA with moderate-to-high risk of stroke recurrence ($ABCD^2$ Score ≥4) within 14 days of symptom onset.

The inclusion criteria were as follows: (1) ≥18 years of any sex or ethnicity; (2) diagnosis of non-cardiogenic AMIS/TIA within 14 days of stroke symptom onset, wherein AMIS was defined as ischemic stroke with an NIHSS score ≤ 3 at the time of enrollment, and TIA was defined as a transient

episode of neurological dysfunction without acute infarction with moderate-to-high risk of stroke recurrence (defined as an ABCD score $\geq$ 4 at the time of enrollment); (3) stable vital signs, with normal cardiac (Class I-II in New York Heart Association Functional Classification), hepatic (normal ranges in blood liver function tests) and renal functions (normal ranges in blood renal function tests); (4) ability consent by themselves or by a legally authorized representative; and (5) agreement to conduct regular RIC by themselves or others.

On the other hand, subjects who met any of the following exclusion criteria were excluded from the study: (1) diagnosis of brain hemorrhage or other pathologies, such as vascular malformation, tumor, abscess, or other non-vascular diseases, based on brain computed tomography (CT) or magnetic resonance imaging (MRI); (2) modified Rankin Scale (mRS) score >2 before the index event; (3) administration of intravenous thrombolytic therapy (Alteplase or Urokinase) or endovascular treatment for the index event; (4) contraindication to aspirin or clopidogrel (e.g., known allergy, severe asthma, heart failure); (5) indication for anticoagulation therapy (cardiac source of embolus); (6) hemorrhagic tendency of any reason (including but not limited to hemostatic disorder, platelet count $< 100 \times 10^9$/L, history of hepatic dysfunction, among others); (7) any hemorrhagic transformation on brain scans (MRI or CT); (8) gastrointestinal bleed or major surgery within 3 months before the index event; (9) stroke or TIA due to medical procedure or other iatrogenic cause; (10) any upper extremity soft tissue disease, vascular injury, or peripheral blood vessel disease, which is contraindicated for RIC; (11) hypertension with a systolic blood pressure $\geq$ 200 mmHg despite medical treatment at the time of enrolment; (12) planned revascularization (any angioplasty or vascular surgery) within the following 3 months; (13) scheduled surgery or intervention within the following 3 months that may affect the study procedure; (14) life expectancy $\leq$ 6 months; (15) pregnant at the time of the study; and (16) ongoing investigational drug or device use by other studies at the time of the study.

Procedures

Eligible subjects were identified from the inpatient service or stroke emergency center of each study site, where a well-trained research physician confirmed the diagnosis of AMIS (NIHSS score $\leq$ 3) or TIA ($ABCD^2$ score $\geq$ 4). If the patient met the criteria and provided written informed consent by themselves or legal proxies, they were instructed how to perform RIC using an electric auto-control device (patent number ZL200820123637.X, China), which can be performed by the patient or family members. RIC consisted of five cycles of 5-min inflation at 200 mmHg and 5-min deflation of cuffs on bilateral upper limbs twice a day (45 min) for 90 consecutive days. Antiplatelet strategies based on the physician's best judgment were as follows: aspirin alone (100–300 mg daily), clopidogrel alone (75 mg daily), or a combination of aspirin and clopidogrel. Study visits were conducted on the day of enrollment (day 1) at the inpatient wards or emergency rooms, and the patients were followed up on day 30 $\pm$7 and 90 $\pm$14 in the outpatient department.

Outcomes

In this study, the primary efficacy outcome was the number (percentage) of patients who had a recurrent ischemic stroke or TIA within 90 days after the index event. The secondary efficacy outcome measures included: (1) number (percentage) of patients who had a second ischemic stroke or TIA within 1 month after the index event; (2) number (percentage) of patients with a new cerebrovascular and coronary artery event within 1 and 3 months, including hemorrhagic stroke, myocardial infarction, and deaths from cardiovascular causes, from all causes, and from the index event; (3) NIHSS score change (continuous) from the baseline to 1 and 3 months; (4) mRS score (continuous), dichotomized by percentage with score $\leq$1 vs. $\geq$2, at 1 and 3 months; (5) Barthel index score (continuous), dichotomized by percentage with score $\geq$95 vs. <95, at 1 and 3 months; and (6) hand grip strength change (continuous) from baseline to 1 and 3 months on the affected side in patients with upper-limb motor deficit who did not have a recurrent vascular event.

Additionally, compliance was assessed with simultaneous records, which were delivered by the electric auto-control device through 4G signals, and the feasibility outcomes were defined as the number (percentage) of patients who completed $\geq$50% or <50% of the long-term, regular, 45-min RIC.

Furthermore, safety outcomes were defined as the risks of expected device-related local or systemic adverse events, including the number (percentage) of patients with upper limb pain assessed by the visual analog scale; redness, swelling, or skin petechiae; palpitation; and dizziness. Any new condition (symptoms, injuries, significant abnormal laboratory values) that was not present at the beginning of the study was also documented as an unexpected adverse event, and the determination of whether these events were associated with the RIC device was adjudicated by research physicians. Serious adverse events (SAEs) in this study included death, life-threatening conditions, inpatient hospitalization or prolongation of existing hospitalization requiring medical/surgical intervention to prevent permanent impairment or damage, and other serious medical events.

Statistical Analysis

The null hypothesis was that the recurrence rate of ischemic stroke/TIA within 3 months would be greater than the largest regression probability of recurrence ($P_0 = 12\%$). The alternative hypothesis was that the recurrence rate of ischemic stroke/TIA within 3 months would be less than the smallest regression probability of recurrence ($P_A = 6\%$) (14). To test our hypothesis, with an assumption of a type 1 error of 5%, a power of 80%, and an attrition rate of 10%, the sample size was estimated to be 165 (15).

Baseline categorical variables were listed as numbers (percentage), baseline normally distributed continuous variables were reported as means (standard deviation, SD), and non-normally distributed continuous variables were reported as medians (interquartile range, IQR).

We used multivariate logistic regression models to assess the association between the odds ratio (OR) of recurrent ischemic stroke/TIA occurring within 3 months with a compliance rate

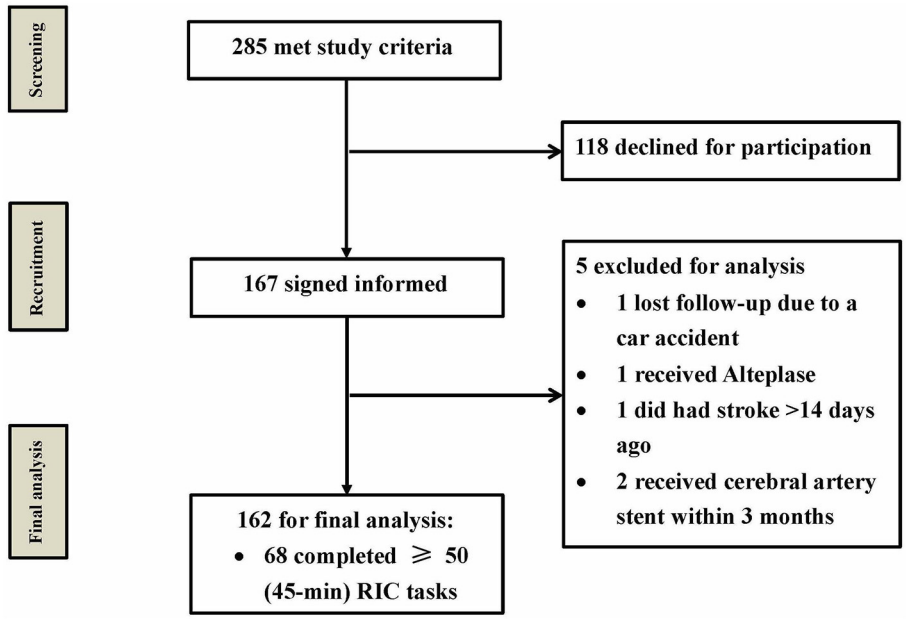

FIGURE 1 | Study patient flow chart. From December 2016 to August 2017, a total of 285 patients met the study criteria, and 167 of them provided informed consent. Only 162 patients were included for final analysis, and five patients were excluded. RIC, remote ischemic conditioning.

of RIC (≥50 vs. <50%), adjusting the antiplatelet strategy (dual vs. single antiplatelet), age (≥60 vs. <60 years), and sex (male vs. female) parameters. Moreover, we used repeated-measures analysis with mixed models to analyze NIHSS or handgrip strength changes associated with the RIC compliance rate (≥50 vs. <50%), antiplatelet strategy (dual vs. single antiplatelet), and follow-up visits (baseline, 1 month, and 3 months). To follow the parsimonious principle in variable selection for regression models, we only included the most crucial covariates that may affect recurrence or neurological behavior results. A *P*-value of <0.05 was considered as statistically significant, and all statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Carry, NC, USA).

RESULTS

Study Patients

From December 2016 to August 2017, 285 patients met the study criteria at the four enrollment centers, and 167 of them provided signed informed consent and were enrolled in the study. Ultimately, a total of 162 patients were included in the final analysis, whereas 118 patients declined to participate for various reasons, including skepticism of the study device or clinical trials, inability to commit to follow-up visits, and other non-specific reasons. Notably, five patients were excluded for the following reasons: one patient lost to follow-up due to a car accident; one patient received Alteplase meeting the exclusion criteria; one patient did not meet the inclusion criteria (had stroke >14 days ago); and two patients received cerebral artery stent within 3 months meeting the exclusion criteria (**Figure 1**).

The median age of the included patients was 58 years, and 74.7% (121/162) of them were male. 106 (65.4%) patients received dual antiplatelet therapy with the combination of aspirin and clopidogrel, 45 (27.8%) patients received aspirin and 11 (6.8%) received clopidogrel. The index event was AMIS in 153 patients (94.4%). The median NIHSS score was 1. $ABCD^2$ score was 5 in the patients with TIA. Regarding medical history, 69.8% (113/162) of the included patients had hypertension, 36.4% (59/162) had diabetes, and 51.2% (83/162) were current or previous smokers. Furthermore, the median time from the onset of the qualifying event to RIC intervention was 7 days. Baseline blood biochemical test results of the included patients are listed in **Table 1**.

Primary Outcomes

Ischemic stroke/TIA occurred in six patients (3.7%) within 3 months. It rejects the null hypothesis and supports the alternative hypothesis. (i.e. the recurrence rate of ischemic stroke/TIA within 3 months is less than the smallest regression probability of recurrence of 6%). Among the six patients, three patients received dual antiplatelet therapy (**Table 2**), two patients received aspirin, and one patient received clopidogrel alone. Regarding compliance to RIC, Three patients are being compliant while the other 3 patients are not compliant.

Secondary Efficacy Outcomes

Ischemic stroke/TIA occurred in one patient (0.9%) under dual antiplatelet therapy, and one patient (1.8%) under single antiplatelet therapy (aspirin, to be specific) within 1 month. None of the included patients had any other vascular events or hemorrhagic stroke. We collected neurological outcomes data from 143 patients who visited the outpatient department in person, showing that 74.8% (107/143) of patients had a favorable outcome (mRS score of 0 or 1) at 3 months, with a median mRS

TABLE 1 | Baseline clinicodemographic characteristics.

Variables	All patients (n = 162)	Dual antiplatelet (n = 106)	Single antiplatelet (n = 56)
Age, median (IQR), years	58 (51–67)	59 (51–67)	58 (52–67)
Male, *n* (%)	121 (74.7)	83 (78.3)	38 (67.9)
Body mass index, median (IQR)	25.0 (23.0–27.7)	25.6 (23.4–27.7)	24.1 (22.3–26.6)
Blood pressure, median (IQR), mmHg			
Systolic	148 (135–162)	150 (135–162)	146 (130–164)
Diastolic	83 (75–94)	85 (80–94)	80 (73–93)
NIHSS, median (IQR)	1 (1–2)	1 (0–2)	2 (1–2)
Hand grip strength, median (IQR), kg[†]	22.5 (13.0–29.0)	23.6 (12.9–30.2)	20.0 (13.0–26.9)
Medical history, *n* (%)			
Hypertension	113 (69.8)	81 (76.4)	32 (57.1)
Hyperlipidemia	81 (50.0)	56 (52.8)	25 (44.6)
Diabetes	59 (36.4)	38 (35.9)	21 (37.5)
Ischemic stroke/TIA	37 (22.8)	20 (18.9)	17 (30.4)
Coronary heart disease	21 (13.0)	17 (16.0)	4 (7.1)
Intracranial hemorrhage	2 (1.2)	1 (0.9)	1 (1.8)
Atrial fibrillation/flutter	0	0	0
Current or previous smoking, *n* (%)	83 (51.2)	47 (44.3)	36 (64.3)
Family history of stroke, *n* (%)	60 (37.0)	45 (42.5)	15 (26.8)
Time to receive intervention, median (IQR), d	7 (5–9)	7 (5–9)	7 (5–10)
Qualifying event			
Acute minor ischemic stroke, *n* (%)	153 (94.4)	99 (93.4)	54 (96.4)
TOAST classification, *n* (%)			
Large-artery atherosclerosis	100 (65.4)	64 (64.7)	36 (66.7)
Cardioembolism	0 (0)	0 (0)	0 (0)
Small-vessel occlusion	50 (32.7)	33 (33.3)	17 (31.5)
Stroke of other determined etiology	0 (0)	0 (0)	0 (0)
Stroke of undetermined etiology	3 (2.0)	2 (2.0)	1 (1.9)
TIA, *n* (%)	9 (5.6)	7 (6.6)	2 (3.6)
$ABCD^2$ (1) score, median (IQR)	5 (4–6)	5 (4–6)	5 (4–6)
Blood Test			
TChol, median (IQR), mmol/L	4.25 (3.70–4.90)	4.30 (3.78–184)	4.20 (3.55–3.92)
HDL, median (IQR), mmol/L	1.04 (0.88–1.30)	1.06 (0.88–1.30)	1.01 (0.85–1.27)
LDL, median (IQR), mmol/L	2.67 (2.07–3.16)	2.75 (2.18–3.19)	2.59 (2.07–3.13)
GLU, median (IQR), mmol/L	5.7 (4.7–7.7)	5.7 (4.9–7.7)	5.5 (4.7–8.0)
HbA_{1C}, median (IQR), %[‡]	7.7 (6.7–9.1)	7.7 (6.7–9.1)	7.7 (6.7–9.1)
HCY, median (IQR), μmol/L	11.6 (10.0–15.9)	11.4 (9.9–16.5)	11.9 (9.8–15.2)

IQR, interquartile range; NIHSS, NIH Stroke Scale; Tchol, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; GLU, glucose; Hb A1C, hemoglobin A1C; HCY, homocysteine.

[†] *Hand grip strength for patients with upper limb weakness without recurrent vascular events.*

[‡] *HbA_{1C} test for patients with diabetes mellitus.*

score of 1 (0–2) and Barthel index of 100 (100–100). The mean change in NIHSS score in the study patients was −0.8 and −1.0 within 1 and 3 months, respectively. The mean change in grip strength at the affected upper limbs was 4.3 and 4.6 Kg within 1 and 3 months, respectively (**Table 2**).

Safety Outcomes

Upper limb pain was reported to be the most common (44/162, 27.2%) local adverse event, followed by upper limb petechia (26/162, 16.0%), heart palpitation (5/162, 3.1%), and superficial venous thrombosis within the upper limb (1/162, 0.6%). No bleeding events occurred. No SAEs were observed.

Compliance

Only 68 patients (42.0%) completed ≥ 50% of the long-term, regular, 45-min RIC sessions using real-time 4G signal data (**Table 2**). They were 43 men (63%) and the median age of the 68 patients was 61 years. Three (4.4%) patients had the primary outcomes within three months, and none had recurrence within 1 month (**Table 2**).

TABLE 2 | Study outcomes.

Outcomes	All patients ($n = 162$)	Antiplatelet group		Compliance to RIC	
		Dual antiplatelet ($n = 106$)	Single antiplatelet ($n = 56$)	≥ 50% compliance ($n = 68$)	<50% compliance ($n = 94$)
Primary efficacy outcome					
Ischemic stroke/TIA within 3 months, *n* (%)	6 (3.7)	3 (2.8)	3 (5.4)	3 (4.4)	3 (3.2)
Secondary efficacy outcomes					
Ischemic stroke/TIA within 1 month, *n* (%)	2 (1.2)	1 (0.9)	1 (1.8)	0	2 (2.1)
Stroke, myocardial infarct or death from cardiovascular causes, *n* (%)	6 (3.7)	3 (2.8)	3 (5.4)	3 (4.4)	3 (3.2)
Hemorrhagic stroke, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Estimated NIHSS change from baseline to 1 month, Mean (SD)†	−0.8 (0.1)	−0.6 (0.1)	−0.9 (0.2)	−0.7 (0.1)	−0.7 (0.1)
Estimated NIHSS change from baseline to 3 months, mean (SD)†	−1.0 (0.1)	−1.0 (0.1)	−1.1 (0.1)	−1.0 (0.1)	−1.0 (0.1)
mRS ≤ 1 at 1 month, *n* (%)†	108 (75.0)	74 (78.7)	34 (68.0)	51 (75.3)	57 (79.8)
mRS ≤ 1 at 3 months, *n* (%)†	107 (74.8)	73 (78.5)	34 (68.0)	50 (74.6)	57 (79.8)
mRS at 1 month, median (IQR)†	1 (0–2)	1 (0–1)	0 (0–2)	1 (0–1)	0 (0–1)
mRS at 3 months, median (IQR)†	1 (0–2)	1 (0–1)	0 (0–2)	1 (0–1)	0 (0–1)
BI ≥ 95 at 1 month, *n* (%)†	133 (89.3)	89 (89.9)	44 (88.0)	65 (89.9)	69 (88.2)
BI ≥ 95 at 3 months, *n* (%)†	132 (97.8)	88 (98.9)	44 (95.7)	64 (98.7)	69 (97.1)
BI at 1 month, median (IQR)†	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)
BI at 3 months, median (IQR)†	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)	100 (100–100)
Estimated hand grip strength change from baseline to 1 month, mean (SD), Kg†	4.3 (0.8)	4.3 (0.9)	4.2 (1.3)	3.4 (1.1)	3.5 (1.1)
Estimated hand grip strength change from baseline to 3 months, mean (SD), Kg†	4.6 (0.8)	4.5 (0.9)	4.7 (1.4)	4.7 (1.5)	4.8 (1.5)
Safety outcomes, *n* (%)					
Pain (upper limb)	44 (27.2)	27 (25.5)	17 (30.4)	25 (36.8)	19 (20.2)
Petechia (upper limb)	26 (16.0)	20 (18.9)	6 (10.7)	8 (11.8)	18 (19.1)
Heart palpitation	5 (3.1)	4 (3.8)	1 (1.8)	1 (1.5)	4 (4.3)
Superficial venous thrombosis (upper limb)	1 (0.6)	1 (0.9)	0 (0)	0 (0)	1 (1.0)
Hand cramps	1 (0.6)	0 (0)	1 (1.8)	1 (14.7)	0 (0)
Any bleeding	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

RIC, remote ischemic conditioning; IQR, interquartile range; SD, standard deviation; NIHSS, NIH Stroke Scale; mRS, modified Rankin Scale; BI, Barthel Index; RIC, remote ischemic conditioning.

† *Tests among patients who did not have a recurrence event and visited the outpatient department in person.*

Subgroup Analysis

No significant difference of recurrent ischemic stroke/TIA within 3 months were detected on comparison of the subgroups completing ≥50% of the 45-min RIC tasks and those who did not (OR: 1.7, 95% CI: 0.3–8.7, $P = 0.55$), after adjusting for age (≥60 vs. <60 years), sex (male vs. female), and antiplatelet strategy (dual vs. single antiplatelet) (**Figure 2**). In the multiple-variable model, age (≥60 vs. <60 years), sex (male vs. female) or antiplatelet strategy (dual vs. single antiplatelet) was not associated with the risks of having a recurrence.

In the repeated-measures models consisting variables of antiplatelet strategy (dual vs. single antiplatelet), the compliance rate of 45-min RIC (≥50 vs. <50%), and follow-up visits (baseline, 1 month and 3 months), NIHSS at 3 months was significantly improved, as compared to baseline (-1.0 ± 0.1, 95% CI: −1.2 to −0.9; $P < 0.001$), although antiplatelet strategy and compliance rate of 45-min RIC were not associated with NIHSS change in this model ($P = 0.60$ and 0.76, respectively). Similarly, handgrip strength was estimated to increase by 4.6 $\pm$ 0.8 kg from baseline to 3 months in patients with unilateral upper limb weakness resulting from the index event without any vascular events (95% CI 3.0–6.0; $P < 0.001$) (**Table 2**); however, antiplatelet strategy and compliance rate of 45-min RIC were not associated with the handgrip strength change in this model ($P =$ 0.24 and 0.98, respectively).

DISCUSSION

The PICNIC-One study found that regular 3-month twice daily RIC could significantly prevent recurrent ischemic stroke or TIA

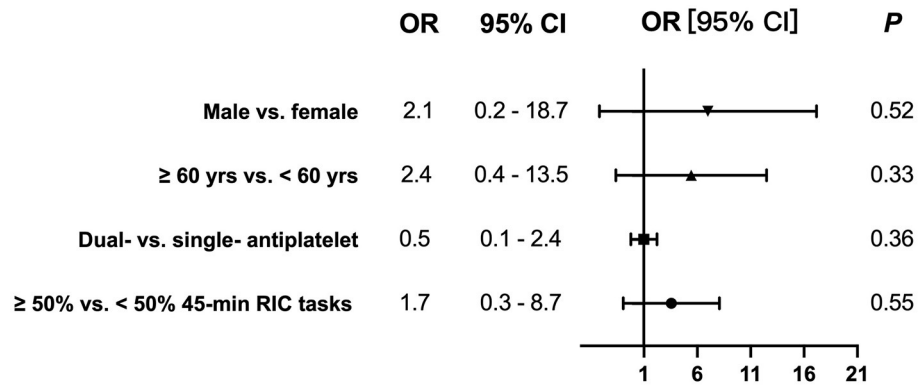

FIGURE 2 | No significant odds ratios (ORs) of recurrent ischemic stroke/TIA within 3 months were detected on comparison of the subgroups completing ≥50% the 45-min RIC tasks and those who did not [OR: 1.7, 95% confidence interval (CI): 0.3–8.7, $P = 0.55$], after adjusting for age (≥60 vs. <60 years), sex (male vs. female), and antiplatelet strategy (dual vs. single antiplatelet).

in patients with AMIS or moderate-to-high risk TIA, showing a recurrence rate of 3.7%. However, the compliance to RIC was not great as only 42.0% of the included patients completed 50% of the study procedures (i.e., 180 sessions of the 45-min RIC), RIC was demonstrated to be safe among the patients due to a low incidence of adverse events.

Only two-thirds of patients with AMIS/TIA were prescribed with dual antiplatelet therapy, despite the fact that the Chinese secondary prevention of stroke guidelines have been in place for 3 years (5). This proved the need to conduct this single-arm study prior to a randomized controlled Phase II study to elucidate the real-world practices of the recommended antiplatelet strategies. In this study, the recurrence rate of ischemic stroke/TIA within 3 months was 3.7%, which appears to be lower than that in the CHANCE study (3). It suggests there is likely a signal using RIC as an adjunctive therapy on top of short-duration use of dual-antiplatelets, but we have to interpret the data with great caution. However, this comparison should be interpreted with caution because the study population in the PICNIC-One study could be different although PICNIC study adopted the inclusion criteria from the CHANCE study. Although age, sex, or medical histories weresimilar between the two studies, only 5.6% of patients suffered TIA as compared to 27.9% patients having TIA in the CHANCE study. The antiplatelet strategy can interfere with the outcomes, and its usage are different between the two studies. For example, aspirin alone was put on 27.8% of patients, clopidogrel alone was prescribed to 6.8% of patients and dual-antiplatelets accounted for 65.4% of patients in the PICNIC-One study. In the CHANCE study, half of the patients were randomized into dual-antiplatelet strategy and the other half were assigned to aspirin alone.

Contrary to our expectations, the compliance of RIC was not good, with fewer than half of the patients completing half of the RIC sessions. It raised a tolerability issue as 27.2% of patients reported pain as the most common side effect. Interestingly the group being more compliant are more likely to report pain as a side effect as compared with the less compliant group (36.8 vs. 20.2%). Notably, long-term RIC has been reported to be well-accepted, with a high compliance rate in 15 prior small clinical trials (10, 11, 16–28), three of which were carried out at the same center (10, 11, 26), however, we have to point out that these compliance rate is based on subjective self-report from patient vs. quantitative objective measures by this study In PICNIC-One study, four study sites actively recruited patients in multiple regions of China. The electric auto-control RIC device had the capacity to upload application data simultaneously through 4G signals, allowing the study team to calculate the compliance rate with objective data. With the objective application data from four centers, the PICNIC-One study advanced the current knowledge regarding the feasibility and compliance of applying long-term regular RIC sessions in this subset of stroke population patients.

Although prior clinical studies have indicated that long-term, regular RIC (twice a day for at least 90 consecutive days or 180 sessions) was effective in reducing stroke recurrence in the patients with symptomatic intracranial artery stenosis (10, 11). There is no statistical difference in primary efficacy outcomes between the more compliant and less compliant group in PICNIC-one study although the study is not powered to demonstrate such difference. We do not know whether less than three months of adjunctive RIC therapy (or <180 sessions) are adequate to prevent stroke recurrence. It remains unknown about the optimal dose of RIC in secondary stroke prevention. As compliance and tolerability is critical issue in this study, it is important to design a dosing-selection study as the next step to better understand the dose, preliminary efficacy and to improve the compliance rate.

In addition to its potential efficacy, the PICNIC-One study also provided evidence of safety profile associated with long-term RIC, reporting no incidences of SAEs. To the best of our knowledge, this is the first study to report RIC-related heart palpitation (3.1%), hand cramps (0.6%), and superficial venous thrombosis (0.6%). We did not find any relationship between adverse events of RIC and functional recovery of patients, which should be tested in the next step.

Despite the important findings of this study, certain limitations were noted. First, while this was a single-arm, not sham-controlled study. The purpose of this study was to get a first-hand experience on using RIC in this stroke subpopulation and to collect preliminary data to better calculate the sample size for the next study. Single-arm study has the advantage of completing the study procedure faster and more cost-effective than a sham-controlled study. Second, the compliance rate needs to be improved. In our next study, we plan to conduct regular daily RIC at home with the support of mobile health in order to improve the patient compliance rate, as described in a previous study (29). Lastly, the present study is not designed to demonstrate the associations between RIC compliance and efficacy outcomes. The optimal dose of RIC remains unknown.

In conclusion, RIC is a safe add-on procedure with a potential benefit in reducing cerebrovascular events in a subgroup of patients with non-cardiogenic AMIS/TIA; however, its compliance is not high and needs to be improved in the future study. Our study provided critical preliminary data to plan a large sample size, randomized controlled clinical study to systematically investigate the safety and efficacy of RIC in this unique stroke patient population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xuanwu Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SLiu: study design and conduct and manuscript preparation. ZG, RM, and HS: study design and conduct. TT, RC, YS, QF, FJ, QZ, JDi, XiaH, QM, KD, SX, ZY, JDu, CC, XC, XinH, and SLi: study conduct. BO and WZ: study design. XJ: study design and conduct and manuscript revise. WF: study design and manuscript revise. All authors contributed to the article and approved the submitted version.

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Remote Ischemic Conditioning in the Prevention for Stroke-Associated Pneumonia: A Pilot Randomized Controlled Trial

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Background: Despite the continuing effort in investigating the preventive therapies for stroke-associated pneumonia (SAP), which is closely associated with unfavorable outcomes, conclusively effective therapy for the prevention of SAP is still lacking. Remote ischemic conditioning (RIC) has been proven to improve the survival in the sepsis model and inflammatory responses have been indicated as important mechanisms involved in the multi-organ protection effect of RIC. This study aimed to assess the safety and the preliminary efficacy of RIC in the prevention of SAP in patients with acute ischemic stroke.

Methods: We performed a proof-of-concept, pilot open-label randomized controlled trial. Eligible patients (age > 18 years) within 48 h after stroke onset between March 2019 and October 2019 with acute ischemic stroke were randomly allocated (1:1) to the RIC group and the control group. All participants received standard medical therapy. Patients in the RIC group underwent RIC twice daily for 6 consecutive days. The safety outcome included any adverse events associated with RIC procedures. The efficacy outcome included the incidence of SAP, changes of immunological profiles including mHLA-DR, TLR-2, and TLR-4 as well as other plasma parameters from routine blood tests.

Results: In total, 46 patients aged 63.1 ± 12.5 years, were recruited (23 in each group). Overall, 19 patients in the RIC group and 22 patients in the control group completed this study. No severe adverse event was attributed to RIC procedures. The incidence of SAP was lower in the remote ischemic conditioning group (2 patients [10.5%]) than that in the control group (6 patients [27.3%]), but no significant difference was detected in both univariate and multivariate analysis ($p = 0.249$ and adjusted $p = 0.666$). No significance has been found in this pilot trial in the level of immunological profiles HLA-DR, TLR4 and TLR2 expressed on monocytes as well as blood parameters tested through routine blood tests between the two groups ($p > 0.05$). The IL-6 and IL-1β levels at day 5 after admission in the RIC group were lower than those in the control group ($p < 0.05$).

Interpretation: This proof-of-concept pilot randomized controlled trial was to investigate RIC as a prevention method for SAP. Remote ischemic conditioning is safe in the prevention of SAP in patients with acute ischemic stroke. The preventive effect of RIC on SAP should be further validated in future studies.

Keywords: stroke, stroke-associated pneumonia (SAP), remote ischemic conditioning (RIC), stroke-induced immunodepression (SIID), immune response

INTRODUCTION

Stroke-associated pneumonia refers to a spectrum of pulmonary infections in patients within 7 days after stroke onset (1). Stroke-associated pneumonia (SAP) is one of the most common complications after stroke, which has been well-known for worsening patient outcomes including the increasing disability and mortality with an estimated incidence ranging from 5–26% in general (2–5). Even though continuing effort in investigating the preventive therapies for SAP especially preventive antibiotics, conclusively effective therapy for the prevention of SAP is still lacking (6, 7).

Remote ischemic conditioning (RIC) is a strategy to protect distant organs against lethal acute ischemia-reperfusion injury which can be applied $\geq$ 1 cycle of brief and non-lethal limb ischemia through simply inflating and deflating a standard blood-pressure cuff placed on the upper arm (5, 8–10). Evidence shows that the survival has been prolonged in the sepsis model of rodents and sheep through applying the RIC procedure (11–13). Even though the mechanisms of the protective effect of RIC are not completely understood, the inflammatory responses have been indicated as important mechanisms involved in the multi-organ protection effect of RIC including the heart and brain (14, 15). However, whether RIC has a beneficial effect on SAP prevention is still not known. In this study, we aimed to assess the safety and the preliminary efficacy of RIC in the prevention of SAP in patients with acute ischemic stroke.

Clinical studies have shown that monocyte human leukocyte antigen-DR (mHLA-DR) and some blood parameters of the routine blood tests are associated with SAP occurrence (16–20). The loss of expression of mHLA-DR is suggested to be closely relevant to stroke-induced immunodepression which predisposes SAP (17–19). Monocytes are of vital significance in acute ischemic stroke (17, 21). In addition, toll-like receptors (TLRs) are pattern recognition receptors of innate immune cells. Inflammatory cells especially macrophages interact with damage-associated molecular patterns (DAMPs) released from destructed neural tissue from a stroke via TLRs, which can activate downstream inflammatory signaling pathways, play a critical role in the pathogenesis of stroke (21). Among TLR families, TLR2 and TLR4 are most studied which are closely relevant to the stroke-induced inflammatory response (17, 21–24). In this study, we examined HLA-DR, TLR-2, and TLR-4 of monocytes as well as routine blood tests to help determine the effects of RIC on SAP prevention.

METHODS

Trial Design

This is a proof-of-concept, pilot open-label, and assessor-blinded randomized controlled trial conducted at Xuanwu Hospital of Capital Medical University. Patients were randomly assigned in a 1:1 ratio to undergo either remote ischemic conditioning or no intervention (control) group. This study was approved by the Ethics Committee of Xuanwu Hospital of Capital Medical University. All subjects or their legally authorized representative provided informed consent before enrollment.

Participants

Eligible patients for enrollment were adults ($\geq$ 18 years of age), have confirmed diagnosis of acute ischemic stroke (AIS) with the onset of symptoms within 48 h at recruitment, have NIHSS score $\leq$ 15, have pre-stroke modified Rankin Scale (mRS) $\leq$ 2, and subjects or their legally authorized representative was able to provide informed consent.

Exclusion criteria were uncontrolled hypertension (defined as systolic blood pressure $\geq$ 200 mmHg), participation in another device or drug trial simultaneously, any vascular, soft tissue, or orthopedic injury (e.g., superficial wounds and fractures of the arm) that contraindicated unilateral arm ischemic conditioning, peripheral vascular disease (especially subclavian arterial and upper limb artery stenosis or occlusion), stroke patients who underwent endovascular therapy for this time of onset, pregnancy, history of malignancies, using remote ischemic conditioning within the preceding 1 week, known dysphagia/nasogastric feeding tubes or infection at admission, a history of infection or the use of antibiotics, immunosuppressants, or steroids within the preceding 3 months, other conditions are not suitable for this trial (evaluated by researchers). Participants underwent follow-up assessment at 3-months through telephone or clinical visits.

Interventions

Standard stroke medical therapy was consistent in all the enrolled patients in both the RIC group and the control group, including antiplatelet, statins, and risk factors management. The antihypertensive and antidiabetic agents were decided by the treating physicians who worked in the stroke unit based on the condition of the patients. Eligible patients allocated in the RIC group received RIC procedures twice daily for 6 consecutive days

and the patients in the control group received standard stroke medical therapy only.

The RIC procedure consisted of five cycles of inflation to a pressure of 200 mmHg and deflation for 5 min alternately within 2 h of enrollment performed unilateral upper arm (the transfusion side was avoided) by an electric auto-controlled device with cuff (patent number ZL201420846209.5, China). This procedure can be stopped at any time if the subject experiences discomfort. The RIC procedure was done with the assistance of the nurses in the stroke unit.

Randomization

All subjects were consecutively enrolled and randomly allocated in a 1:1 ratio to the RIC group (RIC procedure plus standard stroke medical therapy) or the control group (standard stroke medical therapy only) based on a computer-generated randomization code. The randomization of the assignment was sealed and handed to the treating physician who was blinded to the protocol.

Outcome Assessment

Safety Outcome Assessment

The safety outcomes included the following: (1) inability to tolerate RIC procedure that leads to discontinuation; (2) objective signs of tissue or neurovascular injury resulting from RIC procedure; (3) stroke deterioration within 7 days (4); any hemorrhagic transformation within 7 days; (5) any adverse events within 90 days after stroke onset. The inspection was to evaluate especially for any local edema, erythema, skin lesions, and palpation of distal pulses of the upper arm, which was done by observers blinded to the study protocol. Any suspicious adverse event associated with the RIC procedure would be reported to the investigators.

Efficacy Outcomes Assessment

The efficacy outcome included: (1) the incidence of SAP; (2) Favorable outcome (modified Rankin Score (mRS) 0–2); (3) changes of immunological profiles including HLA-DR, TLR-2, and TLR-4 expressed on monocytes; (4) changes of the inflammatory cytokines including IL-1β, IL-6, TNFα, and IL-10. Based on the combination of clinical symptoms, radiological findings, and pathogen detection, SAP was diagnosed by the treating physician according to the modified Centers for Disease Control and Prevention criteria of stroke-associated pneumonia (1). In this study, the diagnosis of SAPs was reaching the definite SAP which means the diagnostic changes on the chest radiograph existed (Detailed diagnostic criteria can be seen in the **Supplementary Material**). Clinical outcome was evaluated according to the mRS. Favorable functional outcome was defined as mRS 0–2 at 90 days through telephone follow-up or clinical visits by certified vascular neurologists.

Flow Cytometry and Routine Blood Test

Baseline blood samples were collected between 5:00 a.m. and 6:30 a.m. after admission to the stroke care unit before any medication was started. Blood samples were collected at baseline, days 2 and 5 after admission.

Immunological profiles including the expression of HLA-DR, TLR-4, and TLR-2 on monocytes were measured through flow cytometry by investigators blinded to clinical endpoints. White blood cells were gated according to CD45+ expression. Monocytes were then gated according to CD14+ expression after removal of cell debris and non-leukocyte particles (**Supplementary Material**). Specifically, 2-ml of blood samples were collected in EDTA-coated tubes. 100 μl of anticoagulated whole blood was added to the mixture of a selected panel of monoclonal antibodies.

The following monoclonal antibodies were used: Fluorescein isothiocyanate (FITC) anti-human CD14 antibody, PerCP anti-human CD45 antibody, phycoerythrin (PE) anti-human CD284 (TLR4) antibody, Alexa Fluor647 anti-human CD282 (TLR2) antibody, allophycocyanin/cyanine7 (APC/Cy7) anti-human HLA-DR antibody (All from BioLegend, San Diego, CA, USA). FITC- mouse IgG2a k Isotype Control, Alexa Fluor647 mouse IgG2a k Isotype Control, and APC/Cy7 mouse IgG2a k Isotype Control were used as quality controls (BioLegend, San Diego, CA, USA). Monoclonal antibodies were mixed with the cell suspension and incubated at room temperature for 20 min in the dark. After erythrocyte lysis of the whole blood/antibody mixture and incubation at room temperature for 10 min. The acquisition was performed by an LSR II flow cytometer (BD Biosciences, San Diego, CA, USA) with Diva version 6.1.3 (BD Biosciences, San Diego, CA, USA), Flowjo software (BD Biosciences, San Diego, CA, USA), Sysmex XE-5000 autoanalyzer (MEK-7222K, Nihon Kohen, Japan), Meso Scale Discovery (MSD, Rockville, Maryland, USA) kit of the cell resuspension solution. Flowjo software was used for analysis.

Venous blood samples were collected into standardized tubes containing an anticoagulant (EDTA) and stored at room temperature. We measured white blood cell, neutrophil, lymphocyte, and monocyte counts through routine blood tests, which were determined using Sysmex XE-5000 autoanalyzer within 1 h after sample collection.

Measurement of Inflammatory Cytokines

Plasma was obtained at baseline, day 2 (24 h after RIC/ no-RIC procedure), and day 5 after admission and stored at −80°C. The plasma inflammatory cytokines including the pro-inflammatory IL-1β, IL-6, and TNFα, as well as the anti-inflammatory IL-10, were measured through ELISA assay conducted with the Meso Scale Discovery (MSD, USA) kit following the manufacturer's instructions.

Statistical Analysis

We compared the data between participants treated with and without RIC. The analysis was based on the per-protocol population, defined as randomly allocated individuals in their assigned group. Continuous variables were exhibited by mean ± *SD* for normal distribution or median while interquartile range (IQR) for skewed distribution. Independent *t*-test or Mann–Whitney *U*-test for continuous variables, and the Chi-Square test or Fisher's exact test for categorical variables were used to detect the differences between two groups. The normality of distributions was tested by the Kolmogorov-Smirnov test.

A multiple logistic regression model was used to adjust the baseline characteristics between the two groups. All data were processed using SPSS 23 for Mac (IBM, Chicago, IL, USA) with a significance level of $P < 0.05$ (two-sided).

Data Availability

Any data not published within the article will be shared in anonymized form by request from any qualified investigator.

RESULTS

Between March 2019 and October 2019, 276 patients were consecutively screened in an advanced stroke center (Xuanwu Hospital of Capital Medical University). Forty seven patients met the inclusion criteria. One patient was reluctant to participate in this study. Forty six patients were enrolled and underwent randomization equally to the RIC group and the control group (23 in each group). 3 cases were lost in the RIC group due to remote transferring where the RIC facilities were not available. Ultimately, a total of 19 patients in the RIC group and 22 patients in the control group completed the observation during admission and 90 days follow-up (**Figure 1**).

Baseline Characteristics

Baseline characteristics of 41 subjects were summarized in **Table 1**. The mean age was 63.1 ± 12.5 years old with a median National Institutes of Health Stroke Scale (NIHSS) score of 6 (4–10). Twenty six patients (63.4%) were men. The age, gender, baseline NIHSS score, and stroke etiology did not differ significantly among these 2 groups. The comorbidities were shown comparable between the RIC group and the control group except the rate of hypertension was higher in the control group ($p = 0.008$).

Safety Outcomes

No severe adverse event was attributed to RIC procedures. The RIC procedures were well tolerated. One patient in the RIC group experienced skin petechiae of the upper arm from repeated pressure cuff applications and decided to discontinue this procedure. 19 (82.6%) patients completed the RIC procedure overall and the per-protocol completion rate was 95%. No other signs of tissue or neurovascular injuries resulting from the RIC procedure including local edema, erythema, or skin lesions were observed. No subjects in both groups experienced hemorrhagic transformation. One patient in the control group showed stroke deterioration which led to death.

Efficacy Outcomes

Clinical Events

Stroke-associated pneumonia (SAP) occurred in 8 (19.5%) of the patients. Two (10.5%) in the RIC group, 6 (27.3%) in the control group. The incidence of SAP was lower in the remote ischemic conditioning group (2 patients [10.5%]) than that in the control group (6 patients [27.3%]), but no significant difference was detected in both univariate ($p = 0.249$) and multivariate analysis (adjusted OR: 0.343, 95% CI: 0.043–2.758, $p = 0.666$) (**Table 2**).

TABLE 1 | Baseline characteristics between the RIC group and control group in patients with acute ischemic stroke.

	All (*N* = 41)	RIC group (*N* = 19)	Control group (*N* = 22)
Age, y (mean ± SD)	63.1 ± 12.5	61.6 ± 10.9	64.3 ± 13.6
Male, *n* (%)	26 (63.4)	11 (57.9)	15 (68.1)
Baseline NIHSS score, median (IQR)	6 (4–10)	6.5 (4.3–7.8)	6 (4–12)
Anterior circulation, *n* (%)	28 (68.3)	13 (68.4)	15 (68.2)
Posterior circulation, *n* (%)	13 (31.7)	6 (31.6)	7 (31.8)
Comorbidities, *n* (%)			
Hypertension	26 (63.4)	8 (42.1)	18 (81.8)
Diabetes Mellitus	18 (43.9)	9 (47.4)	9 (40.9)
Atrial fibrillation	4 (9.8)	1 (5.3)	3 (13.6)
Hyperlipidemia	13 (31.7)	5 (26.3)	8 (36.4)
Previous stroke history	9 (22.0)	5 (26.3)	4 (18.2)
Coronary artery disease	5 (12.2)	2 (10.5)	3 (13.6)
Smoking	16 (39.0)	7 (36.8)	9 (40.9)
Stroke etiology, *n* (%)			
Large-artery atherosclerosis	22 (53.7)	11 (57.9)	11 (50.0)
Cardioembolism	3 (7.3)	1 (5.3)	2 (9.1)
Small-vessel occlusion and other causes	16 (39.0)	7 (36.8)	9 (40.9)
Treatment, *n* (%)			
IV tPA	3 (7.3%)	2 (10.5%)	1 (4.5%)

RIC, remote ischemic conditioning; SD, standard deviation; IQR, interquartile range; NIHSS, National Institutes of Health Stroke Scale; IV tPA, intravenous tissue plasminogen activator.

TABLE 2 | SAP incidence and favorable outcome at 3-month.

	All (*N* = 41)	RIC group (*N* = 19), *n* (%)	Control group (*N* = 22), *n* (%)	*P*
SAP	8 (19.5%)	2 (10.5%)	6 (27.3%)	0.249
mRS: 0–2	25 (61.0)	12 (63.2%)	13 (59.1%)	0.796

RIC, remote ischemic conditioning.

Further results showed that 25 patients (61%) [12 (63.2%) in the RIC group and 13 (59.1%) in the control group] demonstrated favorable outcomes. The favorable outcomes at 3-month between the two groups did not reach a statistical significance ($p = 0.796$) (**Table 2**).

Time Course of the Phenotype of Monocytes After Stroke

The plasma expression of HLA-DR, TLR2, and TLR4 on monocytes was tested on the baseline, days 2 and 5. However, no statistical significance was detected in this pilot trial among these two groups ($p > 0.05$). No significance has been found in this pilot trial in the level of immunological profiles (**Figure 2**) as well as other plasma parameters between the two groups (**Supplementary Material**).

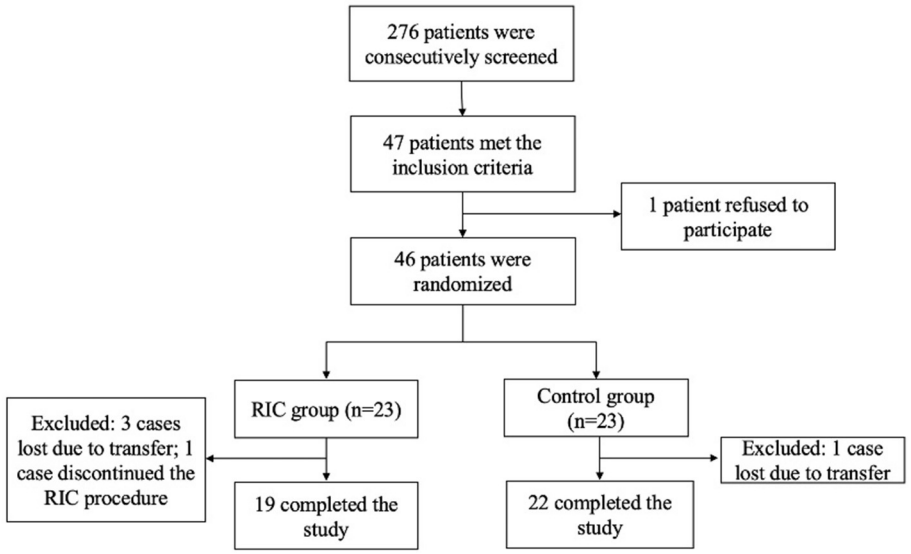

FIGURE 1 | Enrollment and randomization. RIC, remote ischemic conditioning.

Time Course of Inflammatory Cytokines After Stroke

We measured the inflammatory cytokine including the pro-inflammatory IL-1β, IL-6, and TNFα, as well as the anti-inflammatory IL-10. There was no statistical significance between the RIC group and the control group at baseline and day 2 after admission ($P > 0.05$). However, the IL-6 and IL-1β levels at day 5 after admission were higher in the control group than those in the RIC group ($P < 0.05$), while the level of TNFα and IL-10 remained comparable at day 5 after admission (**Figure 3**).

DISCUSSION

This proof-of-concept pilot randomized controlled trial indicated that it was safe to perform RIC in patients with acute ischemic stroke. Even though the incidence of SAP was lower in the RIC group than that in the control group, no significant difference was detected in both univariate and multivariate analyses. RIC seems to be an applicable clinical adjuvant treatment for stroke patients. The efficacy of the preventive effect of RIC on reducing SAP should be further validated in future studies.

This study indicated that RIC was well tolerated by patients with acute ischemic stroke. Despite 1 patient in the RIC group experiencing skin petechiae of the upper arm from repeated pressure cuff applications which lead to the patient's decision the discontinuation of the RIC procedure. No severe local or systematic adverse event was associated with RIC procedures.

The immune responses interacted with the CNS have been indicated in numerous studies of stroke and inflammation has been considered as an important target for stroke therapy (21, 25, 26). Stroke-induced immunodepression caused by neural injury of stroke was widely acknowledged as the underlying mechanism of stroke-associated pneumonia (18, 21, 27). Moreover, monocyte as an important part of our innate immunity plays a significant role in stroke-induced infections, and decreased expression of monocytic HLA-DR has been considered as a predictor of stroke-induced pneumonia (17, 18, 28). An increased number of monocytes can be found in the blood in stroke patients shortly after stroke and the deactivation of antigen-presenting molecules is considered to be a risk factor of poststroke infections (17, 28). TLR4 and TLR2 are pattern recognition receptors that can be sensed by immune innate cells and activate downstream signaling inflammatory cascade, which are the most investigated receptors associated with stroke-induced inflammation response (21).

The mechanisms of the protection of the RIC procedure have not been well elucidated. However, numerous studies have indicated that the RIC exerts its protection effect has been largely associated with its anti-inflammatory effect, not just in neuroprotection, but in multiorgan as well, including changes in the expression of inflammatory genes, attenuating the pro-inflammatory cytokines, and downregulating the pro-inflammatory signaling pathway involving nuclear factor kappa B (NF-κB) (15, 29–33). The survival rates in the sepsis model of rodents and sheep have been shown to improve through applying the RIC procedure (11–13). However, few clinical studies have focused on the effect of RIC on inflammatory responses. In this pilot randomized trial, we particularly focused on the prevention effect of the RIC procedure on reducing SAP, which is a common complication after stroke which is closely relevant to unfavorable outcomes (3, 4).

The clinical outcome indicated that the RIC may have a potential protective effect on the prevention of SAP in patients with acute ischemic stroke. A limited sample size of this pilot trial may be one of the reasons explaining the statistical insignificance of this pilot clinical trial. In this study, we detected the pro-inflammatory cytokines IL-6 and IL-1βat day 5 after admission in

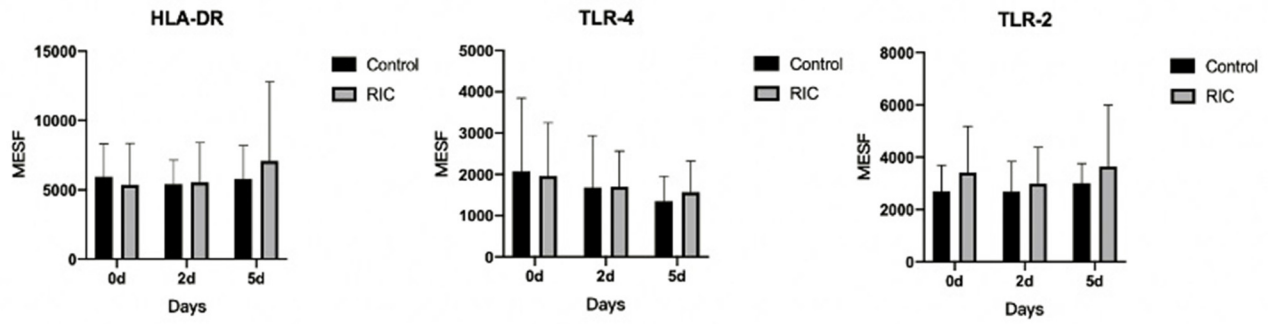

FIGURE 2 | Time course after admission and phenotype of circulating monocytes in stroke patients between RIC group and the control group. RIC, remote ischemic conditioning; MESF, molecules of equivalent soluble fluorochrome.

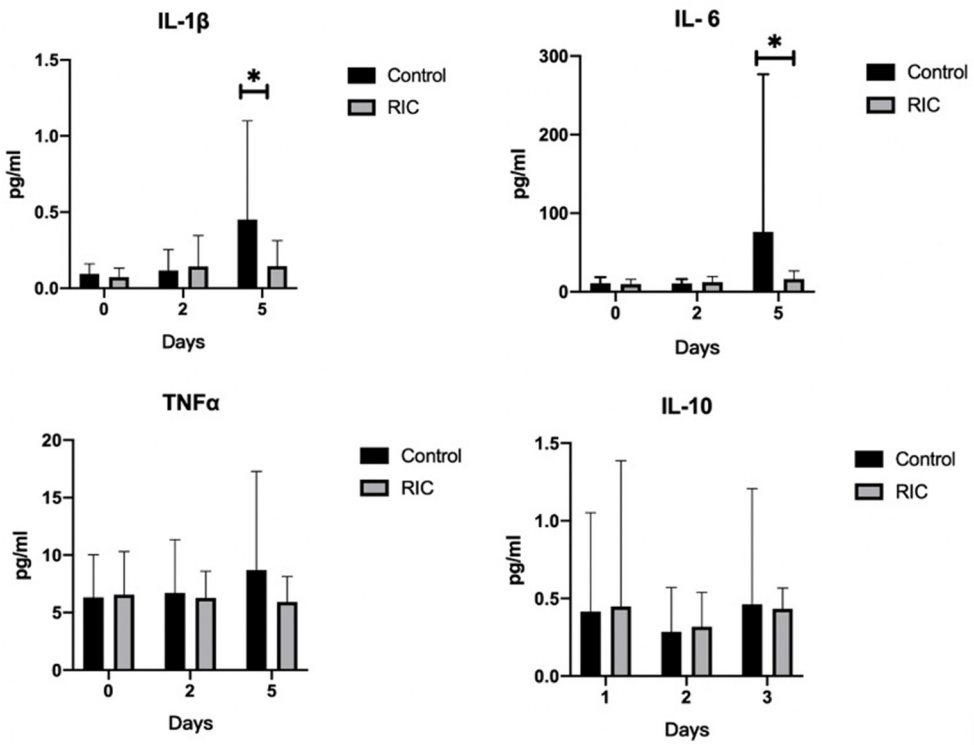

FIGURE 3 | Pro-inflammatory and anti-inflammatory cytokines between RIC group and the control group. IL-1β, IL-6 and TNFα are as pro-inflammatory cytokines and IL-10 is as anti-inflammatory cytokine. Data are expressed as mean ± SD. $^{*}P < 0.05$. RIC, remote ischemic conditioning.

the RIC group were significantly lower than those in the control group but not at day 2. This result was consistent with the animal studies that RIC can attenuate the pro-inflammatory cytokines (11, 13). It suggests that restoring the imbalance between pro-inflammatory and anti-inflammatory cytokines might be an underlying mechanism for RIC to exert its protective effect.

There are several limitations in this pilot trial. First, due to our limited study population and clinical heterogeneity in this exploratory pilot clinical trial, even though the incidence of SAP was lower in the RIC group than that in the control group, no significant difference was detected. Although statistical significances of IL-1βand IL-6 at day 5 after admission were detected between the two study groups, the result should be interpreted with caution and the protective effect of the RIC procedure on SAP should be further validated in future clinical trials in larger populations. Second, the RIC procedure in this study contained twice daily of 5 min ischemia and 5 min reperfusion for 5 cycles, which is a practical dose and may not be the optimal dose of RIC. Studies are still warranted to further investigate the optimal scheme of RIC in the clinical scenario of

the intervention of acute ischemic stroke. Third, the effect of RIC on adaptive immunity was not evaluated in this study so that the specific underlying mechanisms of the protective effect of RIC should be further explored in future studies.

In conclusion, this proof-of-concept pilot randomized controlled trial was to investigate RIC as a prevention method for reducing SAP occurrence. Remote ischemic conditioning is safe in the prevention of SAP in patients with acute ischemic stroke. Attenuating the pro-inflammatory cytokines might be a way for RIC to exert its effect. However, the efficacy of RIC on the prevention of SAP and the underlying mechanisms should be further investigated in future studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Xuanwu Hospital of Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BZ designed the study, collected, and analyzed the data, and drafted the manuscript. WZ and RC participated in the design of the study. HM, YZ, TB, HY, JX, LW, WY, JL, HS, JD, HC, QM, and QZ participated in the coordination of the study. XJ is the corresponding author and participated in the design and coordination of this study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.723342/full#supplementary-material

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Conflict of Interest: XJ is one of the inventors of the electronic auto-control device that has been patented in China (ZL201420846209.5, China).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Daily Remote Ischemic Conditioning Can Improve Cerebral Perfusion and Slow Arterial Progression of Adult Moyamoya Disease—A Randomized Controlled Study

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Background and Purpose: Moyamoya disease (MMD) is a complicated cerebrovascular disease with recurrent ischemic or hemorrhagic events. This study aimed to prove the safety and efficacy of remote ischemic conditioning (RIC) on MMD.

Methods: In total, 34 patients with MMD participated in this pilot, prospective randomized controlled study for 1 year. 18 patients were allocated into the RIC group, and 16 patients accepted routine medical treatment only. RIC-related adverse events were recorded. The primary outcome was the improvement ratio of mean cerebral blood flow (mCBF) in middle cerebral artery territory measured by multidelay pseudocontinuous arterial spin labeling, and the secondary outcomes were the cumulative incidence of major adverse cerebrovascular events (MACEs), the prevalence of stenotic-occlusive progression, and periventricular anastomosis at 1-year follow-up.

Results: In total, 30 of the 34 patients with MMD completed the final follow-up (17 in the RIC group and 13 in the control group). No adverse events of RIC were observed. The mCBF improvement ratio of the RIC group was distinctively higher compared with the control group ($mCBF_{-whole\text{-}brain}$: 0.16 ± 0.15 vs. −0.03 ± 0.13, $p = 0.001$). Stenotic-occlusive progression occurred in 11.8% hemispheres in the RIC group and 38.5% in the control group ($p = 0.021$). The incidence of MACE was 5.9% in the RIC group and 30.8% in the control group (hazard ratio with RIC, 0.174; 95% CI, 0.019–1.557; $p = 0.118$). No statistical difference was documented in the periventricular anastomosis between the two groups after treatment.

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Conclusions: Remote ischemic conditioning has the potential to be a safe and effective adjunctive therapy for patients with MMD largely due to improving cerebral blood flow and slowing the arterial progression of the stenotic-occlusive lesions. These findings warrant future studies in larger trials.

Keywords: moyamoya disease, stroke, remote ischemic conditioning, cerebral blood flow, arterial spin labeling (ASL)

INTRODUCTION

Moyamoya disease (MMD) is a complicated cerebrovascular disease, mainly involving children or young adults characterized by stenosis or occlusion at the bifurcation of the circle of Willis and proliferation of basal collaterals (1). Chronic cerebral hypoperfusion can make patients with MMD suffer from ischemic stroke, transient ischemic attack (TIA), cognitive impairment, and even intracranial hemorrhage (2).

The mechanism of MMD has not been fully elucidated, thereby there is no specific medication for MMD. Antiplatelet therapy is utilized to prevent ischemic stroke and TIA, however, it is controversial for patients with MMD, because antiplatelet agents may increase the risk of intracranial hemorrhage in patients with MMD (3). Revascularization surgery has been shown to improve cerebral perfusion and reduce cerebrovascular events for patients with MMD (4–6), nevertheless, complications like hyperperfusion syndrome, postoperative stroke can occur and may lead to neurological deterioration (7).

Remote ischemic conditioning (RIC) is a non-invasive approach protecting the brain by repeated ischemia-reperfusion on the upper limbs. Neuroprotective factors were produced by the stimulus of RIC and these factors conferred a protective effect on remote target organs (8). It has been confirmed that RIC can improve cerebral perfusion by promoting angiogenesis and arteriogenesis in ischemic animal brains (9–11). In addition, daily RIC is a promising technique to ameliorate injury caused by chronic cerebrovascular diseases such as intracranial atherosclerotic stenosis (ICAS), small-vessel disease (12, 13). Meng et al. revealed that RIC could reduce recurrent stroke in patients with symptomatic ICAS by promoting cerebral perfusion (12). Wang et al. reported that daily RIC for 1 year could improve cognitive function and reduce the volume of white matter hyperintensities of small vessel disease (13). Recently, a single-arm study has reported that RIC relieved ischemic events and improved perfusion in ischemic patients with MMD (14). However, the efficacy of RIC for patients with MMD is still unknown in a randomized controlled setting. As far as we know, two randomized clinical trials about the efficacy of RIC for pediatric patients with MMD are in progress (NCT03546309 and NCT03821181) (15) and thus, we conducted a pilot randomized controlled study to explore the safety and efficacy of RIC treating adult patients with MMD to guide further trials.

METHODS

Study Design

This was a single-center, open-label, prospective, parallel randomized study from 2019 July to 2021 February in patients with MMD with 1-year treatment at Xuanwu Hospital, Capital Medical University. This study was registered at clinicaltrials.gov with the unique identifier NCT04012268. This protocol was approved by the Ethics Committee of Xuanwu Hospital, Capital Medical University. All the subjects have provided written informed consent.

Inclusion Criteria

Patients who participated in this study met all of the inclusion criteria: (1) Patients aged from 18 to 60 years; (2) All of the patients underwent digital subtraction angiography (DSA) and met the current diagnostic criteria recommended by the Research Committee on MMD of the Ministry of Health and Welfare of Japan in 2012; (3) Modified Rankin Scale (mRs) score ≤ 3; and (4) Informed consent obtained from the patient or acceptable surrogate patient.

Exclusion Criteria

Subjects who met any of the exclusion criteria were excluded from this study: (1) patients with acute ischemic or hemorrhagic stroke within 3 months; (2) severe hepatic or renal dysfunction; (3) severe hemostatic disorder or severe coagulation dysfunction; (4) severe cardiac diseases; (5) patients with severe existing neurological or psychiatric disease; (6) patients with moyamoya syndrome caused by autoimmune disease, Down syndrome, neurofibromatosis, leptospiral infection, or previous skull-base radiation therapy; and (7) patients with completed or planned revascularization surgery.

Randomization and Masking

The subjects diagnosed as MMD by DSA who had not been accepted for revascularization surgery were recruited. After baseline assessment, eligible patients signed informed consent. They were randomized in 1:1 ratio to accept either RIC plus routine medical treatment or routine medical treatment only with a computer-generated randomization code. The code was put into an opaque envelope. The investigators would number the eligible patients and open the envelope to determine the treatment plan. Investigators who assessed outcomes were blinded to the allocation.

Sample Size

This was a pilot randomized controlled study. There were no parameters referred to estimate the sample size, and Hertzog (16) has shown that 10–20 subjects per group are adequate to evaluate the feasibility in a pilot study. In addition, Dobkin (17) has suggested that 15 subjects in each group are enough to evaluate whether a larger multicenter trial should be implemented. Thus, we aimed to recruit 15 patients per group, and finally, we recruited a total of 34 patients in this study.

Procedures

Patients in the RIC group accepted bilateral upper limbs RIC performed by an autocontrol device (patent no.: ZL200820123637.X, China), including five cycles of inflating and deflating for 5 min alternately twice daily for 1 year. The inflating pressure was 200 mm Hg. The investigators performed telephone follow-ups every month to assure patients insisting on this treatment. Considering the effect of single-time RIC could sustain 4 days (18), the compliance of 1 month would be considered substandard if the patient discontinued RIC treatment for consecutive 4 days of the month. Both groups accepted routine medical treatment that includes antiplatelet therapy, lipid-lowering therapy, controlling of vascular risk factors, and adjunct drug butylphthalide. Antiplatelet therapy was not available for asymptomatic and hemorrhagic patients in this study.

Outcome Assessment

The primary outcome was the mean cerebral blood flow (mCBF) improvement ratio after 1-year treatment. Secondary outcomes were the progression of stenotic-occlusive lesion, the change of periventricular anastomosis, and the incidence of major adverse cerebrovascular events (MACEs). The outcomes of safety were the incidence of adverse events related to RIC and the change of hematologic indexes. Fasting blood of patients was collected and hematologic indexes were examined including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), and platelet count (PLT). Alanine transaminase (ALT) and aspartate aminotransferase (AST) were examined to reflect hepatic function, creatinine (CREA), and urea nitrogen (UREA) were obtained to assess renal function. Creatine kinase (CK), a biomarker for reflecting skeletal muscle, was also tracked to monitor for injury. Multidelay pseudocontinuous arterial spin-labeling MR (PCASL-MR), time-of-flight MR angiography (TOF-MRA), and hematologic indexes collection were performed within 1 week before and 1 year after the treatment.

Baseline and Follow-Up Evaluations

Demographic characteristics, clinical history including ischemic stroke-related risk factors, symptoms, concomitant diseases, and routine medical treatments were recorded. All participants underwent 3T black-blood T1-weighted intracranial vessel wall imaging to exclude moyamoya syndrome (such as ICAS, vasculitis, and so on).

Cerebral Blood Flow Evaluation

Neuroradiologic outcomes were assessed by two experienced radiologists who were blinded to the allocation, the result would be obtained when a consensus was achieved. The images of cerebral blood flow (CBF) were acquired from PCASL-MR which were performed at 12 ± 0.5 months from baseline. A 3T imaging system (Verio, Siemens Healthcare, Erlangen, Germany) with a 32-channel head coil was applied. The PCASL images were obtained by an acquisition protocol with an acquisition time of 5 min and 40 s, post-labeling delay of 1,500/2,000/2,500/3,000 ms, echo time of 22.67 ms, 26 scan slices, slice thickness of 5 mm, and a 64 × 64 matrix resolution. Data of PCASL in DICOM form were converted to perfusion maps. The CBF map was processed by OsiriX (University of California, Los Angeles, USA). In each hemisphere, the middle cerebral artery (MCA) territory was divided into 10 regions according to Albert Stroke Program Early CT score (ASPECTS). Regions of interest (ROI) were drawn manually in each region of the MCA to determine the absolute CBF values in slices of the corona radiata and the basal ganglia (detailed ROIs were depicted in **Supplementary Figure I**).

Mean CBF was calculated by formula: $mCBF_{-hemisphere}$ = CBF (M1 + M2 + M3 + M4 + M5 + M6 + insula + internal capsule + lentiform nucleus + caudate)/10; mean CBF of the whole brain ($mCBF_{-whole\text{-}brain}$) was defined as the average value of mCBF in the left and right hemisphere of the patient. $mCBF_{-cortex}$ was the average value of CBF values in bilateral M1-M6 and insula. The $mCBF_{-basal-ganglia}$ was defined as the mean value of CBF values in bilateral internal capsule, lentiform and caudate. The improvement ratio of corresponding mCBF was calculated by the formula: the mCBF improvement ratio = (mCBF at 1 year–mCBF at baseline)/mCBF at baseline.

Evaluation of Periventricular Anastomosis and Arterial Lesion Progression

The periventricular anastomosis and arterial lesion progression were evaluated by 3T 3D TOF-MRA. All the subjects were examined on a 3-Tesla system (Magnetom Verio; Siemens Healthineers, Erlangen, Germany), a 32-channel head coil was used for signal reception at 3T. The parameters of 3D TOF-MRA included: axial plane, flip angle 20°, TR/TE = 22/3.99 ms, FOV = 180 × 180 mm^2, slice thickness = 0.8 mm, and matrix = 320 × 320).

The periventricular anastomosis is a connection beginning at the perforating arteries ending at the medullary artery at the lateral corner of the frontal horn or body of the lateral ventricle. The periventricular anastomosis was classified into three types: lenticulostriate anastomosis, thalamic anastomosis, and choroidal anastomosis (19). Each type of anastomosis was recorded as "presence" for "positive" and "absence" for "negative" from TOF-MRA images at sagittal orientation.

Arterial progression could be confirmed according to following conditions: (1) compared to baseline, the stenotic or occlusive lesion extended to another segment of Willis circle (for example, lesion extended from MCA M1 segment to M2 segment); (2) lesion extended from proximal portion to distal portion of one segment (such as extending from proximal portion to distal portion of MCA M1 segment); (3) stenotic lesion

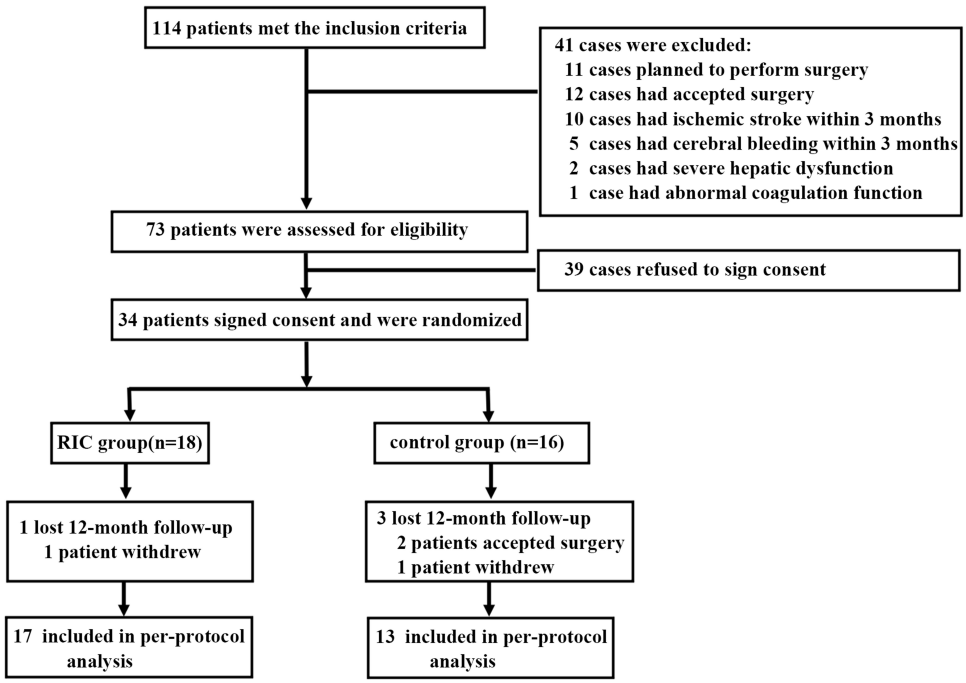

FIGURE 1 | Flowchart.

developed as the occlusive lesion. Houkin's MRA score (20) was used to evaluate the status of the arterial lesion in the Willis circle at baseline and 1 year follow-up.

Major Adverse Cerebrovascular Events Evaluation

Major adverse cerebrovascular events include hospital admissions with the TIA, ischemic stroke, and hemorrhagic stroke in this study. Patients were required to record the adverse events and were telephoned by two experienced neurologists every 3 months.

Statistical Analysis

To compare the characteristics at baseline or follow-up of the RIC group with the control group, continuous variables described as mean ± SD or medians (IQRs) were analyzed by the independent Student's *t*-test or the Mann–Whitney *U* test; categorical variables described as proportions were analyzed by the χ^2 test or the Fisher exact test using SPSS Statistics Version 23 (IBM Incorporation, Armonk, New York, USA). To further evaluate the effect of RIC on the progression of stenotic-occlusive lesion, the binary logistic regression analysis was used to determine the odds ratio (OR) with RIC. For comparing the cumulative incidence of MACEs at 12 months, the Cox proportional-hazards models were used. Interrater agreements of CBF measurements were evaluated by intraclass correlation coefficients (ICC). Consistency between two radiologists is considered good when the ICC value $\geq$ 0.75. $p < 0.05$ was considered to be statistically significant for all the tests. All the tests were 2-sided.

RESULTS

After screening 114 patients diagnosed as adult MMD, 34 patients were recruited. In total, 18 patients were allocated to the RIC group and 16 patients were in the control group. A total of 17 patients in the RIC group completed the 1-year follow-up, while 13 patients in the control group completed the follow-up (**Figure 1**). Thereby, a total of 30 patients (60 hemispheres) were included in this per-protocol analysis. In the RIC group, all the patients complied ≥80% of the months.

Baseline Characteristics

There were no significant differences between the two groups in demographic characteristics, symptoms, concomitant disease, medical treatment (**Table 1**), and hematologic indexes (**Table 2**). mCBF of the whole brain (RIC vs. control: 43.03 ± 8.70 vs. 47.06 ± 8.44 ml/100 g per min, $p = 0.213$), cortex (RIC vs. control: 45.07 ± 9.40 vs. 49.11 ± 10.23 ml/100 g per min, $p = 0.278$), basal ganglia (RIC vs. control: 38.28 ± 8.36 vs. 42.28 ± 10.30 ml/100 g per min, $p = 0.243$) were all not significantly different between the two groups at baseline (**Table 2**). In addition, no significant difference was shown in any type of periventricular anastomosis between the two groups (detailed characteristics were listed in **Table 3**).

Safety Outcome

No severe adverse events related to RIC occurred in 17 patients of the RIC group during the 1-year treatment. The hematologic indexes reflecting hepatic, renal function, and muscle injury

TABLE 1 | Baseline characteristics of the patients with moyamoya disease (MMD).

Characteristic	Control (*N** = 13)	RIC (*N** = 17)	*P*-value
Male sex—no./total no. (%)	4/13 (30.8)	5/17 (29.4)	1.000
Age—yr	36.0 ± 10.7	39.1 ± 10.1	0.422
Suzuki stage			
Median	3	3	0.891
Range	2–4	2–4	
Symptoms—no./total no. (%)			
Ischemic stroke	4/13 (30.8)	4/17 (23.5)	0.698
Hemorrhagic stroke	2/13 (15.4)	0/17 (0)	0.179
Transient ischemic attack	3/13 (23.1)	7/17 (41.2)	0.440
Seizure	0/13 (0)	1/17 (5.9)	1.000
Headache	3/13 (23.1)	4/17 (23.5)	1.000
Prior diagnosis—no./total no. (%)			
Hypertension	4/13 (30.8)	4/13 (23.5)	0.698
Diabetes	0/13 (0)	2/17 (11.8)	0.492
Hyperlipidemia	5/13 (38.5)	3/17 (17.6)	0.242
Lifestyle—no./total no. (%)			
Smoke	2/13 (15.4)	2/17 (11.8)	1.000
Alcohol	1/13 (7.7)	2/17 (11.8)	1.000
Drug treatment—no./total no. (%)			
Antiplatelet drugs	11/13 (84.6)	12/17 (70.6)	0.368
Lipid lowering drugs	7/13 (53.8)	10/17 (58.8)	0.785
Butylphthalide	4/13 (30.8)	4/17 (23.5)	0.698

n, number of subjects.

TABLE 2 | Features of hematologic indexes and cerebral blood flow.

Characteristic	Control (*N** = 13)	RIC (*N** = 17)	*P*-value
Baseline			
mCBF– ml/100 g per minute			
Whole brain	47.06 ± 8.44	43.03 ± 8.70	0.213
Cortex	49.11 ± 10.23	45.07 ± 9.40	0.278
Basal ganglia	42.28 ± 10.03	38.28 ± 8.36	0.243
Hematologic indexes			
WBC—1,012/L	6.34 ± 2.23	5.63 ± 1.37	0.287
RBC—1,09/L	4.57 ± 0.53	4.64 ± 0.57	0.740
HGB—g/L	133.77 ± 18.57	137.29 ± 16.12	0.583
PLT—109/L	241.77 ± 79.46	234.59 ± 61.93	0.783
ALT—IU/L	28.69 ± 17.05	24.18 ± 12.02	0.402
AST—IU/L	26.38 ± 13.14	23.71 ± 5.64	0.455
CREA—μmol/L	62.62 ± 33.86	52.84 ± 16.15	0.303
UREA—mmol/L	7.62 ± 13.38	52.84 ± 16.14	0.384
CK—IU/L	67.31 ± 35.04	59.21 ± 26.96	0.480
1-year follow-up			
mCBF improvement ratio			
whole brain	−0.03 ± 0.13	0.16 ± 0.15	0.001
cortex	−0.01 ± 0.13	0.16 ± 0.18	0.007
Basal ganglia	−0.08 ± 0.16	0.15 ± 0.18	0.001
Hematologic indexes			
WBC—1,012/L	6.12 ± 1.69	7.76 ± 4.14	0.492
RBC—109/L	4.40 ± 0.26	4.67 ± 0.58	0.444
HGB—g/L	135.25 ± 12.61	138.00 ± 15.17	0.790
PLT—109/L	219.50 ± 97.71	224.00 ± 45.59	0.936
ALT—IU/L	26.75 ± 11.93	19.00 ± 10.20	0.361
AST—IU/L	19.75 ± 2.36	18.25 ± 1.89	0.360
CREA—μmol/L	50.46 ± 11.43	62.30 ± 19.53	0.336
UREA—mmol/L	4.60 ± 1.33	4.64 ± 1.32	0.969
CK—IU/L	68.39 ± 21.83	88.12 ± 58.25	0.549

N, number of subjects.
mCBF, mean cerebral blood flow; WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; PLT, platelet count; ALT, alanine transaminase; AST, aspartate aminotransferase; CREA, creatinine; UREA, urea nitrogen; CK, creatine kinase.

remained normal and no statistical difference was documented (**Table 2**).

Efficacy Outcome

Neuroradiologic Outcomes

The inter-rater agreement was good in evaluating CBF by two radiologists. Intraclass correlation coefficients was 0.942 (95% CI, 0.882–0.972), 0.911 (95% CI, 0.821–0.956), 0.930 (95% CI, 0.858–0.966) for improvement ratio of $mCBF_{-whole-brain}$, $mCBF_{-cortex}$, and $mCBF_{-basal-ganglia}$, respectively. The improvement ratio of $mCBF_{-whole-brain}$ in the RIC group was significantly higher than that in the control group (0.16 ± 0.15 vs. −0.03 ± 0.13, $p = 0.001$). The mCBF of the cortex and the basal ganglia in the RIC group were improved by ratios of 0.16 ± 0.18, 0.15 ± 0.18, respectively, which were significantly higher than those in the control group ($p < 0.01$). The mCBF improvement ratios of the cortex and the basal ganglia in the control group were −0.01 ± 0.13, −0.08 ± 0.16, respectively. In addition, there was no significant difference in the improvement ratio between cortex and basal ganglia in the RIC group ($p = 0.830$). Detailed information is shown in **Table 2**; **Figure 2**.

The progression of the stenotic or occlusive lesion in the Willis circle was seen in 10 (38.5%) hemispheres in the control group, while four (11.8%) in the RIC group (OR with RIC, 0.21; 95% CI, 0.06–0.79, $p = 0.021$). In the control group, five hemispheres suffered lesion progression from segment M1 to other segments [four to M2 and one to anterior cerebral artery (ACA)]; three progressed from proximal to distal M1 segment; one progressed from stenotic lesion to occlusive lesion at M1 segment and one progressed from ACA A1 to A2 segment. In the RIC group, three hemispheres suffered progression from segment M1 to other segments (two to M2, one to ACA) and one progressed from proximal to distal M1 segment. The improvement of CBF and stenotic-occlusive lesion of two subjects were shown in **Figure 3**. No significant difference was documented in MRA score at baseline and 1-year follow-up between the two groups (**Table 3**). But, we found MRA score of the control group significantly increased after 1-year medical treatment [3.5 (2–5) vs. 4 (3–5), $p = 0.002$], while the MRA score of the RIC group kept stable [3 (3–4) vs. 3 (3–4), $p = 0.739$].

TABLE 3 | Features of periventricular anastomosis and arterial progression.

Neuroradiologic outcome	Control (*n** = 26)	RIC (*n** = 34)	OR (95%CI)	*P*-value
Baseline				
Anastomosis—no./total no. (%)			NA	
Choroidal	6/26 (23.1)	9/34 (26.5)		0.764
Lenticulostriate	3/26 (11.5)	5/34 (14.7)		0.721
Thalamic	3/26 (11.5)	4/34 (11.8)		0.978
MRA score	3.5 (2–5)	3 (3–4)	NA	0.659
Follow-up				
Lesion progression—no./total no. (%)	10/26 (38.5)	4/34 (11.8%)	0.21 (0.06–0.79)	0.021
Anastomosis—no./total no. (%)			NA	
Choroidal	9/26 (34.6)	9/34 (26.5)		0.495
Lenticulostriate	2/26 (7.7)	5/34 (14.7)		0.402
Thalamic	3/26 (11.5)	4/34 (11.8)		0.978
MRA score	4 (3–5)	3 (3–4)	NA	0.128

**n, number of hemispheres.*
MRA, MR angiography.

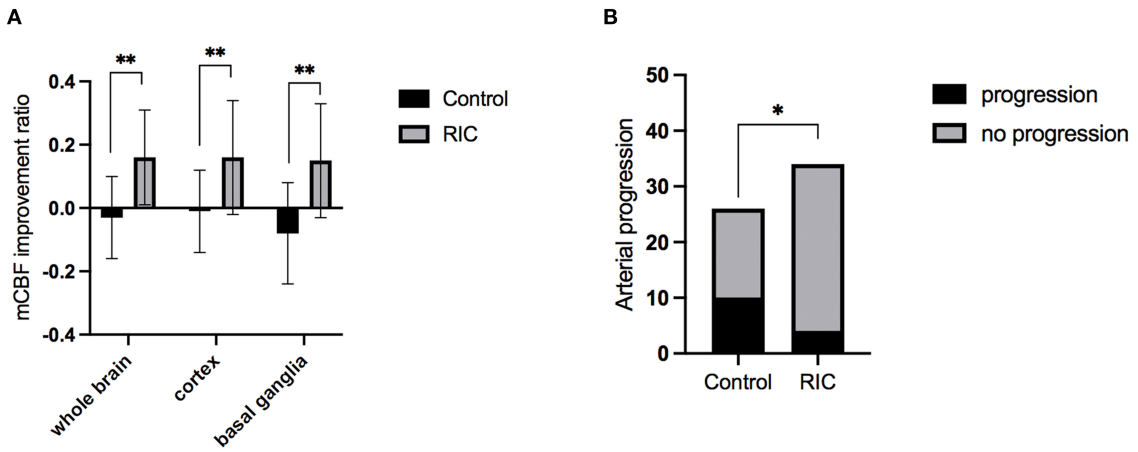

FIGURE 2 | The mCBF improvement ratio and stenotic-occlusive progression of two groups. **(A)** mCBF improvement ratio of whole brain, cortex, and basal ganglia between the two groups at 1-year treatment; **(B)** the number of hemispheres with arterial progression in two groups at 1-year follow-up; mCBF, mean cerebral blood flow; $^{*}p < 0.05$; $^{**}p < 0.01$.

At 1-year follow-up, there were still no differences in three types of periventricular anastomosis between the two groups ($p > 0.05$).

Clinical Outcomes

Major adverse cerebrovascular events occurred in 30.8% of patients in the control group, one suffered TIA, two suffered an ischemic stroke, and one suffered a hemorrhagic stroke. In the RIC group, only one patient (5.9%) suffered TIA and no patients suffered an ischemic or hemorrhagic stroke. However, there was no evidence of a significant difference in cumulative incidence of MACE between the control group and RIC group (hazard ratio with RIC, 0.17; 95% CI, 0.019–1.56, $p = 0.118$) (**Figure 4**). In addition, there was no distinct difference in any components of MACE containing TIA, ischemic stroke, and hemorrhagic stroke (**Supplementary Table I**).

DISCUSSION

Previous studies have shown that daily RIC was well-tolerated and safe in patients with symptomatic ICAS and small vessel disease (12, 13). The present study showed that no patient in the RIC group suffered any RIC-related adverse events and routine blood tests, renal, or hepatic function were normal during 1-year treatment.

Efficacy evaluation indicated that RIC could significantly improve the CBF and alleviate the progression of the stenotic-occlusive lesion in patients with MMD compared with the control

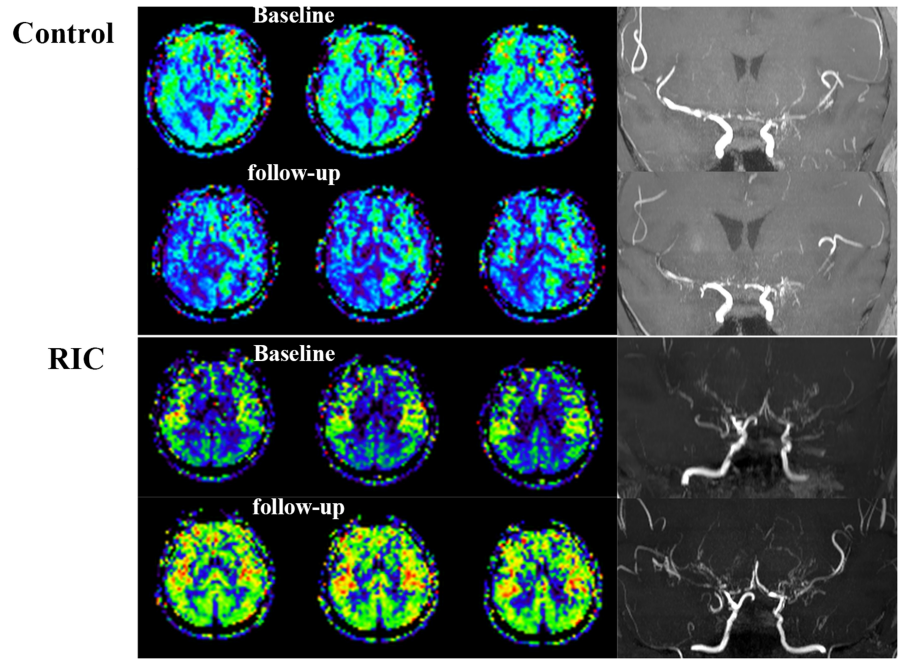

FIGURE 3 | Cerebral blood flow and stenotic-occlusive lesion at baseline and follow-up. ASL images of the patient in the control group showed deterioration of CBF mainly at the right side, which could be reflected by the progression of stenotic-occlusive lesion at right middle cerebral artery (MCA). arterial spin labeling (ASL) images of the remote ischemic conditioning (RIC) group demonstrated improved CBF and more abundant collaterals after RIC treatment in both the sides.

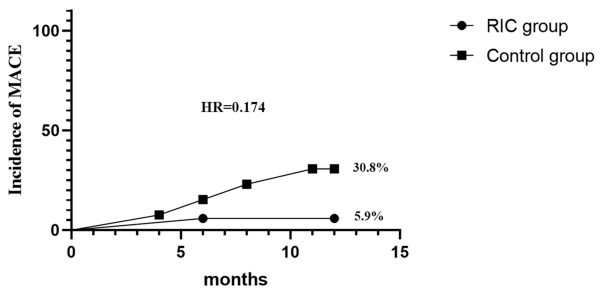

FIGURE 4 | Cumulative incidence of major adverse cerebrovascular events (MACEs) at 12 months.

group. Besides, the presence of periventricular anastomosis was not statistically different between the two groups. Although non-significantly, there existed a tendency that patients in the RIC group had a lower incidence of MACEs.

Patients with MMD with misery cerebral perfusion have a higher risk of subsequent ischemic stroke (21–23). Recent studies also indicated insufficient perfusion may cause future hemorrhagic events (24, 25). Direct surgery has been generally performed to improve the CBF of MCA territory immediately (26). In addition to improving outcomes of ischemic patients with MMD, a Japan Adult Moyamoya (JAM) trial was conducted to reveal that direct surgery could also reduce rebleeding attacks for hemorrhagic patients (6). The improved perfusion at MCA territory supplied by the external carotid artery system can reduce the hemodynamic burden of choroidal or thalamic collaterals, and then prevents subsequent rupture. Other retrospective studies also indicated a lower risk of cerebral infarction or hemorrhage in patients with MMD after revascularization (27–29). Hence, both cerebrovascular events and improved perfusion are major outcomes that need to be evaluated after revascularization surgery (26).

Thus, it is crucial for patients with MMD to improve and maintain cerebral perfusion of MCA territory. RIC has been shown to improve CBF of patients with chronic circulation insufficiency like ICAS by promoting angiogenesis or arteriogenesis (9–11, 30, 31). A recent single-arm study also observed an improvement of cerebral perfusion in patients with MMD by RIC treatment (14). This study showed that mCBF of the MCA region was improved in the RIC group. The control group conversely tends to have a reduced CBF at 1 year from baseline. Several studies reported that revascularization surgery mainly improved cortical perfusion compared with central regions (32). Our results showed no difference in the CBF improvement ratio between cortex and basal ganglia, which demonstrated that the CBF was increased evenly by RIC treatment. The detailed pathway of improving CBF in patients with MMD by RIC is still not clearly understood. Considering unchanged periventricular anastomosis of basal collaterals after RIC treatment in this study, we assumed that the ischemia tissue may be improved by angiogenesis or arteriogenesis from the leptomeningeal system or external carotid artery system and more stable status of Willis circle. Further studies need to be constructed to validate the assumption by DSA.

The absolute CBF was measured by ASL in this study, which has become a validated technique to monitor CBF changes after revascularization surgery without a contrast agent (26). There is a concern that ASL will underestimate the CBF value in patients with MMD, thus multidelay PCASL-MR that can alleviate the underestimation was utilized (33, 34).

Progressive stenosis or occlusion at Willis circle is one of the characteristics of MMD, which can induce a series of symptoms (35, 36). We evaluated the progression of the stenotic-occlusive lesion and found that patients in the RIC group had a lower risk of arterial progression within 1 year, which may be related to the effects of RIC on improving endothelial function (37), ameliorating inflammation (38), and promotion of arteriogenesis or angiogenesis (9, 10). A recent study showed that RIC could ameliorate vascular remodeling in conduit artery and small resistance artery of hypertensive rats by suppressing deposition of the extracellular matrix and hypertrophy of smooth muscle cells (39). It indicated that RIC may prevent arterial progression through ameliorating vascular remodeling in patients with MMD. But the pattern of vascular remodeling is different between MMD and hypertension, the definite mechanism still needs to be further explored.

The regression of dilated and extended anterior choroidal arteries was seen in patients with MMD after revascularization surgery (40, 41), but it was not found after RIC treatment. The method to evaluate choroidal arteries on non-invasive imaging, relatively short-time follow-up, and small sample size in this study may contribute to the result.

The MACE rate of the RIC group was lower than that in the control group, but it was not statistical. In this study, subjects were mostly composed of the ischemic-onset patients with MMD, and the recurrence rate of ischemic events was reported 3–10% which was relatively low (42, 43). A previous retrospective study with a large sample size also could not get a significant difference in the incidence of recurrent stroke between the surgery group and conservative group until 10-year follow-up (44). Long-term follow-up (3–5 years and even more than 10 years) were commonly used in previous studies observing the efficacy of revascularization surgery (6, 44, 45). Thus, we thought that 1-year follow-up in this study with a small sample size was not sufficient to observe a significant difference in MACE rate between the two groups, and long-term follow-up should be implemented in further research.

Although this study could not determine the effect of RIC on the prevention of cerebrovascular events, the improved CBF of patients with MMD which could be an indicator of better long-term outcome was confirmed. Thus, daily RIC could be an alternative therapy for patients with MMD in the future and will need further study.

This study has some limitations. Small sample size may bring selection bias for this study. There existed a discrepancy in the sample size between the two groups which may be due to the demand for further treatment of patients in the control group without a placebo. The mechanism of RIC for MMD needs further studies to reveal.

Our findings demonstrated that daily RIC treatment is safe and well-tolerated for adult MMD patients. RIC can improve the CBF and halt the progression of stenotic-occlusive lesions. Subjects in the RIC group tended to have a lower risk of MACEs. Thus, RIC seems to be a potential treatment approach for MMD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xuanwu Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XJ, SL, QZ, and JX contributed to conceptualization. JX and QZ contributed to writing the original draft. JX, BZ, and WG contributed to the investigation. QY and FW contributed to the methodology. BZ, XX, and WG contributed to data curation. SL, YD, and GR contributed to writing, reviewing, and editing. WZ contributed to the formal analysis. FW contributed to the visualization. JX and SL contributed to project administration. XJ contributed to supervision. SL and XJ contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.811854/full#supplementary-material

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Conflict of Interest: XJ is one of the inventors of the electric autocontrol device that has been patented in China (ZL200820123637.X, China).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Efficacy and Safety of Cilostazol vs. Aspirin for Secondary Stroke Prevention: A Systematic Review and Meta-Analysis

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Background: Cilostazol is often used in Asia-Pacific countries for stroke prevention. The current systematic review and meta-analysis aimed to evaluate the effectiveness, safety, and adverse outcomes of cilostazol monotherapy compared to aspirin monotherapy for secondary stroke prevention.

Methods: The researchers conducted a comprehensive research in multiple databases (PubMed, Embase, and Cochrane library) of randomized controlled trials from conception to December 2020. The primary efficacy outcome was the occurrence of any stroke, the primary safety outcome was the bleeding risk, and the primary adverse outcome was the rate of headache and dizziness. The Mantel-Haenszel method was used to calculate a random-effects prediction. Cilostazol and aspirin were compared using a pooled risk assessment with 95% CIs.

Results: Six studies involving 5,617 patients were included in this review. Compared with aspirin monotherapy, cilostazol was associated with significantly lower rates of any strokes (RR: 0.67; 95% CI: 0.55–0.82) and significantly lower bleeding rates [risk ratio (RR): 0.53; 95% CI: 0.37–0.74]. However, compared with aspirin monotherapy, cilostazol was associated with significantly higher rates of headache (RR: 1.77; 95% CI: 1.41–2.20) and dizziness (RR: 1.28; 95% CI: 1.08–1.52).

Conclusions: Consistent with previous studies, cilostazol monotherapy is superior to aspirin monotherapy in reducing the rate of any strokes and the bleeding risk after having a stroke. However, the use of cilostazol monotherapy is associated with several adverse life outcomes such as headaches and dizziness.

Keywords: stroke prevention, efficacy and safety, cilostazol, aspirin, systematic review and meta-analysis

INTRODUCTION

A stroke has main clinical manifestations of cerebral ischemia and hemorrhagic injury, having a very high mortality and disability rate (1, 2). Antiplatelets are the major therapy for the secondary stroke prevention (3). Aspirin and cilostazol are the most commonly used antiplatelet agents (4). Most patients who have had a stroke are given aspirin (5). According to two major randomized clinical studies of aspirin in acute ischemic stroke, aspirin decreased the risk of early chronic stroke by ∼12% at 2–4 weeks (6). However, aspirin-related cerebral hemorrhage is a complication that is currently of concern (5). Cilostazol was reported to be efficacious for the prevention of stroke recurrence (4), which might be related to the various mechanisms, such as anti-platelet aggregation, anti-atherosclerosis, promotion of vascular endothelial recovery, cell apoptosis inhibition, and practical value for the prevention and treatment of ischemic stroke (5, 7, 8). Studies have shown that cilostazol can be used as a drug to treat ischemic strokes and as a preventive drug for recurrence (9). Shinohara et al. (4) reported that the primary endpoint for prevention of secondary stroke occurred at yearly rates of 2.76% in the cilostazol group and 3.71% in the aspirin group ($p = 0.0357$).

The previous meta-analysis primarily focused on comparing the efficacy and safety of cilostazol monotherapy or dual therapy with clopidogrel and aspirin monotherapy (10–12). However, there is no meta-analysis comparing cilostazol monotherapy to aspirin monotherapy as secondary prevention after stroke and in regard to cilostazol's side effects. Therefore, the researchers conducted a systematic review and meta-analysis to evaluate the efficacy and safety of cilostazol monotherapy compared to aspirin therapy. The researchers will further identify the frequency of the adverse side effects caused by these two treatment arms.

METHODS

Data Sources

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist was used to perform our meta-analysis based on the Preferred Reporting Elements for Systematic Assessments (13). Searches were conducted in the following electronic databases from conception to December 2020: PubMed, Embase, and the Cochrane Library. The researchers searched with the following headings: "stroke," "acute ischemic stroke," "TIA," "secondary prevention," "aspirin," AND "cilostazol." The gray literature was searched through OpenGrey and Google Scholar. After searches, all relevant citations were saved in a bibliographic reference manager (EndNote, x9 version, Thomson Reuters). Duplicated results were considered only one time. The titles and abstracts that did not adhere to the established eligibility criteria were excluded. The resulting articles were evaluated and judged by their full text. Additional citations were sought from the analysis of the reference list of all the articles previously selected. The selection process was conducted by two examiners (EC and CL) and checked by a third examiner (FT) in cases of disagreements.

Selection Criteria and Data Extraction

The inclusion criteria were: (1) randomized controlled studies, (2) a comparison of cilostazol monotherapy with aspirin monotherapy, (3) the efficacy outcomes including recurrent stroke reported, and (4) the adverse outcomes. A total of six studies met the criteria. The exclusion criteria included: (1) non-randomized controlled trials, (2) the cilostazol combination therapy (clopidogrel or aspirin) with an aspirin combination therapy (clopidogrel), (3) only reported efficacy and safety outcomes and no adverse outcomes reported. Two authors independently conducted the research and performed the data extraction (**Figure 1**).

Outcomes Measured

The primary efficacy outcome was the occurrence of any stroke (including ischemic stroke and hemorrhagic stroke); the secondary efficacy outcome includes the occurrence of ischemic stroke. The primary safety outcome was intracranial hemorrhage, including subarachnoid hemorrhage and subarachnoid hemorrhage, and other safety outcomes, including bleeding, vascular death, and all-cause mortality. The primary adverse outcome is the headache; the secondary adverse outcome is dizziness where both outcomes include tachycardia and palpitation.

Assessment of Risk of Bias

Two reviewers (EC and CL) independently evaluated the quality of the included randomized control trials (RCTs) using a modified version of the Cochrane risk of bias tool (RoB2) for randomized trials to address the risk of bias. Any disagreements between the rater of pieces of evidence are resolved by a third examiner (FT) (14). The researchers graded the evidence quality based on random sequence generation, allocation concealment, participant and staff blindness, outcome assessor blinding, missing outcome data (which rated as high risk of bias if missing data exceed 10%), and other biases. The findings were presented using the MAGICapp (15) (**Figure 2**).

Quality of Evidence

The GRADE form was used to evaluate the quality of research (14). We graded the quality of research as high, moderate, low, or very poor for each outcome based on imprecision, inconsistency, indirectness, publication bias, and overall risk of bias.

Statistical Analysis

For dichotomous results, the researchers used the Mantel–Haenszel method to measure overview risk ratios (RRs) and 95% CIs and used a random-effects model to account for the between-study heterogeneity. The researchers further used the Cochrane Q statistics and the I2 test to determine the heterogeneity of

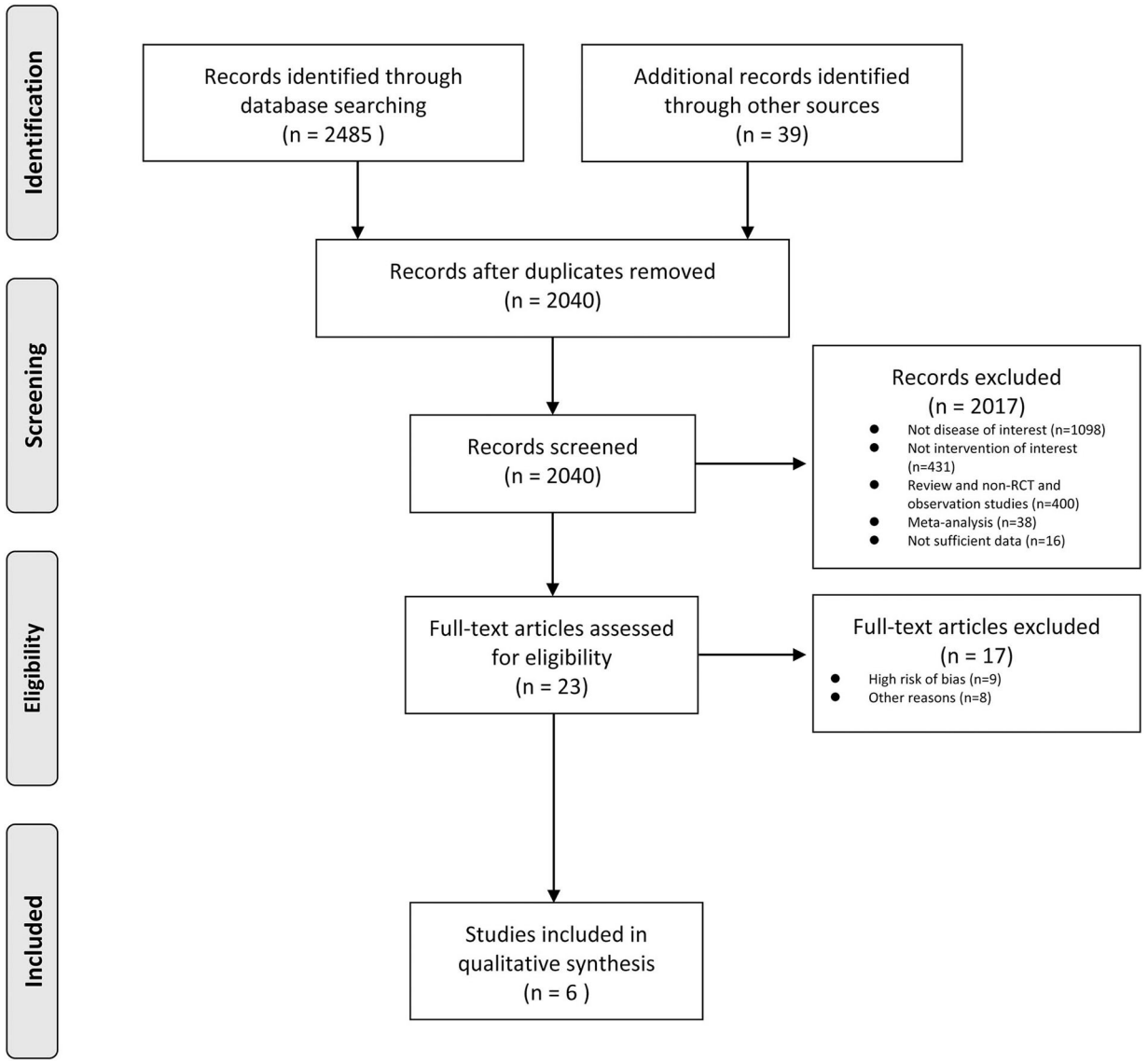

FIGURE 1 | A flowchart.

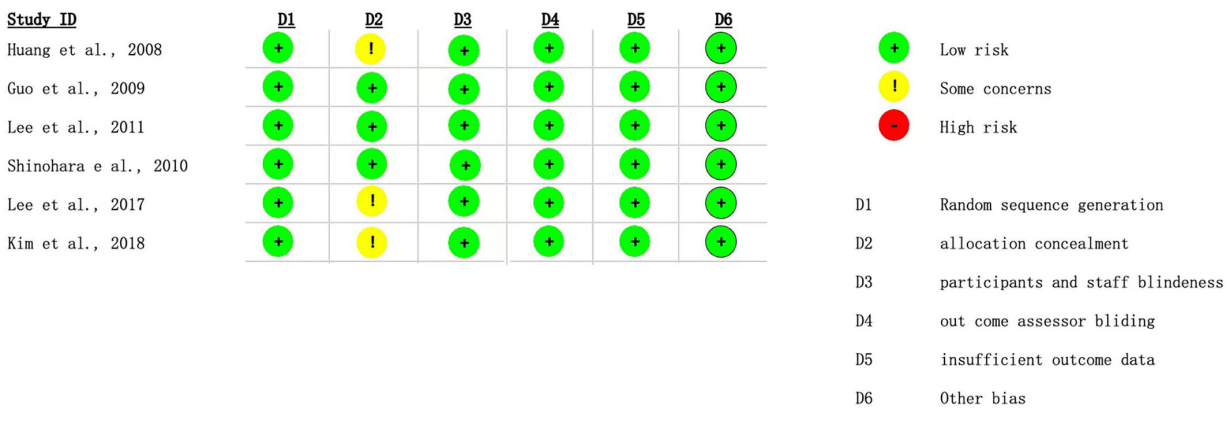

FIGURE 2 | Bias assessment.

TABLE 1 | Characteristics of included studies.

References	Design	Study period	Follow-up (months)	Medications		Total No.	Primary outcome	Cilostazol	Aspirin	*P*-value
				Cilostazol	Aspirin					
Huang et al. (5)	Multicenter Double-blind	May 2004–Dec. 2004	12–18	100 mg twice/day	100 mg/day	*N* = 720 • Ischemic within previous 1–6 months	Occurrence of stroke	0.28	0.85	0.18
Guo et al. (16)		May 2004–Dec. 2005	12	100 mg twice/day	100 twice/day	*N* = 68 • Ischemic within 1–6 months	Cerebro-vascular aggravation	1%	1%	0.90
Lee et al. (8)	Double blind Non-inferiority	Jan.2006–Mar. 2008	3	200 mg/day	300 mg/day	*N* = 458	mRS score of 0–2 at 90 days	173/231	165/227	0.90
Shinohara et al. (4)	Randomized Double-blind Non-inferiority	Dec. 2003–Oct. 2006	29*	100 mg twice/day	81 mg/day	*N* = 2,757 • Non-cardioembolic cerebral infarction previous 26 weeks	Recurrent stroke	82/1,337	113/1,335	0.036
Lee et al. (7)	Double-blind	March 2012–Oct. 2014	3	100 mg twice/day	100 mg/day	*N* = 80 • Acute ischemic stroke/TIA	Serious adverse events	2/40	5/40	0.235
Kim et al. (17)	Multicenter	Aug 2009–Aug 2015	22.8**	100 mg twice/day	100 mg/day	*N* = 1,534 • Non-cardioembolic ischemic stroke/TIA within 180 days	Composite of major vascular events	63/755	80/757	0.008

**Mean follow-up.*
***Median follow-up.*

the included studies and used the RevMan 5.4 to conduct the meta-analysis.

RESULTS

Study Identification and Trial Characteristics

Figure 1 presents the findings of the researchers' included studies. A total of six studies (4, 5, 7, 8, 16, 17) were included in the analysis with a total of 5,617 patients. All studies compared cilostazol monotherapy to aspirin monotherapy. The researchers found 2,524 documents in electronic libraries, 507 of which were duplicates and further reviewed 23 full-text articles with omissions on the 2017 records, depending on the title and abstract.

Table 1 summarizes the characteristics of the six included studies. Four included trials administered with cilostazol at 100 mg two times/day and aspirin at 100 mg/day. One of the trials administered cilostazol at a dose of 200 mg/day and aspirin at 300 mg/day. Another study included a trial administered with cilostazol at 200 mg/day and aspirin at 100 mg/day. Moreover, one included study administered cilostazol at a dose of 100 mg two times daily and aspirin at 81 mg/day. All studies were conducted in Asian countries as a result of cilostazol being mainly used in Asian countries. The patient demographics are summarized in **Table 2**.

Risk of Bias

Figure 2 presented the risks of bias of the six included RCT studies. The appropriateness in estimating the effect of assignment to intervention is unclear in three RCTs. Otherwise, the overall risks of bias are low for the six included RCTs.

Efficacy Outcomes

Compared with aspirin alone, a total of four studies with 5,260 patients showed that cilostazol monotherapy significantly reduced the risk of any stroke (RR: 0.67; 95% CI: 0.55–0.82, $p < 0.0001$) (**Figure 3A**). Four studies with 2,260 patients showed that cilostazol monotherapy was also associated with a lower ischemic stroke rate, however the results recorded were not significantly different (RR: 0.76; 95% CI: 0.54–1.07, $p = 0.11$) (**Figure 3B**).

Safety Outcomes

Compared with aspirin alone, a total of four studies with 2,109 patients showed that cilostazol monotherapy significantly reduced intracranial bleeding (RR: 0.46; 95% CI: 0.22–0.94, $p = 0.03$) (**Figure 4A**) and significantly reduced any bleeding risk (RR: 0.53; 95% CI: 0.37–0.74, $p = 0.0002$) (**Figure 4B**). However, there was no significant difference between cilostazol and aspirin alone for vascular death and all-cause mortality (RR: 1.60; 95% CI: 0.60–4.26 $p = 0.35$) (**Figure 4C**) (RR: 0.91; 95% CI: 0.60–1.37, $p = 0.64$) (**Figure 4D**).

Adverse Outcomes

A total of six studies involving 4,740 patients showed that cilostazol was associated with a higher incidence of headache

TABLE 2 | Included studies patient's demographics.

Studies	Treatment	Age (years)	Male (%)	HTN (%)*	DM (%)*	DLP (%)	Smoker (%)	Systolic BP* (mm Hg)	Diastolic BP* (mm Hg)	HLD (%)*
Huang et al. (5)	Cilostazol	60 ± 10	67	79	18	-	-	135 ± 17	83 ± 9	27
	Aspirin	60 ± 10	70	79	18	-	-	138 ± 18	83 ± 11	31
Guo et al. (16)	Cilostazol	59 ± 11	35	68	6	44	-	-	-	29
	Aspirin	62 ± 11	35	65	12	47	-	-	-	47
Lee et al. (8)	Cilostazol	63 ± 12	64	67	37	-	41	144 ± 25	84 ± 14	39
	Aspirin	63 ± 12	59	63	32	-	40	140 ± 22	82 ± 11	44
Shinohara et al. (4)	Cilostazol	64 ± 9	72	73	29	42	29	-	-	-
	Aspirin	63 ± 9	72	74	29	45	30	-	-	-
Lee et al. (7)	Cilostazol	54 ± 13	72	69	16	-	41	-	-	44
	Aspirin	60 ± 12	59	82	29	-	47	-	-	38
Kim et al. (17)	Cilostazol	66 ± 11	62	89	32	43	19	135 ± 18	80 ± 12	-
	Aspirin	66 ± 11	62	89	33	44	21	136 ± 18	80 ± 12	-

**HTN, hypertension; DM, diabetes mellitus; DLP, dyslipidemia; BP, blood pressure; HLD, hyperlipidemia.*

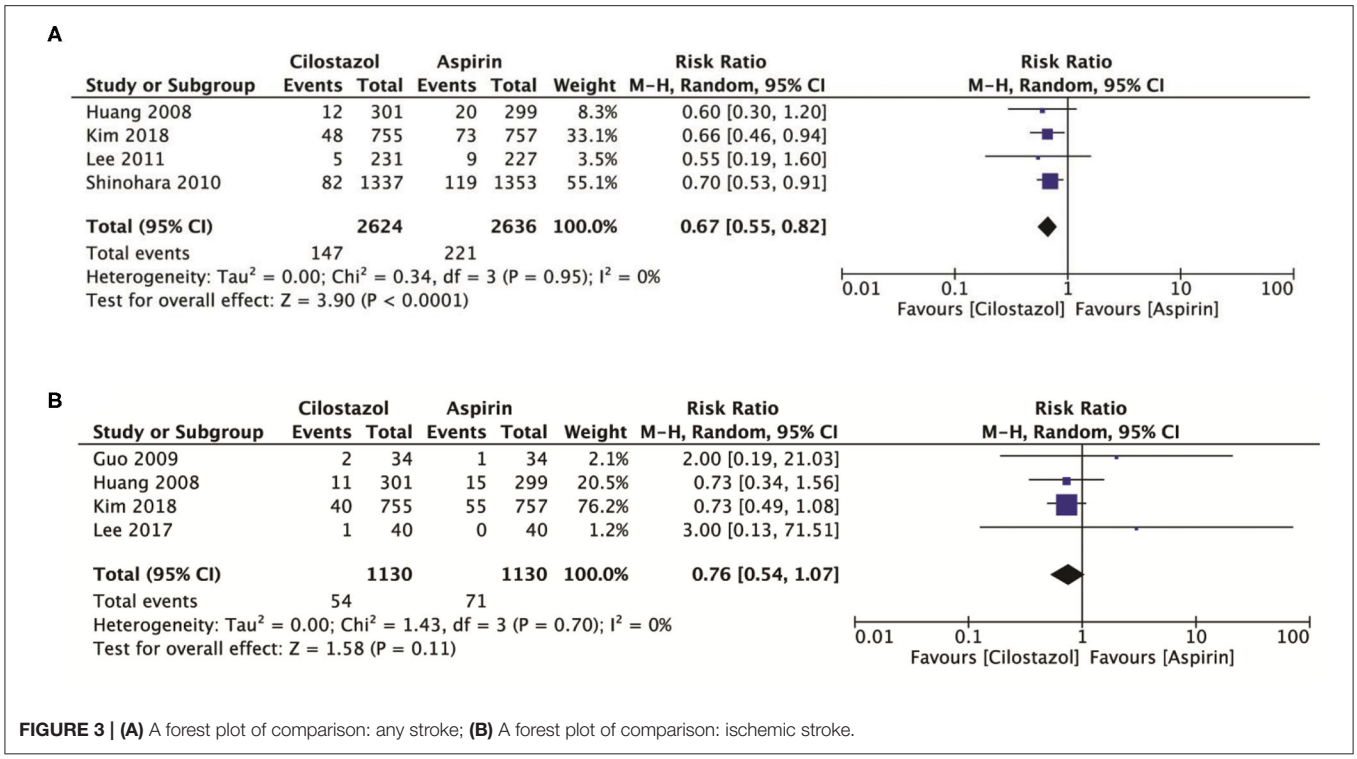

FIGURE 3 | (A) A forest plot of comparison: any stroke; **(B)** A forest plot of comparison: ischemic stroke.

compared with aspirin monotherapy (RR: 1.77; 95% CI: 1.41–2.21, $p < 0.00001$) (**Figure 5A**), while cilostazol also significantly increased the frequency of dizziness (RR: 1.28; 95% CI: 1.08–1.52, $p = 0.005$) (**Figure 5B**). Two studies with 3,391 patients showed that cilostazol monotherapy significantly increased the tachycardia risk compared to aspirin monotherapy (RR: 3.94; 95% CI: 2.62–5.93, $p < 0.00001$) (**Figure 5C**). However, four studies with 4,601 patients showed that cilostazol did not significantly increase the palpitation frequency compared to aspirin monotherapy (RR: 1.47; 95% CI: 0.34–6.31, $p = 0.61$) (**Figure 5D**).

DISCUSSION

The researchers made some potentially valuable findings in this meta-analysis of six RCTs ($n = 29{,}032$) comparing cilostazol monotherapy to aspirin monotherapy. First, in patients with stroke, compared with aspirin, cilostazol significantly reduces any stroke incidence while reducing intracranial bleeding or any bleeding risks and does not significantly increase vascular death or death events. Second, although cilostazol alone is more efficient and safer than aspirin alone, cilostazol increases adverse events, primarily significantly increasing the incidence of headache, dizziness, and tachycardia. Similar results were found showing that the patients who received cilostazol had a 30% lower risk of persistent ischemic stroke, a 59% lower risk of intracranial hemorrhage, and a 29% lower risk of bleeding than patients who received aspirin (18). In addition, the current meta-analysis accounts for the adverse events in the results, which are the strength of this meta-analysis. Additionally, low heterogeneity ($I^2 = 0$-31%) was observed in the evidence.

Stroke is the most common cause of disabilities and death (3). Despite the efforts of researchers and pharmaceutical companies, the risk of stroke recurrence remains high (19). The use of antiplatelet agents is recommended to reduce the long-term risk of non-cardioembolic ischemic stroke or TIA (20). Aspirin is a commonly used antiplatelet agent for secondary stroke prevention, but its benefit must be weighed against its bleeding risks, particularly in the aging population (20). It has been proved that aspirin is safe and beneficial in preventing stroke recurrence, but aspirin can only reduce recurrent vascular events by 20% (21). Previous meta-analyses have evaluated the effect of aspirin combined with clopidogrel on secondary stroke prevention, but, because of the high bleeding complications, no net benefit was found (22–28). Studies have recently found that it is more beneficial for acute high-risk patients treated with ticagrelor and aspirin than aspirin alone (29). Clearly, the optimal antiplatelet regimen, particularly in individuals at high risk for cerebral hemorrhages, such as those with a high burden of cerebral small-vessel disease, remains unclear and needs further investigation in well-designed clinical trials.

This study shows that Kim et al. did not find a significant effect of cilostazol and aspirin on intracranial hemorrhage, which may be due to their fragile small vessels and may lead to a greater incidence of intracranial bleeding (17). Similar findings were reported by Shinohara et al. (4); there was no significant intracranial hemorrhage between cilostazol and aspirin due to a high proportion of patients with a lacunar stroke in their study.

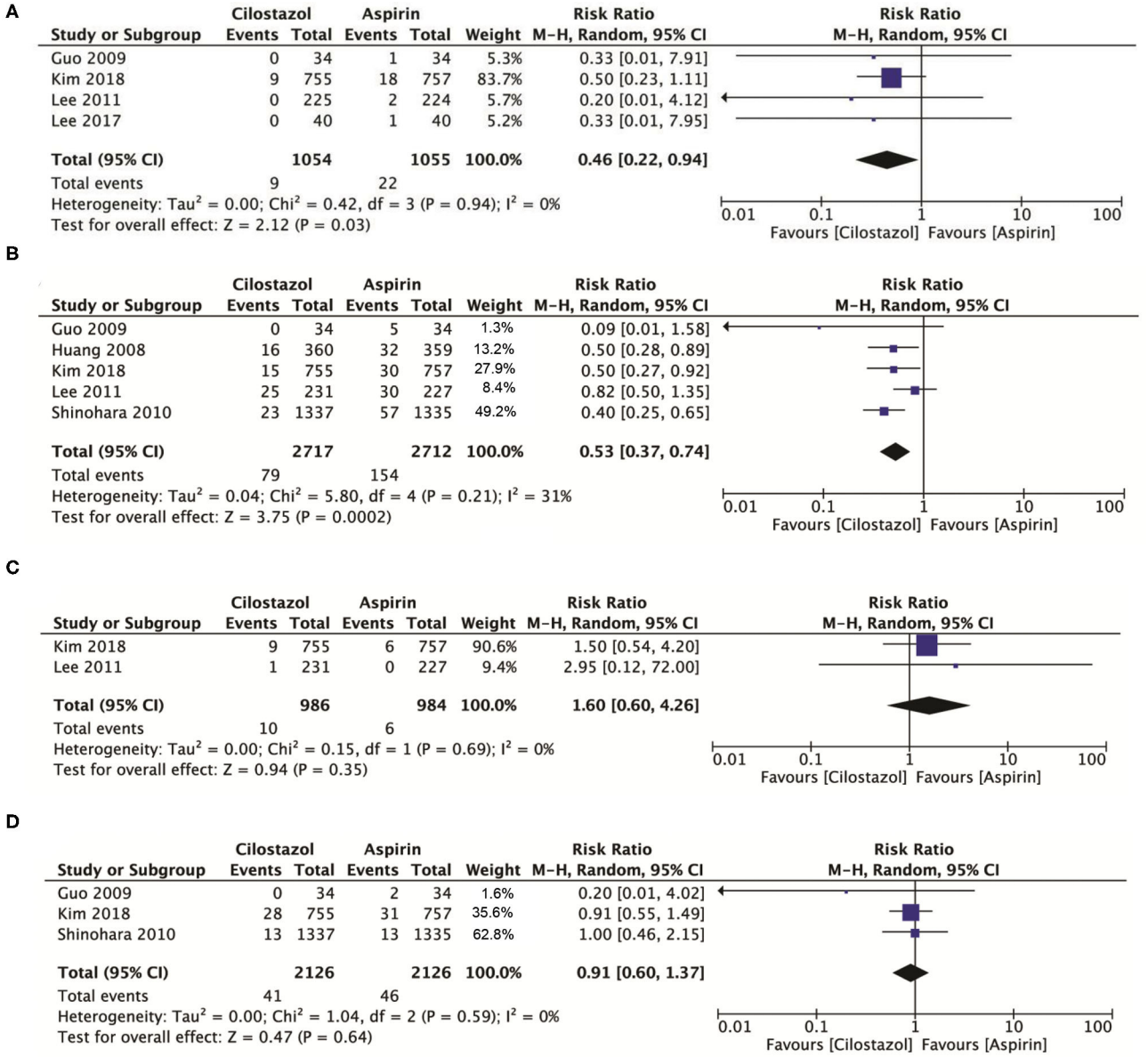

FIGURE 4 | (A) A forest plot of comparison: intracranial bleeding; **(B)** A forest plot of comparison: any bleeding; **(C)** A forest plot of comparison: vascular death; **(D)** A forest plot of comparison: any death.

Adverse events, including headaches and dizziness, occurred more frequently in the cilostazol group than in the aspirin group, but none were severe and all symptoms resolved after discontinuation or dose tapering of cilostazol. A study showed that some patients might avoid the adverse events caused by cilostazol by incremental increases in dose from 50 mg (4).

As a new type of antiplatelet inhibitor, cilostazol has anti-arterial thrombosis, prevents atherosclerosis, and improves vascular endothelial function (30–32). It can also regulate blood lipids and expand arterial blood vessels to stabilize plaques (33). It has a wide range of applications in treating peripheral vascular disease, preventing stent restenosis and thrombosis after PCI (34), and secondary prevention of ischemic stroke (16, 35). It is more suitable for aspirin-resistant or intolerant people, especially Asians (36).

Cilostazol is a selective inhibitor of phosphodiesterase, which increases intracellular activity, thereby inhibiting platelet aggregation (37–39). In some respects, the drug is a potent drug that can replace aspirin. For example, in previous clinical trials and meta-analyses, cilostazol significantly reduced the risk of stroke recurrence and lower bleeding events compared to aspirin (40, 41). Moreover, our current meta-analysis is in line with previous meta-analyses that found cilostazol to be more beneficial in patients with ischemic stroke (18, 42).

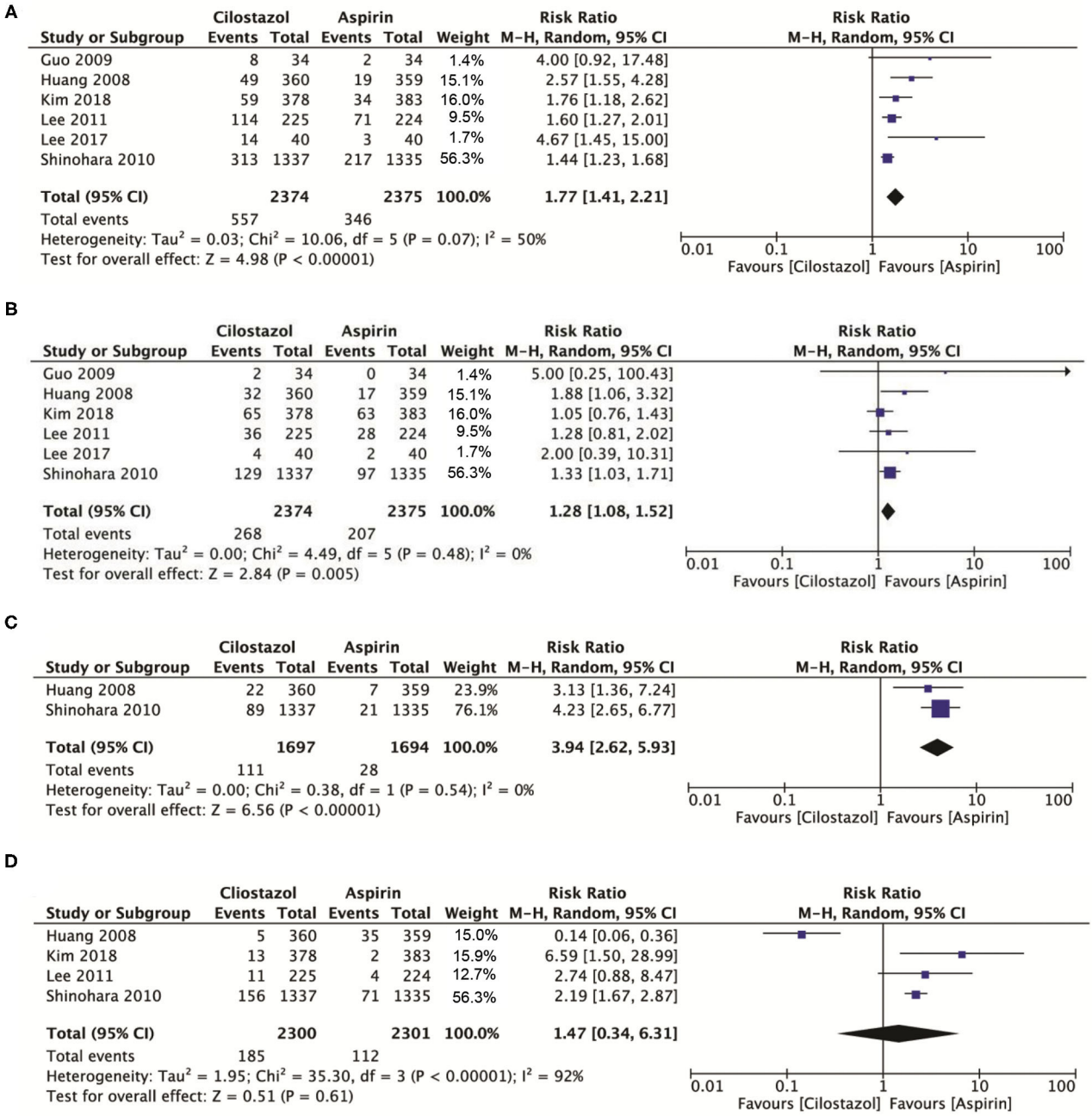

FIGURE 5 | (A) A forest plot of comparison: headache; **(B)** A forest plot of comparison: dizziness; **(C)** A forest plot of comparison: tachycardia; **(D)** A forest plot of comparison: palpitation.

Although current meta-analysis and previous studies have shown the effectiveness and relative safety of using cilostazol as secondary prevention of stroke, research also shows that, even in non-Asian populations, cilostazol may have a significant potential for secondary stroke prevention. Patients with bleeding tendencies, such as small vessel disease and numerous microbleeds, or those who have hemorrhagic strokes, are likely to benefit from cilostazol treatment (43). However, compared with Asians, cilostazol is relatively uncommon in Western populations. Several reasons may explain this uncommonness. First, intracranial atherosclerosis (ICAS) is the leading cause of stroke, and Asians more often have ICAS than Caucasians (44). Second, the absorption, metabolism, and excretion of cilostazol may be modified by race/ethnicity (45). For instance, common polymorphisms in the CYP2C19 gene for clopidogrel metabolism vary by race/ethnicity, noted

in ∼30% of Caucasians, 40% of blacks, and more than 50% of East Asians (46). The pharmacogenetic of cilostazol is less well described, but it has been observed that genetic polymorphisms in CYP2C19 genes influence cilostazol pharmacokinetics (47). This is, therefore, possible that race/ethnicity may influence the effect of cilostazol on lowering ischemic stroke, ICH, and bleeding in non-Asian populations, but more studies are needed to examine how genetics and environment may affect the metabolism of cilostazol (18). Third, due to the lack of sufficient RCTs to study the effectiveness and safety of cilostazol as secondary prevention of stroke in Western populations, non-Asian physicians are not inclined to use cilostazol (44). Therefore, further pieces of research on the effect of cilostazol on different groups of people and ethnicity are needed.

The current meta-analysis has several limitations. First, the patients included in the studies were mainly from the Asian region, which will lead to regional deviations in the results. Large-scale research is required to determine whether the researchers' results are valid and similar in non-Asian populations. Second, the present meta-analysis did not conduct subgroup analysis to assess the impact of time to randomization following a stroke and the length of time spent taking the research drug on effectiveness and safety outcomes. Also, sensitivity analysis was not performed. Third, the follow-up length is different, ranging from 3 months to 29 months. Finally, in MI, there was inter-study variability in the outcomes. Such inherent variations between the researchers' included trials, such as sample demographics, non-cardioembolic infarction inclusion/exclusion requirements, stroke occurrence, treatment, follow-up duration, drug compliance rates, and other factors, are not considered.

CONCLUSIONS

Cilostazol is more effective than aspirin alone in reducing the recurrence rate of stroke without increasing the risk of bleeding and death. However, when using cilostazol, the significantly increased probability of adverse events cannot be ignored.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EC, JC, CL, XZ, ZF, and FT conceived and designed the study. JC, SY, KZ, and WL selected the studies and collected the data. EC, JC, ZX, YZ, and FT analyzed the data. EC and FT drafted and revised the article. All authors interpreted the results, read, and approved the final version of the manuscript.

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