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NEUROPROTECTION

ORIGINAL ARTICLE

Post-recanalization administration of rapamycin improves functional outcome and reduces infarct size in a rat model of ischemic stroke

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Abstract

Aim: Despite advances in endovascular recanalization for ischemic stroke, many patients experience poor outcomes. Adjunct cerebroprotective therapies are needed to improve recovery. The mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin has shown neuroprotective effects in preclinical stroke models. However, most studies administered rapamycin prior to or during stroke onset, limiting translational relevance. The aim of this study was to determine whether rapamycin administered immediately after recanalization improves infarct size and functional outcome, and whether these effects are associated with changes in cerebral blood flow (CBF) or blood-brain barrier (BBB) integrity.

Methods: Male Wistar Han rats were subjected to transient middle cerebral artery occlusion (tMCAO) for 90 min. Animals were randomized using the sealed envelope method to receive intravenous rapamycin (250 μg/kg, $n = 9$) or vehicle ($n = 9$) immediately after recanalization. Infarct volume, CBF, and BBB integrity were assessed using magnetic resonance imaging (MRI) at 72 h, alongside validated neurological tests. Group comparisons were performed using unpaired Student's *t*-tests.

Results: Rapamycin significantly reduced infarct volume compared with vehicle ($44.77 \pm 30.93\ mm^3$ vs. $113.44 \pm 60.19\ mm^3$, $p = 0.0114$) and improved Garcia neurological scores (12.78 ± 1.04 vs. 11.67 ± 0.87, $p = 0.0295$). In the adhesive removal test, rapamycin-treated animals showed shorter time to notice the stimulus (45.04 ± 11.91 s vs. 72.33 ± 12.17 s, $p = 0.0002$). Rapamycin had no significant effect on CBF, BBB disruption, or edema at 72 h (all $p > 0.05$). The p-mTOR/mTOR ratio did not differ significantly between groups at day 3 (0.55 ± 0.32 vs. 0.90 ± 0.41, $p = 0.1880$).

Discussion: Rapamycin administered after recanalization improves functional outcomes and reduces infarct size, without altering sustained perfusion or BBB permeability. These findings highlight a perfusion-independent, time-sensitive cerebroprotective mechanism and support rapamycin's development as an adjunctive therapy in ischemic stroke.

KEYWORDS

cerebral infarction, ischemic stroke, magnetic resonance imaging, neuroprotection, sirolimus

Alastair M. Buchan and Daniel J. Beard contributed equally to this study.

Highlights

- Post-recanalization administration of rapamycin significantly reduced infarct volume in a rat model of ischemic stroke.
- Rapamycin-treated animals demonstrated improved somatosensory function, particularly in the adhesive removal test.
- Rapamycin showed no effect on acute or subacute cerebral blood flow, suggesting cerebroprotective benefits independent of perfusion enhancement.
- Magnetic resonance imaging-based assessments confirmed no significant changes in blood-brain barrier permeability or edema at 3 days post-stroke with rapamycin treatment.
- These findings support the potential of rapamycin as an adjunct cerebroprotective therapy administered immediately after recanalization.

1 | INTRODUCTION

Stroke treatment has recently been revolutionized by recanalization therapy and better patient selection thanks to improved imaging modalities. Despite high recanalization rates (60%–70%), half of patients are still left with poor functional outcomes, and only about 10% are symptom-free at 3 months.[1] Therefore, an adjunct pharmacological treatment that has neuronal and vascular protective (i.e., cerebroprotective) effects may help improve outcomes post-recanalization. Pharmacological inhibition of (mammalian target of rapamycin complex 1) mTORC1 with the FDA-approved immunosuppressive drug rapamycin has been shown to protect brain tissue during experimental stroke.[2,3] However, the mechanism by which rapamycin exerts its cerebroprotective effects and which cerebral cells and anatomical structures it targets are still being explored. A large body of literature reports that rapamycin can reduce infarct volume and improve neurological function in stroke models.[4–19] However, only a few studies have investigated the effects of rapamycin on structural changes of the blood-brain barrier (BBB) and cerebral blood flow (CBF) following transient middle cerebral artery occlusion (tMCAO).[2,8,9,13,14,18,19] Of these, five reported reduced BBB disruption after rapamycin treatment.[9,13,14,18,19] More recently, we showed rapamycin can alleviate pericyte-mediated capillary constriction and improve microvascular reperfusion post-recanalization, further supporting its cerebrovascular protective potential.[20] It is unclear whether rapamycin's benefits rely on sustained CBF increases or alternative mechanisms such as inflammation modulation or BBB stabilization. Evidence suggests its effects depend on administration timing relative to ischemia and reperfusion. The aims of this study are threefold: first, to assess rapamycin's effects on immediate post-recanalization CBF; second, to determine if these effects persist to 3 days and influence lesion volume and function; and third, to evaluate BBB integrity using contrast-enhanced MRI. Results will be interpreted alongside literature suggesting early treatment may enhance reperfusion, whereas later treatment may limit secondary injury, such as BBB breakdown and inflammation.

2 | METHODS

2.1 | Ethics and animal care

All experimental procedures were approved by the UK Home Office (1986 Animal Act, Scientific Procedures; licence number PP7444704 granted 23.12.22), conducted in accordance with the Clinical Medicine Ethical Review Guidelines of the University of Oxford, the ARRIVE and the IMPROVE guidelines for animal and pre-clinical stroke work.[21,22] Male Wistar Han rats (250–320 g, 8–11 weeks old, Envigo Research Model Services in Blackthorn, England) were housed in groups in individually ventilated cages under a 12-h light/12-h dark cycle with *ad libitum* access to food (standard food pellets) and water. Animals remained group-housed both before and after surgery. Single housing would only have been implemented if required for welfare reasons, which was not the case for any animal in this study. The main investigator (AMS) began daily animal handling, weighting, and training the rats for the adhesive removal test (see below) 3 days before the surgery. During the 3 days following surgery, the rats underwent daily welfare scoring.

2.2 | Study design

2.2.1 | Controls and exclusion criteria

Appropriate control groups were included in all experiments. For the rapamycin comparison, vehicle groups were used. Since naïve groups were included in similar animal studies undergoing the same intervention, and the investigators tried to minimize the number of animals used, no sham groups were included in this study.[9] Where possible, within-subject controls were used, such as when comparing stroke expansion on the ipsilateral versus contralateral hemisphere. Pre-defined exclusion criteria during surgery were a laser Doppler flow (LDF) signal below 30% of baseline and confirmed subarachnoid hemorrhage (SAH), a rare but known complication of the focal ischemic stroke model used.

2.2.2 | Randomization and blinding

Animals were randomized using the sealed envelope method to receive either rapamycin (Sigma Aldrich, Catalog Number R0395-1MG, 250 µg/kg, $n = 9$) or vehicle (<5% ethanol in saline, $n = 9$), administered by the main investigator blind to treatment allocation (AMS). Blinding continued throughout the whole experiment and until data acquisition was complete.

2.3 | Anesthesia and monitoring

Rats were initially anaesthetized with 5% isoflurane in 70% N_2O and 30% O_2 and maintained at 1%–2% isoflurane in 70% N_2O and 30% O_2. During surgery, the core body temperature was maintained at 37.0 ± 0.5°C using a rectal thermometer connected to a feedback-controlled heating pad (Harvard Apparatus, Cambourne, UK). Body temperature and respiratory rate were recorded every 15 min throughout the procedure. Respiration was kept between 50 and 60 breaths per minute by adjusting the isoflurane concentration.

2.4 | Cerebral blood flow measurements

A fibreoptic probe connected to an LDF apparatus (Oxyflo 2000 Optronix, Oxford, UK) was used to continuously monitor cerebral perfusion in the lateral MCA territory, corresponding to the core area after MCA occlusion, as described previously.[23] Briefly, the animal's head was secured with ear bars in a stereotaxic frame, and the probe was placed + 4 mm lateral of the midline and – 2 mm posterior of Bregma. LDF traces were recorded on a Windows XP workstation running with WinDaq Data Acquisition Software (Dataq Instruments, Akron, Ohio, USA). MCA perfusion was recorded throughout the experiment. The 70% threshold was chosen from previous studies reporting an infarct threshold of 20%–30% from baseline.[24,25]

2.5 | CBF analysis

LDF was used to assess CBF in the MCA territory before, during, and after MCAo and reperfusion. For LDF analysis, continuous recordings were summarized over predefined experimental phases (baseline, occlusion, and post-recanalization) to enable comparison between treatment groups. Raw CBF data were exported as time-series files and processed in Excel. To reduce signal noise, values were averaged in 10-min blocks using the OFFSET function. Outliers were identified and removed using the interquartile range (IQR) method. In addition, a small number of individual outliers were excluded manually after visual inspection of the raw signal, based on abrupt signal loss or artifacts unrelated to physiological changes.

2.6 | Focal cerebral ischemia and rapamycin treatment

This study used 22 rats. All procedures were conducted under sterile conditions. Focal brain ischemia was induced by transient occlusion of the MCA (tMCAO) using the Longa filament model for 90 min, as previously described.[26–28] Immediately after reperfusion, rapamycin (250 µg/kg in vivo) or vehicle was administered through intravenous tail vein injection. The dose of rapamycin and the route of administration were chosen based on a meta-analysis of our laboratory group on the dose-dependent effects of rapamycin in stroke.[2] The CBF was recorded for another 90 min from the time of reperfusion.

2.7 | Behavioral assessment

Neurological assessments to measure post-stroke impairments were performed on all 3 days following surgery. Tests included Bederson,[29] Garcia,[30] and the adhesive removal test.[31] The Bederson scale assesses post-stroke behavioral deficits by evaluating forelimb flexion, resistance to lateral push, and circling behavior, using a 0 to 3 grading scale (0 = not affected, 3 = severely affected).[29] The Garcia battery consists of 6 tests to evaluate sensorimotor deficits, graded on a 0–3 scale (0 = not affected, 3 = severely affected), with a total score range of 0 to 18 points.[30] The adhesive removal test examines somatosensory deficits by placing a 1 cm × 1 cm adhesive on the left or right forepaw and measuring the time to first touch the tape ("time-to-contact") and the time to remove it ("time-to-remove"), indicative of contralesional forepaw sensory and motor function. The order of application was randomized, and the stimulus was applied with equal pressure. To minimize inter-individual differences, training sessions (2 trials per day) were conducted 3 days before surgery, and the time (in seconds) to notice and remove the tape was compared between rapamycin and vehicle groups. The test was conducted immediately before MCAo surgery and repeated 3 days later, just before the MRI session.

2.8 | Magnetic resonance imaging (MRI)

MRI was performed 72 h after rapamycin administration using a 9.4 T horizontal-bore scanner (Agilent Technologies, USA) with a 72 mm transmit coil and a 4-channel receive array (Rapid Biomedical, Germany). Anesthesia was induced with 5% isoflurane in 70% N_2 and 30% O_2, and maintained at 1%–2% isoflurane in the same gas mixture. The rats were placed in a cradle with a stereotaxic holder, rectal thermometer, and pressure probe to monitor respiration. Core body temperature was maintained at 37.0 ± 0.5°C using a feedback-controlled heating pad (Harvard Apparatus, USA), and respiration was kept between 50 and 60 breaths per minute by adjusting isoflurane concentration. Physiological parameters were recorded regularly during the imaging session.

Quantitative maps for T1 and T2 relaxation times (seconds), cerebral blood flow (CBF, mL/100 g/min) via arterial spin labeling (ASL), and apparent diffusion coefficient (ADC, μm²/ms) were acquired. T1w and T2w images were acquired with a spin-echo echo-planar imaging (EPI) sequence with a FOV = 32 × 32 mm², matrix = 256 × 256 mm², thickness = 1 mm, and 10 slices. T1w and T2w images were acquired using a spin-echo EPI readout with FOV = 32 × 32 mm², matrix = 256 × 256 mm², thickness = 1 mm, 10 slices. T1w anatomatic images were acquired with a scan repetition time (TR) and echo time (TE) of 500 and 20 ms, and enhanced images were obtained post-injection of 150 μL of gadolinium-based contrast agent (GBCA; gadodiamide [Omniscan, Germany]) via tail vein. T1w maps (TR = 1000 ms, TE = 30–160 ms), repetition time/echo time [TR/TE] = 1000 ms/27.16 ms) were obtained using an inversion recovery sequence (TI = 13.14, 29.3, 65.3, 145, 324, 723, 1610, 8000 ms). T2w anatomical imaging scans (TR = 3000 ms, TE_{eff} = 51.26 ms) were obtained with an echo spacing (ESP) of 8.54. ASL and ADC data were acquired using a spin-echo EPI readout with a field of view (FOV) = 32 × 32 mm², matrix = 64 × 64 mm², thickness = 1 mm, 10 slices. Fast spin-echo multi-slice localization sequences (TR = 1000 ms, TE_{eff} = 40, ESP = 10 ms) were acquired using a midline and axial orientation with FOV = 50 × 50 mm², matrix = 256 × 256 mm², thickness = 2 mm, single slice. Based on the acquired FSMES-images, the labeling plane for ASL imaging (6.2 mm thickness) was placed in the rat's neck at a 45° angle to the animal's rostrocaudal axis. CBF maps were generated by multiphase pseudo-continuous arterial spin labeling (briefly: blood labeling used a pulse train of Hanning-shaped pulses, each 600 μs long at 40° flip angle, separated by 600 μs; multiphase images were acquired by arying the phase increments of pulses in the labeling train from 0° to 315° in eight steps of 45°; TR = 7600 ms, TE = 28 ms; full details available in.[32] ADC maps were generated from diffusion-weighted images acquired in three orthogonal directions for b = 0/mm² and b = 1000 s/mm². DW imaging scans were obtained using a two-dimensional spin-echo echo-planar imaging sequence (TR = 3 s, TE = 43.2 ms).

2.8.1 | MRI analysis

Segmentation and analysis were performed using ITK-SNAP (Version 3.8.0, 2019)[33] and MATLAB (Mathworks, Natick, MA). On T2w maps, ischemic areas were identified as hyperintense regions.[34] On T1w maps, areas of BBB breakdown were identified as hyperintense after subtracting pre- and post-Gd images. Regions of interest (ROIs) for ischemia and BBB breakdown were manually segmented in the transverse sections. Infarct volume was corrected for edema using Kaplan's formula[35]: Corrected infarct size = infarct volume × (volume of contralateral hemisphere/volume of the ipsilateral hemisphere). Corrected infarct size = infarct volume × (contralateral hemisphere volume/ipsilateral hemisphere volume). On ADC maps, ischemic areas were identified as regions with ≥23% reduced diffusion relative to the contralateral hemisphere. On ASL maps, hypoperfused areas were defined as regions with ≥57% reduced blood flow.[36] The contralateral hemisphere was defined by drawing a line from the central sulcus to the base of the brain, reflecting the ipsilateral hemisphere across this line. Relative diffusion and perfusion of the pre-defined cortical area were calculated as follows: Diffusion and perfusion ratios were calculated as diffusion/perfusion of ipsilateral ROI ÷ diffusion/perfusion of contralateral ROI. All ROIs were manually selected by the main investigator (AMS), who was blinded to treatment.

2.9 | Waxholm brain atlas

The Waxholm Space Atlas is an open-access volumetric atlas of rat brain anatomy based on isotropic magnetic resonance and diffusion tensor images based on ex vivo of 80-day-old male Sprague Dawley rats (Waxholm Space Atlas of the Sprague Dawley Rat Brain (RRID:SCR_017124).[37] Spatial referencing is provided by the Waxholm coordinate system. The atlas was used to overlay T2w MRI images acquired in the same stereotaxic space, using the distance from bregma and lambda for coordinates.

2.10 | Western blotting

After imaging, rats were euthanized with intraperitoneal pentobarbital (800 mg/kg).[38] Brains were collected and sliced into 2 mm-thick coronal sections using an ice-cold stainless-steel matrix (Kent Scientific). 1 mm² areas from the striatum and cortex of both hemispheres were taken from one section and snap-frozen. Proteins were extracted from the ipsilateral cortical lesion using RIPA buffer with a protease inhibitor cocktail. Protein quantification was performed using the BCA assay (Pierce BCA Protein Assay Kit, 23225, Thermo Fisher Scientific). 50 μg of protein was denatured (95°C for 5 min) with lysis and Laemmli sample buffer (161-0737, Bio-Rad, with DTT). Samples were separated on a 10% gradient gel (Criterion TGX Precast Gel, 5671033, Bio-Rad) using an electrophoresis unit (Bio-Rad, Cressier, Switzerland). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. Nonspecific binding was blocked with PBS containing 5% BSA and 0.1% Tween-20 for 1 h at room temperature, followed by overnight incubation in primary antibodies (mTOR and phospho-mTOR (Ser 2448), Cell Signaling, dilution for both 1:1000) in 5% PBS-T. After 3 ×5-min-washes in PBS-T, the membrane was incubated in secondary antibody goat anti-rabbit (IgG H&L (HRP), ab6721, Abcam, dilution 1:2000) in 5% BSA PBS-T for 1 h at RT. After 3 ×5-min-washes in PBS-T, the membrane was developed using enhanced chemiluminescence (ECL; 12644055, Fisher Scientific) for 5 min and immediately imaged. Western Blot analysis and quantification were performed using densitometry and normalized to β-tubulin. The membrane was probed

for antibodies twice, using the following mild antibody-stripping protocol: The membrane was stripped and re-probed using a mild stripping solution (7.5 g glycine, 0.5 g SDS, 5 mL Tween-20, pH to 2.2, and diluted up to 500 mL distilled water) for 2 ×7 min, following by 2 ×10-min-washes in PBS, and 2 ×5-min washes in PBS-T, before blocking again in 5% BSA and 0.1% Tween-20.

2.11 | Statistical analysis

The experimental design and animal numbers were optimized to address the hypotheses and adhere to scientific reporting standards.[39] Statistical analyses were conducted after the study's conclusion, with no additional animals included. The study was powered with $n = 9$/group, based on an estimated 30% effect size (± 20% variability) in lesion volume, the primary outcome, providing 80% power to reject the null hypothesis ($\alpha = 0.05$). Secondary outcomes, including behavioral subtests, MRI-derived measures, and Western blot analyses, were considered exploratory and were not individually powered to detect smaller effect sizes. Accordingly, the absence of statistical significance in selected secondary outcomes should be interpreted in the context of limited power and biological variability. Statistical analysis was performed using GraphPad Prism 8.42 (La Jolla, USA). Data normality was assessed with the D'Agostino and Pearson tests, and parametric tests were only applied when normality assumptions were met. An unpaired Student's *t*-test was used to compare treatment groups. Correlations between imaging parameters and functional outcomes were assessed using linear regression, classified as tiny (<0.05), very small (0.05–0.1), small (0.1–0.2), medium (0.2–0.3), large (0.3–0.4), or very large (≥0.4) according to Funder and Ozer.[40] A p-value < 0.05 was considered statistically significant, with data presented as mean ± SD.

3 | RESULTS

3.1 | Excluded animals

A total of 22 rats underwent surgery. Four animals were excluded intraoperatively due to insufficient LDF drop ($n = 3$) or subarachnoid hemorrhage ($n = 1$), resulting in 18 animals available for post-surgical analyses. Behavioral assessments were performed in all remaining animals ($n = 18$). For MRI-based analyses, one animal did not undergo MRI and was excluded from all MRI-derived outcomes ($n = 17$). One additional animal did not have T2-weighted imaging and was therefore excluded from infarct volume analysis ($n = 16$). For T1-weighted contrast-enhanced imaging, five animals were excluded due to insufficient image quality. For BBB analysis, a further four animals were excluded due to insufficient gadolinium contrast, resulting in a final sample size of $n = 12$. For CBF analysis, six animals were excluded due to absence of a reliable signal, resulting in $n = 16$. Diffusion and perfusion MRI analyses were performed in 17 animals. Western blot analysis was performed in a subset of animals ($n = 10$; vehicle $n = 6$, rapamycin $n = 4$), as several samples were excluded due to insufficient image quality; exclusions were based solely on predefined quality criteria and were unrelated to treatment allocation.

3.2 | Rapamycin significantly reduces infarct volume

Rapamycin significantly reduced infarct volume at 72 h after stroke (rapamycin: 44.77 ± 30.93 mm³ vs. vehicle: 113.44 ± 60.19 mm³, $p = 0.0114$; Figure 1). Overlay of the Waxholm Space Atlas onto T2-weighted MRI sections indicated involvement of multiple cortical and subcortical regions, including the somatosensory cortex (Supplementary Figure 1, Supplementary Table 1).

3.3 | Rapamycin significantly improves functional outcomes

Rapamycin significantly improved Garcia test performance (rapamycin: 12.78 ± 1.04 points vs. vehicle: 11.67 ± 0.87 points, $p = 0.0295$, Figure 2A). It did not improve performance on the Bederson test (rapamycin: 1.56 ± 0.73 vs. vehicle: 2.11 ± 0.60 points, $p = 0.0962$, Figure 2B). Rapamycin-treated animals took less time to notice the adhesive tape on their forepaw, indicating improved sensory function (rapamycin: 45.04 ± 11.91 s vs. vehicle: 72.33 ± 12.17 s, $p = 0.0002$, Figure 2C). They also removed the tape faster (rapamycin: 49.89 ± 23.09 s vs. vehicle: 76.56 ± 12.92 s, $p = 0.0146$, Figure 2D). A strong correlation was observed between infarct volume and neurological test outcomes, with larger lesions leading to worse function (Infarct volume

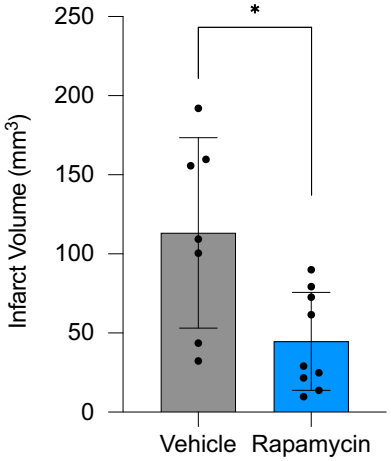

FIGURE 1 Rapamycin significantly reduces infarct volume at 72 h after stroke. Infarct volume was assessed using T2-weighted magnetic resonance imaging (MRI) at 72 h after transient middle cerebral artery occlusion, with correction for edema. Infarct volume was significantly reduced in the rapamycin-treated group compared with vehicle. Data are presented as mean (SD), $n = 7$ for vehicle and $n = 9$ for rapamycin (*$p < 0.05$). Statistical analysis was performed using unpaired Student's *t*-tests. Representative coronal sections are shown in Supplementary Figure 1.

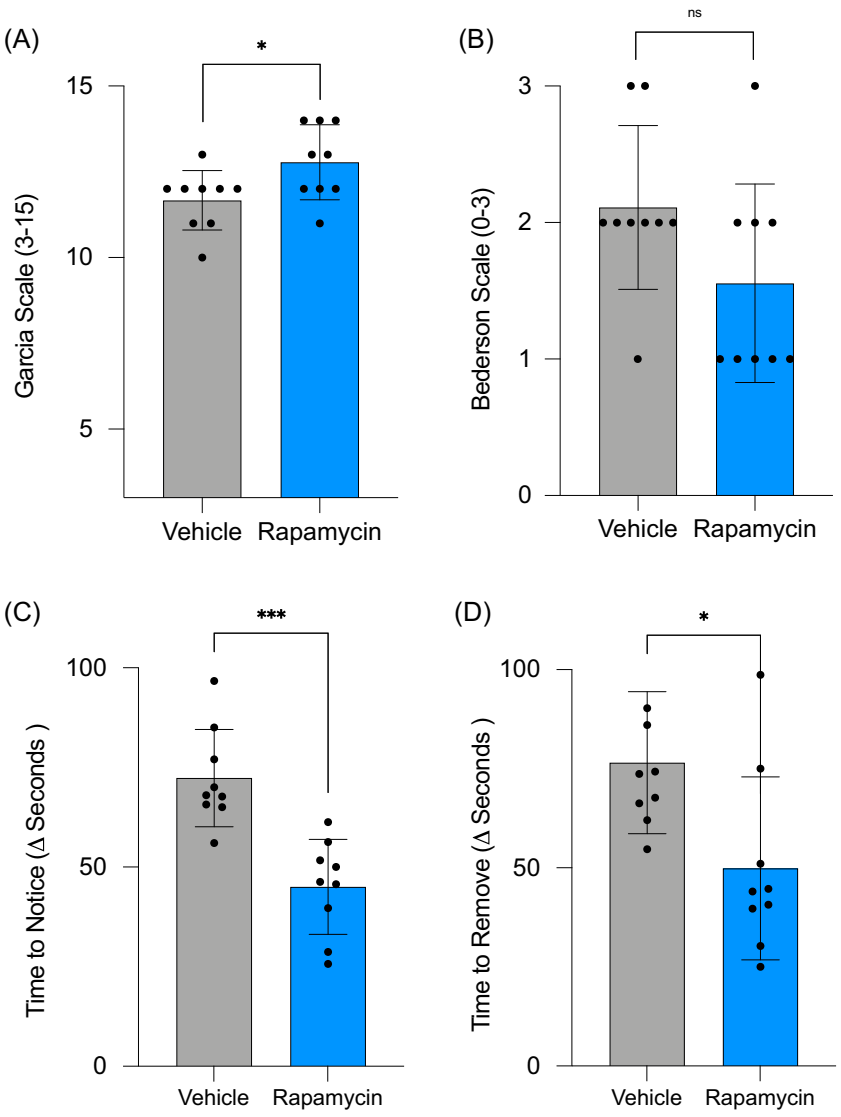

FIGURE 2 Rapamycin significantly improves functional outcomes. (A) Neurological assessment with Garcia scale 72 h post-stroke (3–15 points). (B) Neurological assessment with Bederson scale 72 h post-stroke (0–3 points). (C) Rapamycin significantly reduces sensory deficits assessed by the adhesive removal test. Time to notice the left paw (contralateral to stroke side), calculated as the difference between baseline and 72 h post-stroke (seconds). (D) Rapamycin significantly reduces motor deficits assessed by the adhesive removal test. Time to remove the adhesive tape from the left paw (contralateral to stroke side), calculated as the difference between baseline and 72 h post-stroke (seconds). Data are mean (SD), $^{*}p < 0.05$, $^{***}p < 0.001$, when compared to vehicle. $n = 9$ for all groups.

vs. Garcia: $r = -0.5502$, $p = 0.0272$; Figure 3A; Infarct volume vs. Bederson: $r = 0.6138$, $p = 0.0114$, Figure 3B; Infarct volume vs. time to notice forepaw in adhesive removal: $r = 0.5486$, $p = 0.0278$, Figure 3C).

3.4 | Rapamycin does not improve immediate post-recanalization blood flow

There was no difference in blood flow before or during MCAo, both time points being before rapamycin treatment. (**before**: vehicle: 91.73 ± 9.97% vs. rapamycin: 82.21 ± 6.87% pre-MCAo baseline, $p = 0.0784$, Figure 4A; **during**: vehicle: 40.28 ± 13.32% vs. rapamycin: 46.25 ± 9.11% pre-MCAo baseline, $p = 0.3774$, Figure 4B). Rapamycin did not change average MCA territory blood flow in the first 90 min post-recanalization (**after**: vehicle: 96.73 ± 10.6% vs. rapamycin: 94.82 ± 16.37% pre-MCAo baseline, $p = 0.8386$, Figure 4C).

3.5 | Rapamycin does not alter diffusion or perfusion

After assessing rapamycin's effect on blood flow in the hyperacute phase, we investigated its impact on spatially matched dynamic changes in the subacute phase.

At 3 days, no rat had a large enough diffusion deficit to define the cortical MCA region as ischemic. Rapamycin did not significantly alter relative diffusion (rapamycin: 1.12 ± 0.05 vs. vehicle: 1.11 ± 0.09, $p = 0.5765$, Figure 5A) or absolute diffusion in the ipsilateral ROI (rapamycin: 0.00081 ± 0.0001 × 10^4 mm²/s vs. vehicle: 0.00080 ± 0.0001 × 10^4 mm²/s, $p = 0.6099$, Figure 5B).

One rat showed a significant perfusion abnormality between hemispheres. However, rapamycin did not significantly change relative perfusion (rapamycin: 0.9997 ± 0.3364 vs. vehicle: 1.065 ± 0.1856, $p = 0.6340$, Figure 5C) or absolute perfusion (rapamycin: 94.60 ±

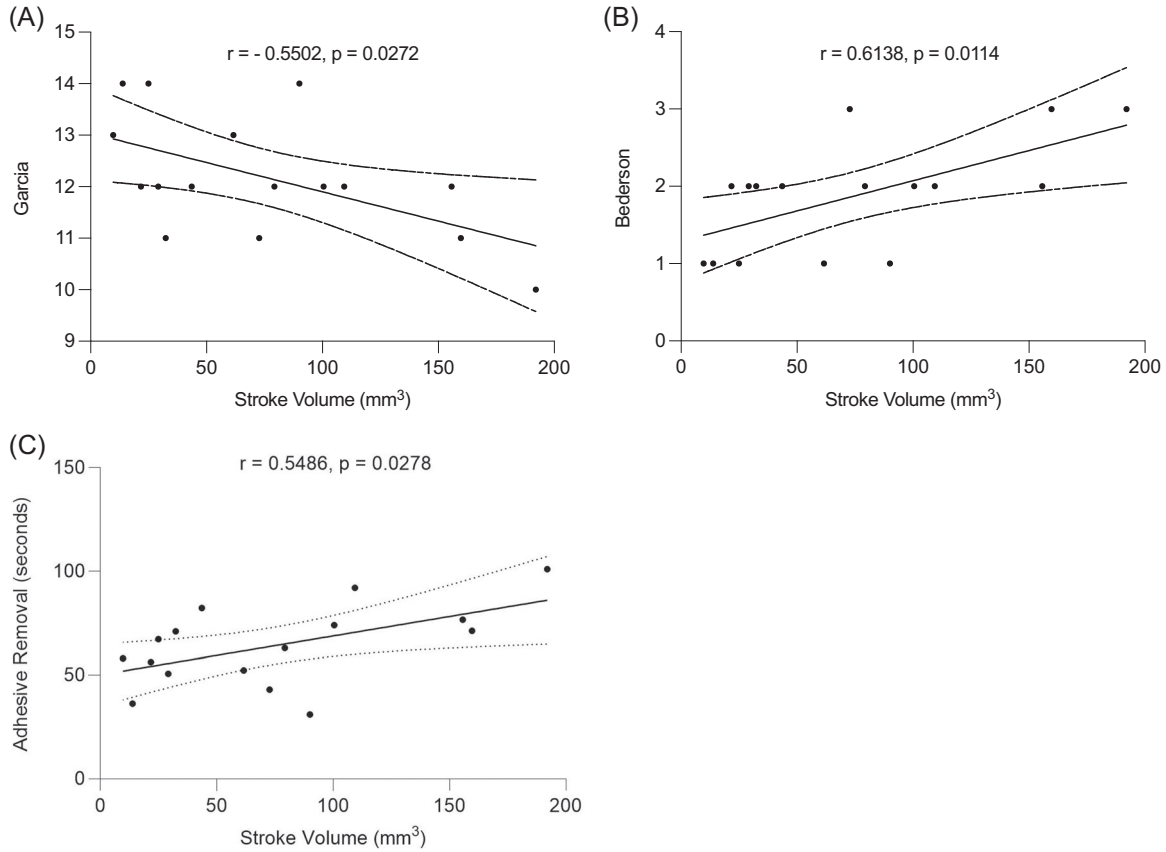

FIGURE 3 There is a very strong correlation between infarct volume and neurological test results, with larger lesions leading to worse functional outcomes. (A) Stroke volume versus Garcia $r = -0.5502$, $^*p < 0.05$; (B) Stroke volume versus Bederson $r = 0.6138$, $^*p < 0.05$; (C) Stroke volume versus time to notice of contralateral forepaw in adhesive removal test $r = 0.5486$, $^*p < 0.05$. $n = 16$ for all groups.

34.67 mL/100 g/min vs. vehicle: 139.10 ± 87.92 mL/100 g/min, $p = 0.4807$, Figure 5D).

3.6 | The effect of rapamycin on edema formation and BBB integrity

Edema formation on T2w images was assessed using the calculation from Kaplan[35]: Extent of edema = (volume of ipsilateral hemisphere − contralateral hemisphere volume)/contralateral hemisphere volume. Rapamycin did not significantly affect edema volume (rapamycin: 9 ± 5.66 mm³ vs. vehicle: 14.25 ± 7.19 mm³, $p = 0.1129$, Figure 6A). T1w imaging with gadolinium injection assessed BBB integrity, For BBB analysis, five animals were excluded due to insufficient T1-weighted image quality and a further four animals due to insufficient gadolinium contrast, resulting in a final sample size of $n = 12$. Rapamycin did not significantly alter BBB breakdown (rapamycin: 3.27 ± 2.34 mm³ vs. vehicle: 7.65 ± 4.50 mm³, $p = 0.0606$, Figure 6B).

3.7 | mTOR activity was not reduced at 3 days

The ratio of phosphorylated (p-mTOR) to total mTOR (mTOR) protein was used as a measure of pathway activity. The p-mTOR/mTOR ratio did not differ significantly between rapamycin-treated and vehicle-treated animals at 72 h after stroke (rapamycin: 0.55 ± 0.32 vs. vehicle: 0.90 ± 0.41, $p = 0.1880$; Supplementary Figure 1).

4 | DISCUSSION

This study assessed postischemic mTOR inhibition on CBF, BBB integrity, and stroke outcome in rats. Rapamycin given immediately after recanalization reduced infarct volume and improved function by Day 3, alleviating somatosensory deficits without affecting BBB integrity. It did not increase CBF in the MCA territory within 90 min post-recanalization or at 3 days, contrasting with earlier work showing enhanced microvascular reperfusion when given at or before reperfusion via reduced pericyte-mediated capillary constriction.[20]

These findings suggest that when administered after recanalization, rapamycin's cerebroprotection occurs via mechanisms other than acute or sustained perfusion—likely inflammation modulation or NVU stabilization. This is consistent with studies showing BBB preservation[9,13,14,18,19] and microglial modulation (10, 15, 16). Clarifying its cellular targets, mechanism, and optimal dosing remains important for translation.

Rapamycin reduced lesion volume at 3 days, exceeding our prior meta-analysis estimate of a 22%

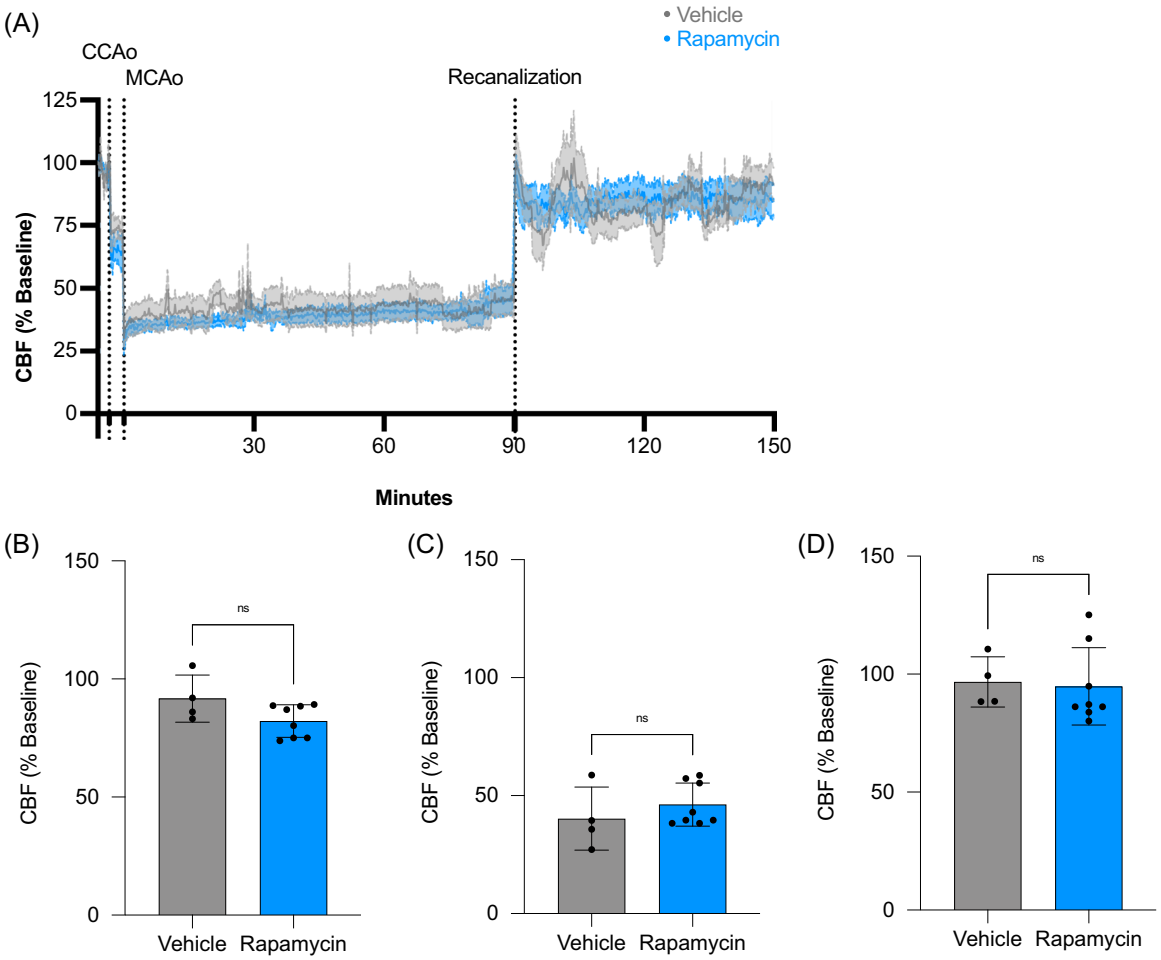

FIGURE 4 Rapamycin does not improve immediate post-recanalization blood flow. (A) Cerebral blood flow (CBF) expressed as % of baseline immediately before middle cerebral artery occlusion (MCAo). (B) Average CBF before MCAo. (C) CBF during MCAo. (D) Mean CBF during the first 90 min after recanalization. Rapamycin was administered immediately after recanalization. No significant differences were observed between vehicle-treated ($n = 7$) and rapamycin-treated animals ($n = 9$) at any time point (all $p > 0.05$). Data are presented as mean (SD). Statistical analysis was performed using unpaired Student's *t*-tests.

infarct reduction across 30 rodent studies[2] with a mean 39% reduction here. MRI showed no difference in subacute diffusion or perfusion between groups at 3 days, and no DPM in either, suggesting no salvageable tissue at this time point. Accordingly, diffusion–perfusion mismatch was not presented as a separate outcome, as predefined mismatch criteria were not met in any animal at this time point. DPM volume declines rapidly post-MCA occlusion, dropping to a quarter of its initial size within 45–210 min.[24,40] Few studies have examined DPM after recanalization in temporary MCAo; Meng et al. found it highest within the first 2 h after reperfusion.[41] The early reperfusion period is thus optimal for adjunct therapies, as most salvageable tissue remains. Although rapamycin was given at recanalization, its benefits appeared independent of sustained perfusion, suggesting actions on other mechanisms such as immune modulation, BBB stabilization, or broader neurovascular protection. However, BBB integrity did not differ between rapamycin and control animals.

Subtle BBB impairment can occur within 30 min to 6 h after ischemia-reperfusion (I/R) in mouse tMCAo, allowing small macromolecule leakage (≤3 kDa) into brain parenchyma.[42–44] After 3 h, larger macromolecules (≥40 kDa) extravasate, and tight junctions—intact during the hyperacute phase—begin to fail around 48 h[43,45] due to cytokine-activated MMP-3, MMP-9, and cyclooxygenase-2. This loss underlies vasogenic edema.[42,46] Rapamycin has reduced BBB breakdown in previous studies.[8,9,13,14,19,47] The lack of benefit here may reflect limited statistical power, as BBB breakdown was a secondary outcome: the mean difference was ~50%, but high variability (SD ~ 72%, $n = 6$) reduced significance. Another possibility is reduced rapamycin levels by Day 3, as shown by our Western blot, coinciding with peak BBB disruption. These findings align with our earlier post-stroke fasting work, where early reperfusion reduced inflammation and BBB disruption.[48] As fasting also inhibits mTOR, both pharmacological and metabolic mTOR inhibition may protect the BBB, with timing critical to maximize benefit. Consistently, our T2w edema analysis showed no significant edema reduction at 3 days.

Rapamycin improved somatosensory function after stroke. Behavioral testing is essential for evaluating cerebroprotective therapies, and 3 days post-stroke is optimal for detecting persistent deficits without surgical recovery confounds. Our battery assessed motor, somatosensory, and overall function.

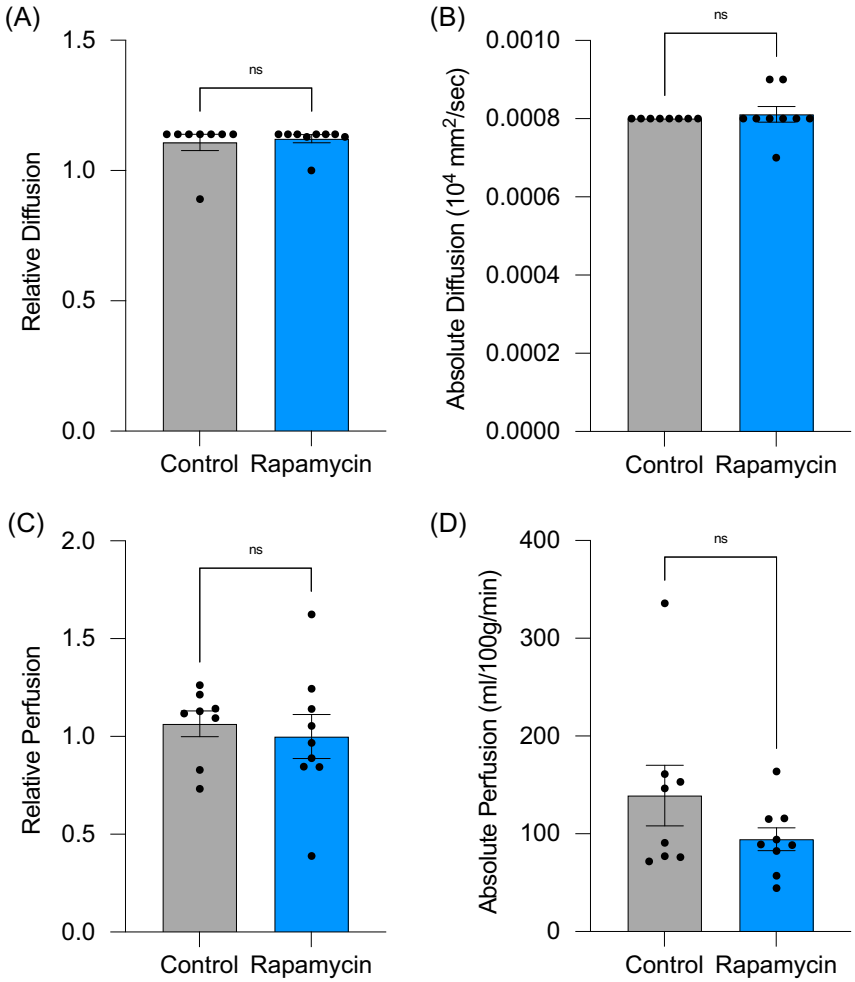

FIGURE 5 Rapamycin does not alter diffusion or perfusion. (A) Ipsilateral diffusion within the lesion area relative to the corresponding contralateral region, assessed using magnetic resonance imaging (MRI) with apparent diffusion coefficient (ADC) mapping. (B) Absolute diffusion values of the ipsilateral hemisphere. (C) Ipsilateral perfusion within the lesion area relative to the corresponding contralateral region, assessed using arterial spin labeling (ASL) MRI. (D) Absolute perfusion values of the ipsilateral hemisphere. All measurements were obtained at 72 h after transient middle cerebral artery occlusion (tMCAo). No significant differences were observed between vehicle-treated ($n = 8$) and rapamycin-treated animals ($n = 9$). Data are presented as mean (SD). Statistical analysis was performed using unpaired Student's *t*-tests (all $p > 0.05$).

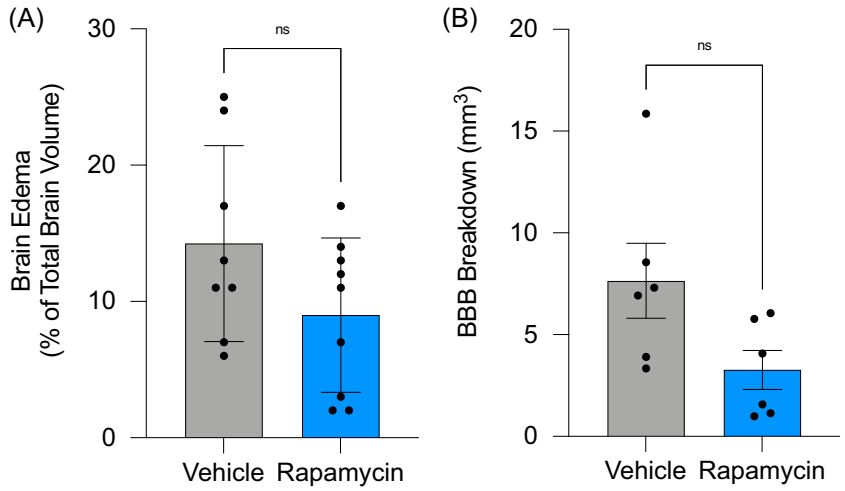

FIGURE 6 The effect of rapamycin on edema formation and blood-brain barrier (BBB) integrity. (A) Brain edema expressed as a percentage of whole-brain volume, assessed using T2-weighted magnetic resonance imaging (MRI) at 72 h after transient middle cerebral artery occlusion. No significant difference was observed between vehicle-treated ($n = 8$) and rapamycin-treated animals ($n = 9$). (B) Blood-brain barrier permeability assessed using contrast-enhanced T1-weighted spin echo MRI before and after intravenous administration of a gadolinium-based contrast agent (GBCA), with values corrected for edema formation. No significant difference was observed between groups ($n = 6$ per group). Data are presented as mean (SD). Statistical analysis was performed using unpaired Student's *t*-tests (all $p > 0.05$).

Rapamycin improved both time to notice and time to remove adhesive in the adhesive removal test, with greater improvement in time to notice (somatosensory) than time to remove (sensorimotor).[49] Time to notice correlated more strongly with infarct volume, reflecting its sensitivity to somatosensory cortex injury and partial rescue by rapamycin, as seen in Figure 2B and Supplementary Table 1. Differences might also result from surgery-induced ischemia in masticatory muscles, hindering adhesive removal.[50] Rapamycin did not improve Bederson or Garcia scores, which assess sensorimotor deficits without mouth use.[51] Overall, rapamycin appeared more effective for sensory than motor recovery, consistent with prior studies,[5,6,10,11,13,14,19,52] our earlier adhesive test results,[23] and our meta-analysis showing a 30% neurological score improvement and significant correlation with infarct volume.[2]

Western blot of ipsilateral cortex showed unchanged mTOR activity (p-mTOR:mTOR ratio) at 3 days, suggesting rapamycin's effects had already occurred. No rat data exist beyond 3 days, but in Sprague Dawley rats, levels rapamycin fall to ~1 nM at 72 h after a 1 mg/kg dose (half-life ~ 25 h), below the effective neuronal culture concentration.[53,54] Rapamycin's Half-live is 58 h in mice[55] and 79 h in healthy males,[56] indicating species differences. Future studies should include earlier endpoints to assess effects on mTOR activity, microvasculature, infarct volume, and function. Testing later administration could clarify whether benefits depend on hyperacute blood flow (within 2 h). Further work should examine lesion organization and BBB stabilization at later stages, as BBB disruption evolves from hypoxic injury within 72 h to inflammation.[57,58] Understanding temporal effects of post-ischemic mTOR inhibition, per STAIR criteria, could optimize rapamycin's clinical use.[59]

While this study was prospectively powered for infarct volume as the primary outcome, several secondary measures—including Western blot analysis and MRI-based assessments of BBB disruption and perfusion—showed substantial variability and were likely underpowered to detect more subtle effects. In addition, different techniques were used to assess related biological processes (e.g., perfusion measured by LDF and ASL-MRI at different time points), which capture distinct spatial and temporal aspects of cerebrovascular function. These factors should be considered when interpreting the absence of significant effects in selected secondary outcomes.

While rapamycin is a well-characterized inhibitor of mTORC1, the present study was not designed to perform in-depth mechanistic analyses. Importantly, tissue was collected at 3 days post-stroke, a time point at which mTOR activity had returned to baseline levels, suggesting that the window of direct rapamycin-mediated pathway modulation had already passed. As such, additional molecular or cellular analyses at this stage would be unlikely to capture the primary mechanisms underlying the observed neuroprotective effects. Future studies should therefore focus on earlier post-recanalization time points to delineate the temporal dynamics of mTOR inhibition and to investigate downstream pathways, including cell survival signaling, metabolic stress responses, and immune modulation in the peri-infarct region.

This preclinical trial of rapamycin, incorporating key translational factors, shows that mTOR inhibition at reperfusion improves function and reduces infarct volume without sustained CBF increases. At 3 days, lesion size was smaller and outcomes better, indicating mechanisms beyond vascular effects. This time-dependent, multimodal action supports further development of rapamycin as an adjunct cerebroprotective therapy in ischemic stroke. Validation in larger models and mapping temporal dynamics will be critical for translation.

AUTHOR CONTRIBUTIONS

Anna M. Schneider: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); visualization (lead); writing—original draft (lead). **Yvonne Couch**: Conceptualization (equal); resources (equal); supervision (equal); validation (equal); writing—review and editing (equal). **James Larkin**: Methodology (equal); visualization (equal); writing—review and editing (equal). **Alastair M. Buchan**: Conceptualization (equal); resources (equal); supervision (equal); validation (equal); writing—review and editing (equal). **Daniel J. Beard**: Conceptualization (equal); supervision (equal); validation (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

Alastair M. Buchan is cofounder of BRAINOMIX. Daniel J. Beard is cofounder of ShearFlow. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All experimental procedures were approved by the UK Home Office (1986 Animal Act, Scientific Procedures; licence number PP7444704 granted 23.12.22), conducted in accordance with the Clinical Medicine Ethical Review Guidelines of the University of Oxford, the ARRIVE and the IMPROVE guidelines for animal and pre-clinical stroke work.

INFORMED CONSENT

This study did not involve human participants, and informed consent was therefore not required.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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