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A systematic review and meta-analysis of erythropoietin in experimental stroke

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Erythropoietin (EPO) has shown promise as a neuroprotectant in animal models of ischemic stroke. EPO is thought not only to protect neurons from cell death, but also to promote regeneration after stroke. Here, we report a systematic review and meta-analysis of the efficacy of EPO in animal models of focal cerebral ischemia. Primary outcomes were infarct size and neurobehavioral outcome. Nineteen studies involving 346 animals for infarct size and 425 animals for neurobehavioral outcome met our inclusion criteria. Erythropoietin improved infarct size by 30.0% (95% CI: 21.3 to 38.8) and neurobehavioral outcome by 39.8% (33.7 to 45.9). Studies that randomized to treatment group or that blinded assessment of outcome showed lower efficacy. Erythropoietin was tested in animals with hypertension in no studies reporting infarct size and in 7.5% of the animals reporting neurobehavioral outcome. These findings show efficacy for EPO in experimental stroke, but when the impact of common sources of bias are considered, this efficacy falls, suggesting we may be overestimating its potential benefit. As common human co-morbidities may reduce therapeutic efficacy, broader testing to delineate the range of circumstances in which EPO works best would be beneficial.

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Keywords: animal studies; experimental; focal ischemia; neuroprotection; neuroregeneration; trophic factors

Introduction

Of hundreds of interventions that improve outcome in experimental stroke, only recombinant tissue plasminogen activator has found a place in routine clinical practice (O'Collins *et al*, 2006). This discrepancy between animal and human studies might be due to bias in the conduct or reporting of animal experiments leading to an overstatement of neuroprotective efficacy; to a failure of clinical trials to test interventions under circumstances where efficacy has been observed in animals; or to a failure of animal studies to model human diseases with sufficient fidelity. Systematic review and meta-analyses are techniques that allow an assessment

of the likelihood that research conclusions are distorted by bias, and a clear description of the circumstances under which efficacy has been observed in animals.

Erythropoietin is a hematopoietic growth factor used clinically to treat anemia, which has shown promise as an acute treatment in *in vitro* and *in vivo* studies in cerebral ischemia (Ruscher *et al*, 2002; Sinor and Greenberg, 2000; Siren *et al*, 2001). The mechanisms of this reported protection are not clear, but may include activation of endogenous survival pathways that inhibit apoptosis (Fliser *et al*, 2006) and decrease inflammatory responses (Villa *et al*, 2003). Erythropoietin has also been shown to support regeneration by recruiting stem cells and stimulating neoangiogenesis (Brines and Cerami, 2008). To date, the single published clinical trial of EPO in acute ischemic stroke showed limited success in its results (Ehrenreich *et al*, 2002), the Multicenter-Erythropoietin-Stroke Trial is awaiting analysis and the REGENESIS study (of EPO with recombinant human chorionic gonadotrophin) is in follow-up. The benefits of EPO in human stroke are therefore not known at the present time.

Here, we report a systematic review and meta-analysis of the use of EPO in experimental stroke.

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Our aim was to investigate whether elements of study design or quality have a significant impact on outcome. These data may assist the development of further preclinical hypothesis to test in animals and in the design of future large-scale clinical trials.

Materials and methods

Systematic Review

We searched three electronic databases (Pubmed, ISI Web of Science, and Embase, in July 2009) using the terms (EPO or erythropoietin) and (stroke or ischemia or ischaemia or cerebrovascular or middle cerebral artery or MCA or MCAO or ACA or ACAO or anterior cerebral artery), limiting results to animals.

Two investigators (MJ, KF) assessed the titles and abstracts of studies and obtained copies of articles that described controlled studies of EPO or EPO analogues in animal models of focal cerebral ischemia and that measured outcome as infarct size or neurobehavioral outcome. The latter was defined to include all methods measuring neurobehavioral outcome or functional behavior where a baseline of normal or pre-stroke function could be clearly established. Articles in foreign languages were translated. Reference lists of the articles were assessed to locate additional studies not identified in the initial search.

Data Extraction

From included studies, we extracted data on study design including method of ischemia induction, species, dose, time and route of administration, and time of assessment of outcome. The number of animals used, mean and variance of treatment and control groups were also extracted. Where data required for meta-analysis were missing, we contacted authors to request additional information. If data were only expressed graphically, values were requested from the authors, and where a response was not received, we measured data from the graphs using digital ruler software. Where data required for meta-analysis were not presented or obtainable, the studies were excluded from the analysis.

Study quality was assessed based on the CAMARADES study quality checklist (Macleod *et al*, 2004) comprising (1) publication in a peer-reviewed journal, (2) statement of control of temperature, (3) statement of monitoring of physiologic parameters, (4) randomization to treatment or control, (5) blinded induction of ischemia, (6) blinded assessment of outcome, (7) anesthetic without marked intrinsic neuroprotective properties, (8) use of co-morbid animals (9) sample size calculation, (10) statement of compliance with animal welfare regulations, and (11) statement regarding possible conflicts of interest.

Statistical Analysis

A random effects weighted mean difference meta-analysis was used for statistical analysis (DerSimonian and Laird, 1986). The data were stratified according to elements of

study quality and aspects of study design to assess their impact on efficacy. We assessed the impact of continuous variables (dose, time of administration, and assessment) on the efficacy of EPO using meta-regression. To allow for multiple comparisons, we set our significance level using Bonferroni correction; $P < 0.003$ for both infarct volume and neurobehavioral outcome.

Results

Our search identified 1565 unique publications of which 19 met our inclusion criteria and were included in this analysis (Tables 1–3). One publication was excluded from the analysis because the results were not presented in figures or graphs and another publication was excluded because the number of animals used was not stated (Chang *et al*, 2008; Yu *et al*, 2005). In both circumstances, we were unable to obtain this information from the authors.

For treatment initiated after induction of ischemia, infarct volume was reported in 23 experiments using 346 animals and neurobehavioral outcome was reported in 30 experiments using 425 animals. For treatment initiated before induction of ischemia, infarct volume was reported in 6 experiments using 109 animals and neurobehavioral outcome was not studied.

Overall Efficacy and Impact of Study Design

Erythropoietin and EPO analogues improved infarct volume by 30.0% (95% CI: 21.3 to 38.8) and neurobehavioral outcome by 39.8% (33.7 to 45.9). There was substantial between-study heterogeneity for the analysis of both infarct volume ($\chi^2 = 63.5$, $df = 22$, $P < 0.003$) and neurobehavioral outcome ($\chi^2 = 67.5$, $df = 29$, $P < 0.003$).

For infarct volume, meta-regression suggested that efficacy was reduced by 0.90% (0.39 to 1.41, $P < 0.003$) per hour of delay in treatment, and efficacy was reduced by 2.41% (1.51 to 3.51, $P < 0.003$) per hour of later assessment. No relationship was identified for neurobehavioral outcome.

For infarct volume, efficacy was highest when EPO was administered once rather than multiple times (41.6% (32.9% to 50.2%), $\chi^2 = 33.9$, $df = 1$, $P < 0.003$; Figure 1A) and when the method of middle cerebral artery occlusion was not stated (48.0% (41.3% to 54.6%), $\chi^2 = 35.4$, $df = 5$, $P < 0.003$; Figure 1B).

For neurobehavioral outcome, efficacy was highest with continuous infusion of EPO rather than single or multiple injections (55.3% (21.1% to 89.4%), $\chi^2 = 26.7$, $df = 2$, $P < 0.003$; Figure 1C); when the method of middle cerebral artery occlusion was not stated (68.8% (26.8% to 110.9%), $\chi^2 = 28.9$, $df = 5$, $P < 0.003$; Figure 1D) and in permanent rather than temporary or thromboembolic models of ischemia (49.1% (41.0% to 57.2%) $\chi^2 = 18.0$, $df = 2$, $P < 0.003$; Figure 1E).

Table 1 Studies describing the use of EPO administered after induction of focal cerebral ischemia

Author, year of publication	Drug	Species	Stroke model	Method of administration	Outcome measures, n (treated/control)	Quality score
Sadamoto et al (1998)	EPO	Rat	Permanent MCAO	Immediately after occlusion, intracerebroventricular infusion, 0.73, 3.64, or 18.18 U/kg daily for 28 days	Morris water maze (24/8)	6
Brines et al (2000)	EPO	Rat	Permanent MCAO	Immediately, 3, 6, or 9 h after occlusion, intraperitoneal injection, 5,000 U/kg	Infarct volume (32/9)	4
Dahlberg et al (2004)	EPO	Rat	MCAO (2 h)	5 mins after occlusion, intraperitoneal injection, 5,000 U/kg	Infarct volume (10/10)	3
Wang et al (2004)	EPO	Rat	Embolic MCAO	24 h after occlusion, intraperitoneal injection, 5,000 or 10,000 U/kg	Infarct volume (16/8) Foot Fault test (28/14) Corner test (28/14)	3
Faure et al (2006)	EPO	Gerbil	Permanent CCAO	2 and 48 h after occlusion, intraperitoneal injection, 5,000 U/kg	Morris water maze (19/6) Object recognition (19/6)	5
Aluclu et al (2007)	EPO	Rat	MCAO (2 h)	2 h after occlusion, intraperitoneal injection, 5,000 U/kg	Neurological score (15/15)	3
Kolb et al (2007)	EPO	Rat	Permanent focal devascularization	72 h after occlusion, intracerebroventricular infusion for 7 days, total 927 U/kg	Cylinder test (6/15) Swimming task (6/15) Tray reaching test (6/15)	3
Villa et al (2007)	EPO	Rat	Permanent MCAO	3 h after occlusion, intravenous injection, 5,000 U/kg	De Ryck somatosensory score (7/7) Foot-fault test (7/7)	3
Wang et al (2007)	EPO	Rat	Embolic MCAO	6, 24, and 48 h after occlusion, intravenous injection, 50, 500, 1150, or 5,000 U/kg	Infarct volume (36/12) Foot-fault test (40/10)	4
Esneault et al (2008)	EPO	Rat	MCAO (1.5 h)	24 and 48 h after occlusion, intraperitoneal injection, 1,000 U/kg per injection	Infarct volume (10/12) Neurological score (10/12) Limb placing (10/12)	5
Belayev et al (2009)	EPO	Rat	MCAO (1.5 h)	6, 7, and 8 days after occlusion, intravenous injection, 1,440 U/kg per injection	Infarct volume (12/13) Neurological score (12/13)	7
Fletcher et al (2009)	EPO	Mouse	MCAO (1 h)	Immediately after occlusion, intranasal, 100 U	Infarct volume (16/16) Neurological score (16/16)	6
Li et al (2009)	EPO	Rat	Embolic MCAO	24 h after occlusion and then daily for 7 days, intraperitoneal injection, 5,000 U/kg	Infarct volume (11/7) Neurological score (11/7)	5

A separate meta-analysis of the efficacy of the eight experiments using EPO analogues only was also performed (Table 2). Infarct volume was reported in 8 experiments using 116 animals and neurobehavioral outcome was reported in 8 experiments using 114 animals. Erythropoietin analogues improved infarct volume by 45.4% (38.7 to 52.2, $\chi^2=8.11$, $df=7$, $P=ns$) and neurobehavioral outcome by 49.4% (40.5 to 58.3, $\chi^2=11.0$, $df=7$, $P=ns$).

For treatment initiated before induction of ischemia, EPO improved infarct volume by 33.8% (18.2 to 49.5, $\chi^2=19.5$ $df=5$, $P<0.003$). Efficacy was highest when EPO was administered once rather than multiple times (50.6% (35.7 to 65.4), $\chi^2=9.5$, $df=1$, $P<0.003$).

Impact of Study Quality

Overall, studies had a median quality score of 4 out of 11 for both outcomes (interquartile range 3 to 5). Study quality accounted for a significant amount of between-study heterogeneity; for infarct volume, studies that reported random allocation to group

were associated with lower estimates of efficacy than those that did not (18.7% (10.3 to 27.1, $n=146$ animals) versus 36.4% (25.6% to 47.2%, $n=195$), $\chi^2=19.7$, $df=1$, $P<0.003$; Figure 2A). Neurobehavioral outcome studies that reported blinded assessment of outcome were associated with lower estimates of efficacy (30.8% (23.7% to 38.0%, $n=211$) versus 47.3% (40.3% to 54.4%, $n=186$), $\chi^2=24.3$, $df=1$, $P<0.003$; Figure 2B). Hypertensive animals were only used in studies assessing neurobehavioral outcome (32 animals out of 425), and there was no difference in efficacy between these groups. Significant differences between high and low quality studies were observed, with low quality studies reporting the highest efficacy for both outcomes ($P<0.003$; Figures 3A and 3B).

Discussion

Our analysis of 18 publications identified a significant improvement in experimental stroke after EPO

Table 2 Studies describing the use of EPO analogues administered after induction of focal cerebral ischemia

Author, year of publication	Drug	Species	Stroke model	Method of administration	Outcome measures, n (treated/control)	Quality score
Erbayraktar <i>et al</i> (2003)	Asialo EPO	Rat	MCAO (1.5 h)	1.5 h after occlusion, intravenous injection, 44 µg/kg	Infarct volume (6/6)	4
Leist <i>et al</i> (2004)	Carbamylated EPO	Rat	MCAO (1 h)	1 or 4 h after occlusion, intravenous injection, 5 or 50 µg/kg	Infarct volume (8/16 and 8/8)	1
Belayev <i>et al</i> (2005)	Darepoetin alfa	Rat	MCAO (2 h)	2 h after occlusion, intraperitoneal injection, 10 µg/kg	Infarct volume (16/13) Limb placing (16/13)	7
Villa <i>et al</i> (2007)	Caranesp	Rat	Permanent MCAO	1 h after occlusion, intravenous injection, 50 µg/kg	Infarct volume (9/9) De Ryck somatosensory score (9/9)	3
	Carbamylated EPO	Rat	Permanent MCAO	1 h after occlusion, intravenous injection, 50 µg/kg	Infarct volume (8/9) De Ryck somatosensory score (8/9)	3
	Carbamylated EPO	Rat	Permanent MCAO	3 or 3, 24, and 48 h after occlusion, intravenous injection, 50 µg/kg per injection	De Ryck somatosensory score (5/7 and 5/7) Foot fault (5/7 and 5/7)	3
	Carbamylated EPO	Rat	Permanent MCAO	24 and 48 h after occlusion, intravenous injection, 50 µg/kg per injection	De Ryck somatosensory score (5/5) Foot fault (5/5)	3
	EPO S-100E	Rat	Permanent MCAO	3 h after occlusion, intravenous injection, 50 µg/kg	De Ryck somatosensory score (8/8)	3

Table 3 Studies describing the use of EPO administered before induction of focal cerebral ischemia

Author, year of publication	Drug	Species	Stroke model	Method of administration	Outcome measures, n (treated/control)	Quality score
Bernaudin <i>et al</i> (1999)	EPO	Mouse	MCAO (1.5 h)	24 h before occlusion, intracerebroventricular infusion, 50 U/kg	Infarct volume (8/9)	3
Brines <i>et al</i> (2000)	EPO	Rat	Permanent MCAO	24 h before occlusion, intraperitoneal injection, 5,000 U/kg	Infarct volume (8/9)	4
Dahlberg <i>et al</i> (2004)	EPO	Rat	MCAO (2 h)	Immediately before occlusion and then once daily for 7 days, subcutaneous injection, 5,000 U/kg	Infarct volume (11/10)	3
Li <i>et al</i> (2007)	EPO	Mouse	Permanent MCAO	0.5 h before occlusion and then once daily, intraperitoneal injection, 5,000 U/kg	Infarct volume (6/6)	5
Wakida <i>et al</i> (2007)	EPO	Mouse	Permanent MCAO	24 and 1 h before and immediately after occlusion, intraperitoneal injection, 30,000 U/kg per injection	Infarct volume (11/13 and 7/11)	5

treatment. However, these results must be interpreted with caution due to potential sources of bias, such as the lack of allocation concealment and randomization to group of some of the included studies.

Erythropoietin was most effective the earlier it was administered after ischemic stroke induction, although efficacy is still maintained up to treatment initiation of 24 h post-ischemia. This supports the

hypothesis that EPO not only has neuroprotective properties but may also support regeneration.

There are limited data using EPO in animals with co-morbidities associated with human stroke; only 7.5% of the animals in the neurobehavioral dataset were hypertensive, all of which were spontaneously hypertensive rats. The efficacy of EPO in the presence of such co-morbidities may have implications for the potential use of EPO in stroke patients.

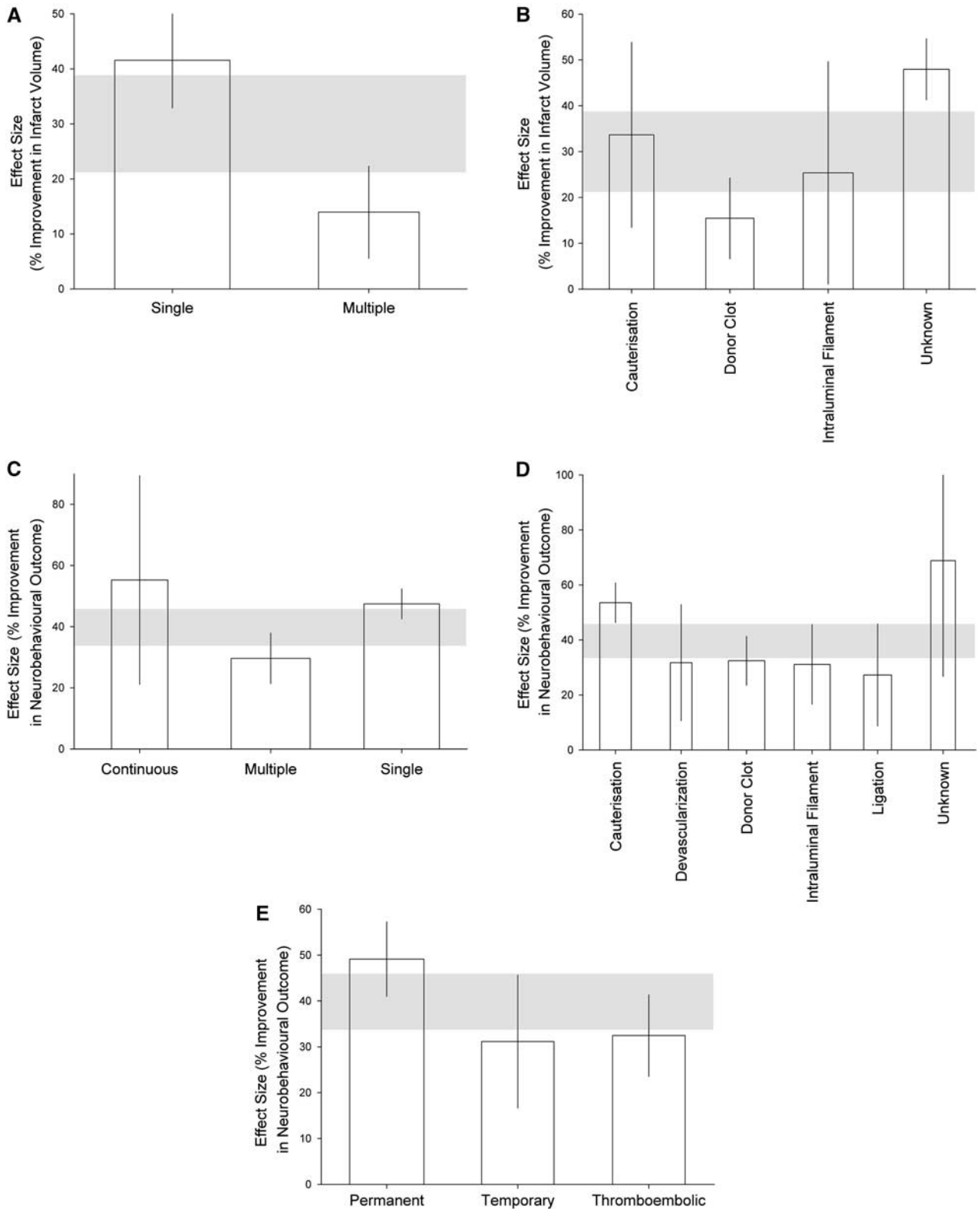


Figure 1 Effect on the estimate of efficacy for infarct volume of (A) type of delivery, (B) method of artery occlusion, and for neurobehavioral outcome of (C) type of delivery, (D) method of artery occlusion, and (E) model of ischemia, $P < 0.003$. The shaded gray bar represents the 95% confidence limits of the global estimate. The vertical error bars represent the 95% confidence intervals for the individual estimates. The width of each bar reflects the log of the number of animals contributing to that comparison.

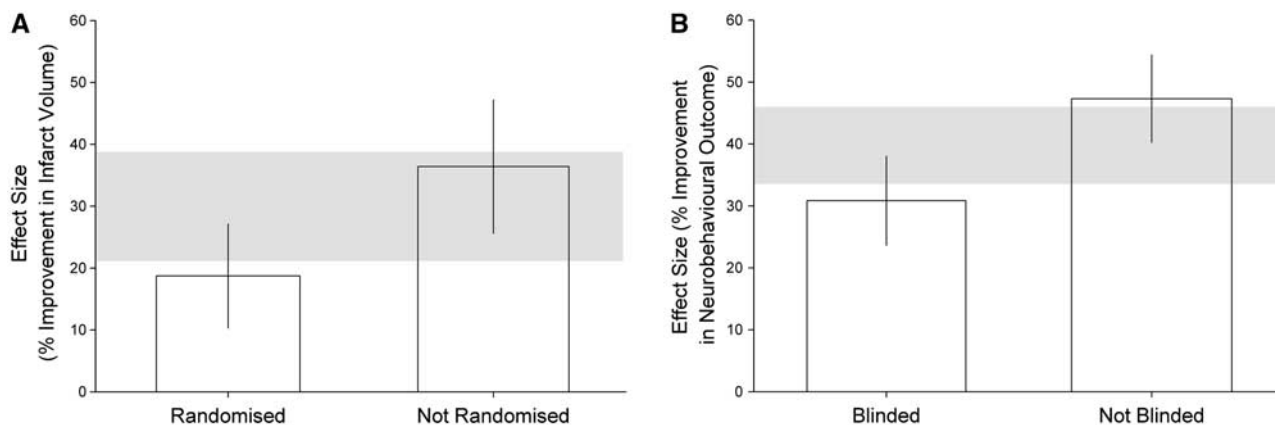


Figure 2 Effect on the estimate of efficacy for infarct volume of (A) random allocation to treatment or control group, and for neurobehavioural outcome of (B) blinded assessment of outcome, $P < 0.003$.

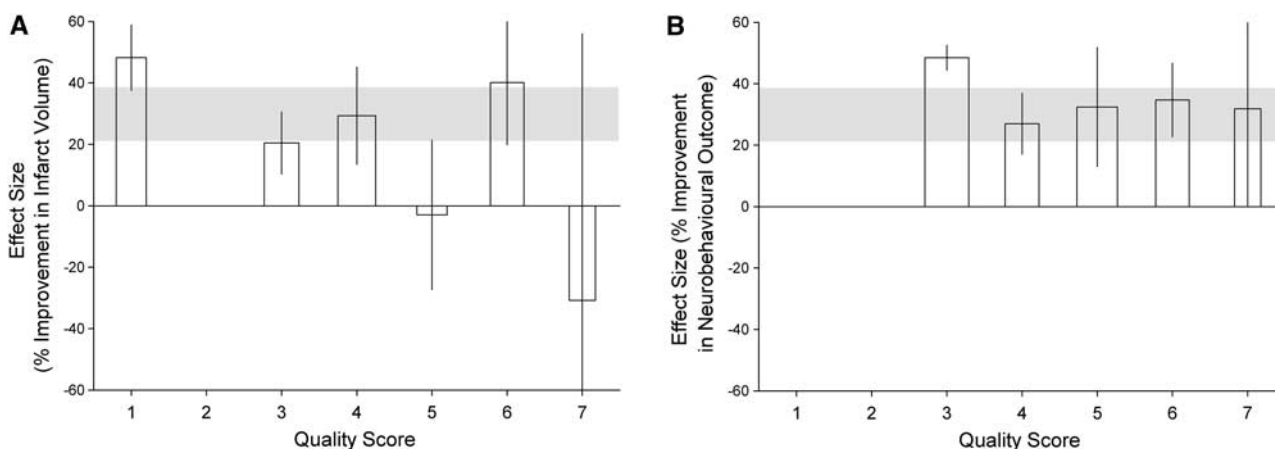


Figure 3 Effect on the estimate of efficacy of quality score (1 = worst, 11 = best) for (A) infarct volume and (B) neurobehavioral outcome, $P < 0.003$.

This review is subject to possible methodological weaknesses. First, the dataset is too small to reliably assess publication bias. In a review of seven different growth factors including EPO (unpublished data), publication bias was identified using a funnel plot and Egger regression, and it is reasonable to expect that this bias extends to these data. Second, analysis can only include data described in the identified articles. This might relate not only to the reporting of items described in the CAMARADES quality checklist (although recent guidelines now recommend this (Macleod *et al*, 2009), but also incomplete presentation of data, for instance of efficacy at later time points or at lower doses.

This study is a comprehensive systematic review of the use of EPO therapies in experimental stroke. The only clinical trial published to date reported that EPO did not significantly affect stroke mortality, although it did report improvements in functional outcome on the Barthel Index and beneficial trends on the NIH and Scandinavian stroke scales as well as on infarct size as a secondary outcome. The limited animal and human data on the safety and efficacy of

EPO in ischemic stroke suggests further testing of EPO in experimental models is warranted before further clinical trials. Elucidating the conditions in which the most benefit is seen in studies with minimal potential sources of bias may provide potential hypotheses to test in clinical trials.

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Conflict of interest

The authors declare no conflict of interest.

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