The effect of rapamycin treatment on cerebral ischemia: A systematic review and meta-analysis of animal model studies.

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Abstract

Background: Amplifying endogenous neuroprotective mechanisms is a promising avenue for stroke therapy. One target is mammalian target of rapamycin (mTOR), a serine/threonine kinase regulating cell proliferation, cell survival, protein synthesis and autophagy. Animal studies investigating the effect rapamycin on mTOR inhibition following cerebral ischemia have shown conflicting results.

Aim: To conduct a systematic review and meta-analysis evaluating the effectiveness of rapamycin in reducing infarct volume in animal models of ischemic stroke.

Summary of review: Our search identified 328 publications. Seventeen publications met inclusion criteria (52 comparisons: 30 reported infarct size, 22 reported neurobehavioral score). Study quality was modest (median 4 out of 9) with no evidence of publication bias. The point estimate for the effect of rapamycin was a 21.6% (95% CI, 7.6% - 35.7% \( p < 0.01 \)) improvement in infarct volume and 30.5% (95% CI 17.2% - 43.8%, \( p < 0.0001 \)) improvement in neuroscores. Effect sizes were greatest in studies using lower doses of rapamycin.

Conclusion: Low dose rapamycin treatment may be an effective therapeutic option for stroke. Modest study quality means there is a potential risk of bias. We recommend further high quality preclinical studies on rapamycin in stroke before progressing to clinical trials.
**Introduction**

Amplifying the brain’s intrinsic neuroprotective pathways is a promising avenue for developing new treatments for stroke. The serine/threonine kinase Mammalian Target of Rapamycin Complex 1 (mTORC1) plays a role in neuronal degeneration following global ischemia.\(^1\) If cellular energy is abundant, mTORC1 will be activated leading to protein synthesis, cell proliferation and cytoskeletal formation.\(^2\) When energy supplies are depleted, mTORC1 is inhibited through the action of the tuberous sclerosis complex (TSC) made up of hamartin (TSC1) and tuberin (TSC2).\(^3, 4\) Following global ischemia, resistant cells within the cornu ammonus 3 (CA3) subfield of the hippocampus are able to upregulate the expression of hamartin leading to mTORC1 inhibition, limiting protein synthesis and increasing productive autophagy to preserve energy stores and promote cell survival.\(^1\)

Pharmacological inhibition of mTORC1 can be achieved using rapamycin (sirolimus). Rapamycin is an FDA approved immunosuppressant/antirejection treatment for kidney transplantation\(^5\) and in drug eluting coronary stents preventing intimal hyperplasia and occlusion.\(^6\) Post-hoc analysis of preclinical data justifying previous candidate neuroprotectants, NXY-059 and Erythropoietin identified significant experimental deficits.\(^7, 8\) Our systematic review and meta-analysis aims to measure the overall efficacy of rapamycin in experimental stroke. This will serve to guide further preclinical studies or if significant neuroprotection is detected with no effects of bias, rapamycin may be rapidly re-purposed as a stroke treatment.

**Methods**

Methods were pre-specified in a study protocol (http://www.dcn.ed.ac.uk/camarades/research.html#protocols) and followed the Systematic Review Protocol for Animal Intervention Studies.\(^9\)

**Search Strategy**

The Ovid interface was used to search MEDLINE (1946-May 2017) and EMBASE (1974-May 2017) with no language limits.

The search focused around two keywords “ischemic stroke” and “rapamycin” with respective thesaurus terms and free-text terms. Full search strategies are in supplementary material.
Inclusion and Exclusion Criteria

Included studies reported the effects of rapamycin using *in vivo* animal model of cerebral ischemia with the primary outcome of infarct volume by any method. Secondary outcome measures included neurobehavioral scores, cerebral blood flow (CBF) and blood brain barrier (BBB) permeability. Experiments involving co-treatments with additional drugs or using rapamycin to rescue a genetic knock-down were excluded.

Quality Assessment

We assessed risk of bias using a 9-item quality checklist (adapted from Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES))\(^\text{10}\).

Data Extraction

Titles and abstracts were screened independently by 2 reviewers (D.J.B and G.H). Full texts were obtained for eligible studies and screened independently (D.J.B and G.H). A third reviewer (B.A.S) arbitrated any differences of opinion. Data were extracted by G.H and D.J.B for study design elements including: species, strain, age, sex of animals used; co-morbidities; anaesthetic, ischemia duration, model of ischemia; dose, route, timing of drug administration, time of outcome assessment; methods of outcome assessment and type of outcome measurement (infarct volume, behaviour, CBF, BBB permeability). For extraction of dose for each comparison, a standardised dose in mg/kg was calculated based on the dose of rapamycin administered and the number of times that dose was administered.

For each comparison, data describing the number of animals per group, the mean outcome, and the standard deviation for both the control and treatment group were extracted to enable calculation of a normalised mean difference (NMD). Where studies compared multiple doses with a single control group, individual comparisons were made between each specific dose and the control, with control n numbers divided by the number of doses. Where data were reported graphically, digital ruler software ascertained values, and where data for a single animal group are reported at different time points, data were extracted for the final time point only.
**Meta-analysis**

All meta-analyses and meta-regression were conducted using Stata/SE 15.0 (StataCorp LLC, College Station, TX, USA). Means and standard deviations for infarct volume and behavioral outcomes from each study were used for all meta-analyses. A $p < 0.05$ was considered statistically significant. To determine the overall effect of rapamycin treatment on each outcome measure, a meta-analysis was performed using the metan function. For the effect of rapamycin on both infarct volume and neurological deficit behavioural scores in each individual study, NMD was calculated using Equation (1):

$$NMD_i = 100\% \cdot \frac{\bar{x}_R - \bar{x}_C}{\bar{x}_C}$$ (1)

A pooled effect size was determined using the random effects model of DerSimoninan and Laird (estimated heterogeneity taken from the Mantel-Haenszel model). A heterogeneity of $I^2 > 50\%$ indicated significant heterogeneity. To assess correlation between infarct volume and behavioural outcome for studies including both outcomes, a linear regression was performed. To identify which variables contributed to any heterogeneity observed in the infarct volume meta-analysis, meta-regression was conducted using the metareg function. For each independent variable, an adjusted $R^2$ value was calculated determining the proportion of between-study variance explained by the variable. A trim and fill analysis was performed using the metatrim function assessing publication bias.

**Results**

A total of 528 records were retrieved (Ovid Medline, 243; Ovid EMBASE, 285), following removal of duplicates, 328 records remained. A further 278 articles were excluded on title and abstract, leaving 64 full text articles. 42 articles were excluded after reading the full text. The remaining 22 full text articles used rapamycin in the treatment of brain ischemia (17 focal cerebral ischemia, 3 neonatal hypoxia-ischemia and 2 global ischemia) (Supplementary Figure 1). Our pre-defined study design stipulated that quantitative assessment would only be carried out on >10 observations leaving only focal cerebral ischemia studies. These 17 publications, $^{11-26}$ contained 52 comparisons: 30 comparisons reported data on infarct size and 22 comparisons reported data neurobehavioral score. All studies investigating the effect of rapamycin in either neonatal hypoxia ischemia or global ischemia showed that rapamycin reduced brain damage $^{27-31}$ (Supplementary Tables 1 and 2).
Efficacy

The point estimate for the effect of rapamycin was a 21.6% (95% CI 7.6-35.7%, \( p < 0.05 \)) improvement in infarct volume (Figure 1A) and 30.5% (95% CI 17.2-43.8%, \( p < 0.0001 \)) improvement in neuroscores (Figure 1B). There was significant statistical heterogeneity within comparisons of infarct volume (\( I^2 = 92\% \)) and neurobehavioral scores (\( I^2 = 97.8\% \)). 22 of the 30 comparisons of infarct volume also had associated neurobehavioral assessment, enabling a correlation analysis between the two outcome measures. Infarct volume and neurobehavioral scores were highly correlated \( (R^2 = 0.7069, p < 0.0001, \text{Figure } 1C) \), showing consistency of improvement by rapamycin as determined by these two outcome measures. In line with our pre-defined selection criteria, we did not perform meta-analysis on other secondary outcome measures of BBB breakdown and CBF, as there were <10 comparisons for each variable. However, 5 out of 6 studies reporting BBB breakdown showed that rapamycin treatment reduced BBB permeability.\(^ {15, 16, 21, 22, 26, 32} \) A single study reporting CBF showed that rapamycin non-significantly improved CBF following reperfusion\(^ {14} \) (Supplementary Table 3).

Effect of study characteristics on efficacy

Study design characteristics are in Table 1. Only the dose of rapamycin explained a significant amount of between-study heterogeneity \( (R^2 = 47\%, p < 0.001) \). Lower doses of rapamycin resulted in a greater effect size (Figure 2A). The range of doses used was substantial \( (0.000001 - 40 \text{ mg/kg}) \) and so visualization of this correlation was difficult due to the cluster of studies using a 0 - 1 mg/kg dose of rapamycin. The dose was log transformed and there is a clear association, with lower doses of rapamycin providing greater protection against injury following cerebral ischemia (Figure 2B). Important to note the two studies using the highest dose of rapamycin (40mg/kg) also had the worst outcomes.\(^ {14, 15} \) Other design characteristics such as species, route of administration, dose timing, ischemia model, permanent vs. transient ischemia, ischemia duration, outcome assessment time, comorbidities, randomization, blinded ischemia induction or blinded outcome assessment did not account for a significant portion of between study heterogeneity (Supplementary Table 4 contains all R\(^2 \) and \( p \) values).

Study quality and publication bias
The median number of study quality checklist items scored was 4 out of a possible 9 (range 3-7) (Supplementary Table 5). Classifying studies by quality score did not account for a significant portion of study heterogeneity ($R^2 = 2.94\%$, $p > 0.05$) (Figure 3A). The funnel plot with trim and fill analysis did not suggest publication bias (Figure 3B).

**Discussion**

This systematic review and meta-analysis supports the overall efficacy of rapamycin improving both histological and functional outcomes in animal models of acute ischemic stroke. Infarct volumes and behavioral scores were highly correlated. There was no evidence of publication bias. The highest levels of efficacy were at lower doses of rapamycin. No other variables had a significant bearing on the efficacy of rapamycin. These findings suggest that low dose rapamycin has the potential to be utilized as a neuroprotective strategy in patients with ischemic stroke.

A global estimate of 30 comparisons revealed that rapamycin significantly reduced infarct volume by 22%. This is slightly lower than a 31% reduction of infarct volume reported in a systematic review and meta-analysis (with median quality score of 4 and publication bias) of FK506 (Tacrolimus), another immunosuppressive compound. Unlike rapamycin, FK506 binding to FKBP12 does not induce mTOR inhibition and changes in autophagy. Rather, the FK506-FKBP12 complex blocks the activation of calcineurin and downstream signaling pathways, resulting in neuroprotection. These different mechanisms of action may explain the difference in efficacy between the two treatments. FK506 has been trialed in human stroke but failed due to adverse side effects. Though our dataset does not indicate an influence of confounding factors such as randomization or blinding on rapamycin effect size, there may still be a high risk of bias given the smaller number of studies and moderate study quality.

The dose of rapamycin accounted for significant variability in the model. Specifically, lower doses of rapamycin showed greater efficacy at reducing infarct volume than higher doses. Doses used in this study ranged from < 1mg/kg to 40mg/kg. If one takes into account allometric scaling between rats and humans, a 40mg/kg dose in a 300g rat would equate to 10mg/kg in a 70kg human. This dose is 50 times higher than that used in humans for kidney transplantation (0.2 mg/kg/day). This is in contrast with FK506, in which animal studies used doses that more closely resembled doses used clinically. FK506 efficacy showed a slight trend towards an inverted U shaped dose response curve. Studies using higher doses of rapamycin were published in 2016. The authors do not to give a
rationale for using such high doses of rapamycin. Such an increase in the dose used may be as a result of a drop in the price of rapamycin following the patent (US5100899A) expiring in 2013.

Though our dose findings seem counterintuitive, there are two sets of observations that suggest they make sense from a biological perspective. Inhibition of mTORC1 increases autophagy. Basal levels of autophagy are necessary for neuronal health (neurodegeneration and ubiquitinated protein aggregates occur in animal models lacking this process). At low doses, rapamycin may be beneficial but harmful at high doses because of unregulated autophagy. A second possible explanation is that prolonged rapamycin treatment can affect the assembly and function of mTORC2. mTORC2 plays an important role in cell survival during stress. Studies utilizing low doses of rapamycin may also have stimulated autophagy enough without inhibiting mTORC2 to enhance neuronal survival following ischemia. Higher dose studies may have over-stimulated autophagy and/or inhibited mTORC2 leading to increased infarct volume. A number of studies have shown that adverse effects such as immunosuppression and glucose intolerance are mediated by mTORC2 inhibition due to prolonged rapamycin treatment. Experimental evidence from rodents suggests that a one off bolus of 1mg/kg of rapamycin reduces peripheral mTORC1 activity for 1-3 days without suppressing mTORC2 and ultimately improves immunological function and reduces glucose intolerance in these animals. Use of rapamycin as an acute therapy inhibiting mTORC1 in brain without peripheral suppression of mTORC2 may improve its side effect profile. Future preclinical trials of rapamycin in stroke and potential safety trials in humans should assess mTORC1 and mTORC2 activity levels in peripheral immune cells, overall immune cell numbers and glucose tolerance tests.

Efficacy of rapamycin was not affected by timing of drug administration though many of the studies provided therapy before or early after onset of stroke (Table 1) and equally efficacious in permanent and temporary ischemia models. Rapamycin could be a stroke treatment not bound by time or imaging constraints, used for patients who are outside the time window for thrombolysis/thrombectomy or as an adjunctive therapy to this gold standard treatment. Inhibiting mTORC1 affects numerous cell death, CBF, BBB and inflammatory pathways that develop within minutes to days after stroke onset. Patients with transient ischemic attacks (TIAs), who have an increased 90 day stroke risk may also benefit. In 313 Mayo Clinic heart transplant patients on Sirolimus (rapamycin) followed over 20 years, not a single patient in this inherently high-risk group had a stroke or TIA. In a similar sized study at the same
institution, 5% of patients not taking sirolimus developed cerebrovascular events. Rapamycin may therefore have applications in the prevention and treatment of ischemic stroke.

Further high quality pre-clinical studies of rapamycin in stroke are needed before progressing to clinical trial. No studies in this review provided a sample size calculation. Only 8 of 17 studies conducted blinded outcome assessment with a trend toward increased effect size in blinded studies. The only co-morbidity reported in this review was hypertension (3 studies) with a trend towards lower efficacy in hypertensive animals. Rigorous multi-centre preclinical trials have been used previously to assess candidate preclinical drugs before progression to clinical trial (Interleukin-1 Receptor Antagonist and anti-CD49d antibodies). The design of such a trial of rapamycin, learning from the shortcomings of previous preclinical studies of rapamycin, will improve the overall study quality, improve our confidence in taking this therapy forward to clinical trials and ultimately increase the translational impact of preclinical rapamycin stroke research.

Systematic reviews and meta-analyses have limitations. Negative studies are less likely to be published overestimating efficacy. Previous meta-analyses have shown that average neuroprotective efficacy is ~31%, which is often adjusted to an actual estimate of efficacy of around 20%, accounting for publication bias. Interestingly, the effect size of rapamycin was a 21% reduction in infarct volume, with trim and fill analysis suggesting no evidence of publication bias in studies identified. It must be noted that the small number of studies in this analysis means that there may be limited power to detect subgroup effects and publication bias. However, the lack of publication bias seems in line with the general observation that 4 comparisons showed that rapamycin was not neuroprotective, making infarct volume worse, yet these studies were still published. Our analysis has revealed that 3 of these 4 studies utilized supra-therapeutic doses of rapamycin (5-40mg/kg), the most likely explanation for exacerbated injury seen in these studies.

In conclusion rapamycin is neuroprotective in animal models of ischemic stroke. Reduced infarct volumes were highly correlated with improvements in neurological outcome. Clinically relevant lower doses of rapamycin showed the greatest efficacy in reducing infarct volume. The modest quality of the studies and large heterogeneity between
studies means that further high quality (multicenter randomized) preclinical trials are required to determine the suitability of rapamycin as a treatment for human stroke.

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Conflicts of Interest

AMB is a senior medical science advisor and co-founder of Brainomix, a company that develops electronic ASPECTS (e-ASPECTS), an automated method to evaluate ASPECTS in stroke patients. All other authors declare no conflict of interest.

References


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**Figure Legends.**

**Figure 1.** Efficacy of rapamycin treatment on infarct volume and behavioral scores. Point estimate and 95% CIs for global estimate of (A) infarct volume for each of 30 comparisons and (B) neurobehavioral score for each of 22 comparisons. Effect size is improvement in treated animals expressed as a proportion of outcome in control animals. The size of the filled square for each study indicates the weighting in the global estimate. Diamond and dashed vertical line indicate the global estimate. Solid vertical line represents where treatment and control are equal. (C) Linear regression of infarct volume versus neurobehavioral score. Broken horizontal line represents where the treatment and control are equal for infarct volume. Broken vertical line represents where the treatment and control are equal for neurobehavioral score.

**Figure 2.** Meta-regression of the effect of rapamycin dose on outcome. (A) Meta-regression of infarct volume effect size plotted against standardized dose of rapamycin. Dose of rapamycin accounted for a significant portion of the
heterogeneity of infarct volume effect size. Lower doses of rapamycin having a greater reduction in infarct volume. 

(B) Due to the clustering of studies using doses of rapamycin between 0 – 1 mg/kg, infarct volume was plotted against the log standardized dose of rapamycin, where the association is more apparent. Weighting of studies represented by size of the circles.

**Figure 3.** Quality score and publication bias. (A) Point estimates of infarct volume effect size by reported study quality score (adapted from CAMARADES checklist). Weighting of each study for meta-regression is indicated by the size of the circle. Study quality score did not significantly account for effect size. (B) Filled funnel plot of a trim and fill analysis of point estimates of infarct volume effect size to determine any publication bias. Global estimate of effect size as well as pseudo 95% confidence limits are presented. There is no evidence of publication bias.
Tables.

Table 1. Design characteristics of included studies.

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<th>Publications</th>
<th>Year</th>
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<th>N (C)</th>
<th>N (Rx)</th>
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<th>Species</th>
<th>Time to treatment</th>
<th>Anesthetic</th>
<th>Temp/Perm</th>
<th>Route of Delivery</th>
<th>Outcome</th>
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Number of animals in control group (n(C)); number of animals in experimental group (n(Rx)); dose range; species; interval from onset of ischemia to start of treatment; anaesthetic used; permanent or temporary ischemia; route of drug delivery and outcomes measured. N.K, not known; i.v., intravenous; i.c., intracerebral; i.c.v., intracerebroventricular; i.p., intraperitoneal; o., oral, Infarct, infarct volume measurement; behaviour, neurobehavioral outcome; BBB, blood brain barrier permeability, CBF, cerebral blood flow