

## A systematic review of nitric oxide donors and L-arginine in experimental stroke; effects on infarct size and cerebral blood flow

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### Abstract

**Background:** Nitric oxide (NO) is a candidate treatment for acute ischaemic stroke, however published studies in experimental stroke have given conflicting results.

**Methods:** We performed a systematic review of published controlled studies of L-arginine (the precursor for NO) and NO donors in experimental stroke. Data were analysed using the Cochrane Collaboration Review Manager software. Standardised mean difference (SMD) and 95% confidence intervals (95% CI) were calculated.

**Results:** Altogether, 25 studies(s) were identified. L-Arginine and NO donors reduced total cerebral infarct volume in permanent (SMD  $-1.21$ , 95% CI  $-1.69$  to  $-0.73$ ,  $p < 0.01$ ,  $s = 10$ ) and transient models of ischaemia (SMD  $-0.78$ , 95% CI  $-1.21$  to  $-0.35$ ,  $p < 0.01$ ,  $s = 7$ ). Drug administration increased cortical CBF in permanent (SMD  $+0.86$ , 95% CI  $0.52$ – $1.21$ ,  $p < 0.01$ ,  $s = 8$ ) but not transient models (SMD  $+0.34$ , 95% CI  $-0.02$  to  $0.70$ ,  $p = 0.07$ ,  $s = 4$ ).

**Conclusions:** Administration of NO in experimental stroke reduces stroke lesion volume in permanent and transient models. This may be mediated, in part, by increased cerebral perfusion in permanent models. These data support clinical trials in stroke patients, although the presence of a narrow therapeutic time window may be a limiting factor.

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**Keywords:** Nitric oxide donors; Stroke; Animal models; L-Arginine

Nitric oxide (NO) is a multimodal endogenous mediator that is synthesised from its precursor L-arginine by the action of nitric oxide synthase (NOS) [1]. It is implicated in the pathophysiology of acute ischaemic stroke, although its precise role remains to be determined [2,3]. In stroke, NO produced by the neuronal and inducible isoforms of NOS (nNOS, iNOS) can be neurotoxic [4,5], partly as a consequence of the formation of peroxynitrite, a free radical, which leads to direct damage to mitochondrial enzymes and DNA [6,7]. In contrast, NO produced by the endothelial isoform of NOS (eNOS) is beneficial in acute stroke [8].

Endothelial-derived NO may limit neuronal damage through effects in vascular beds or within the brain itself [9]. In the intravascular space NO acts as a powerful vasodilator that can modulate blood flow. Also, NO inhibits leukocyte adhesion/migration to endothelium [10,11] and has antiplatelet effects [12]. In the brain NO may be neuroprotective through several mechanisms including: scavenging of reactive oxygen species [13–15], anti-inflammatory effects [10,11], and possibly through attenuation of NMDA receptors [16]. Furthermore, in experimental stroke exogenous NO limits metabolic derangement [17,18], reduces apoptosis [19], and stimulates neurogenesis [20]. Consequently, administration of NO has been considered to be a candidate treatment for acute stroke.

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Useful sources of NO for study in animal models are its essential amino acid substrate L-arginine and pharmacological donors. Exogenous L-arginine appears to increase NO levels partly via the NOS pathway, but also by the release of other vasoactive substances and arginase enzyme [21,22]. In contrast, NO donors are drugs that generate NO through mechanisms that are independent of NOS. Commonly used agents are the organic nitrates (e.g., glyceryl trinitrate, isosorbide dinitrate), sodium nitroprusside (SNP), sydnonimines (e.g., molsidomine, SIN-1), S-nitrosothiols (e.g., S-nitrosoglutathione), NONOates (e.g., SPERMINE-NONOate, DETA-NONOate), and hybrid donors (e.g., nitroaspirins, nicorandil). Pre-clinical studies of these agents have given variable results for effects on lesion size and cerebral blood flow (CBF) in animal models of cerebral ischaemia [23]. The aim of the present investigation was to determine systematically what effect NO donors have on these parameters.

## Methods

### Study identification

Experimental studies of L-arginine and NO donors were sought and included if they reported the effect on infarct volume or CBF in ischaemic stroke models (transient or permanent, global or focal). Systematic searches of 'Pubmed,' 'EMBASE,' and 'Web of Science' were made by MW for articles published from 1980 to 2002. The search strategy employed four primary keywords (nitric oxide, cerebro\*, brain, and ischaemia) combined with a fifth word chosen from a list of NO donors and L-arginine, and limited to animal studies. Additional articles were identified from previous non-systematic reviews [2] and reference lists by CG, SM, and PB. On the basis of title and abstract, articles of interest were selected for a review of the full publication by MW. Decisions on inclusion or exclusion were then made by MW and PB. Exclusion criteria were: non-stroke model, NO donor not administered, outcomes other than infarct volume or CBF, no control group, insufficient data given, or duplicate publication.

### Data extraction

Data were extracted on infarct volume and CBF from the selected articles. Infarct volume was recorded in cubic millimetre or as a percentage of normal brain. Measurements from the longest period of follow up were used. CBF was recorded as millilitre per minute per gram or percentage of baseline readings or baseline control. In permanent models, the CBF measurement closest to one hour after onset of occlusion was used.

For transient models CBF measurements were used after one hour of reperfusion. Where articles gave infarct volume or CBF readings for different brain regions the data were classified as sub-cortical, cortical, or total. If the location was not specified then it was assumed to be total brain. When studies used multiple groups of animals to assess dose–response relationships or optimal timing of administration, the data from each group were individually extracted for separate analysis. If the number of animals in a specific group was given as a range then the lowest figure quoted was used. Occasionally, numerical data were not available in print and it was necessary to extract data directly from enlarged, photocopied graphs. All discrepancies were resolved by PB. Methodological quality of studies was assessed according to published recommendations [24] using an 8 point 'STAIR' rating scale [25]. One point was given for written evidence of each of the following factors; (i) presence of randomisation; (ii) monitoring of physiological parameters; (iii) assessment of dose response relationship; (iv) assessment of optimal time window; (v) blinded outcome measurement; (vi) assessment of outcome at days 1–3; (vii) assessment of outcome at days 7–30; and (viii) combined measurement of infarct volume and functional outcome.

### Analysis

Data were analysed using the Cochrane Collaboration Review Manager (RevMan version 4.1) software. Fig. 1 shows a typical forest plot and illustrates the layout of studies. Published recommendations suggest that candidate neuroprotectants should be effective in multiple brain regions, at different times of administration, and in permanent and transient stroke [24,26]. Hence, infarct volume and CBF data were grouped 'a priori' for analysis in several ways: (i) by experimental model—permanent or transient; (ii) by outcome location—total brain, cortex, sub-cortex; and (iii) by timing of administration—NO administered before (pre-treated), 0–1 h after (early treatment) or >1 h after (late treatment) onset. Results are given as standardised mean difference (SMD, which allows data measured on different scales to be merged) with 95% confidence intervals (CI). A random effects model was used since heterogeneity was likely to be present due to the use of different protocols, animal species, drugs, and administration timings. Statistical heterogeneity was assessed with a  $\chi^2$  test. The likely causes of heterogeneity were explored using subgroup analyses based on drug type (L-arginine, organic nitrates, SNP, sydnonimines, S-nitrosothiols, NONOates, and hybrid donors) and timing of administration. Publication bias was assessed using Egger's asymmetry test [27] (Stata function 'metabias') and visual assessment of a funnel plot. Significance was set at  $p < 0.05$ .

**Comparison: 14 Permanent, total  
Outcome: 01 Lesion volume**

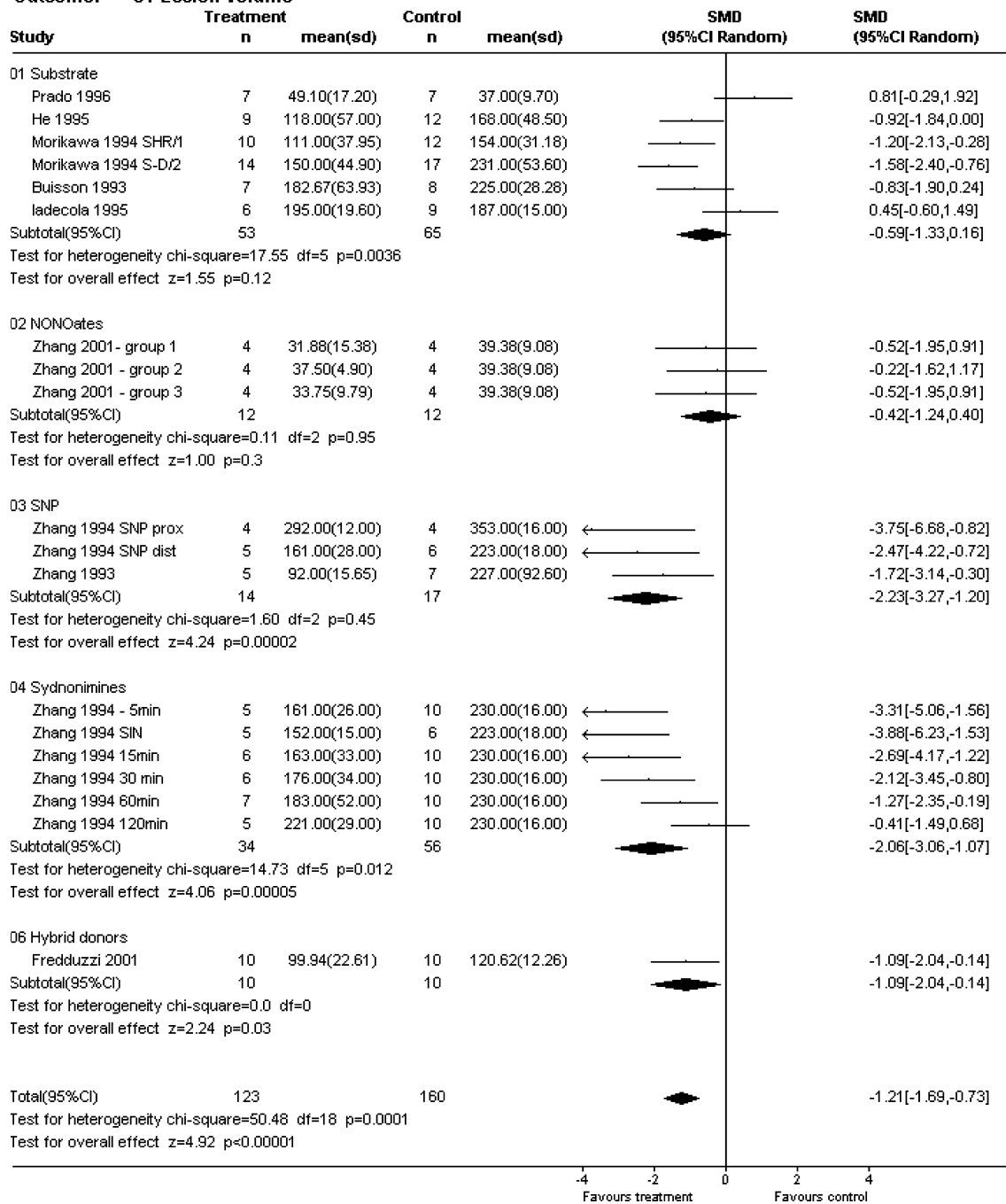


Fig. 1. Effect on total lesion volume by different NO donor classes for permanent models of ischaemia.

**Results**

*Design of studies*

The literature search identified 440 potential articles, although most of these were excluded (Fig. 2). The characteristics of the remaining 25 studies (median 41 animals) are given in Table 1. Most of these studied NO administration in permanent/focal ischaemia (15 articles). Transient models utilised both focal (6 articles) and global (4

articles) ischaemia. Rat species (Sprague–Dawley, Wistar, Spontaneously Hypertensive, and Long Evans) were used in 23 studies (Table 1), whereas only 1 chose a rabbit model [28] and 1 used both [14]. Methodological design was variable as far as drug administration was concerned. Several different routes (intra-venous, intra-arterial, and intra-peritoneal) were used at timings ranging from 18h before to 24h after induction of ischaemia. Likewise, dosage regimens differed between studies, e.g., L-arginine 3.0–300mg/kg, SNP 0.1–3.0mg/kg, and SIN-1 0.1–10.0mg/kg.

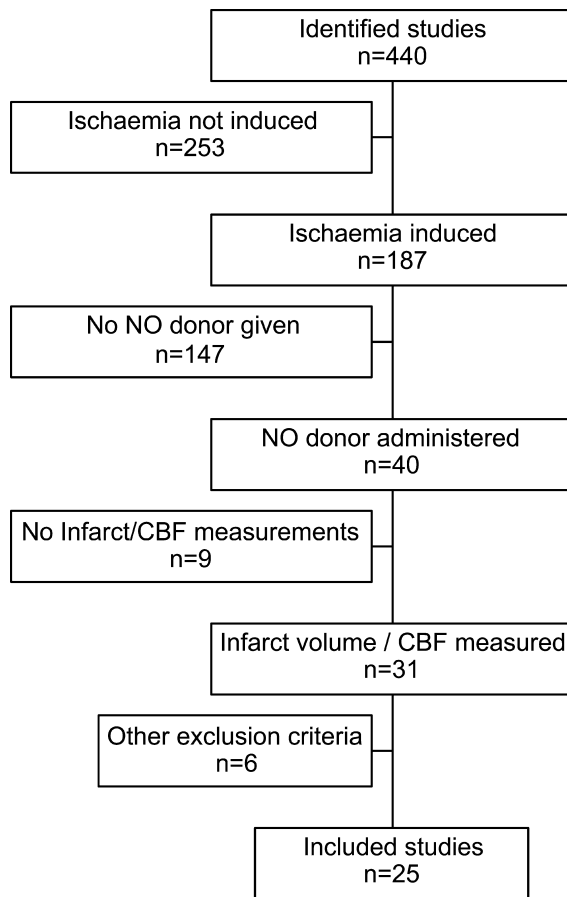


Fig. 2. Search process showing reasons for excluding studies.

In three studies [9,29,30], the hypotensive effect of high doses of SNP and SIN-1 were countered by co-administration of phenylephrine.

Study design was more consistent for outcome measures. Infarct volume was mostly measured in cubic millimetre, apart from two instances when it was given as percentage of total brain [31] or hemisphere [20]. Volumes were assessed by histological staining techniques and image analysis of sequential coronal brain sections in all studies. Regional CBF was commonly measured as percentage of baseline values in the cerebral cortex using laser doppler flowmetry [9,14,29–37]. In addition, several studies analysed CBF as millilitre per minute per gram using [<sup>14</sup>C]iodoantipyrine [38,39] or hydrogen clearance [17,18,28] techniques.

The median STAIR rating for the included articles was 2 points (range 1–7 out of 8). Animals were allocated treatment by randomisation in 8 articles [14,19,20,28,37,39–41]. In 9 studies [20,30–32,35,36,40–42], different dosage regimens were tested, but only 4 assessed the optimal timing window for administration [9,20,34,40]. Most studies examined outcome measures between days 1–3; only one did this blinded to treatment [14] whilst two looked at functional outcome as well as infarct volume [20,40].

### Infarct volume and CBF

Overall, NO administration resulted in significant reductions in total, cortical, and sub-cortical infarct volume in permanent models of ischaemia (Table 2). NO administration also reduced total infarct volume in transient models; data were limited to single studies for cortical and sub-cortical stroke. At approximately one hour of permanent occlusion CBF was significantly increased by NO donors in the cerebral cortex (Table 2). There was no significant effect at 1 h of reperfusion in transient stroke. Data were lacking for other comparisons. Publication bias was present for studies reporting the effect of NO administration on lesion volume in permanent (Egger's test  $p = 0.02$ ), but not transient (Egger's test  $p = 0.39$ ) models. Heterogeneity was present in several of the analyses and so infarct volume and CBF were further examined by drug type (Table 3, Fig. 1). Likewise, the effects of different timings of administration were examined separately in permanent and transient models (Table 4).

### L-Arginine (NO substrate)

L-Arginine did not significantly alter lesion volume in both permanent and transient stroke. This was despite evidence of a beneficial effect on cortical CBF in permanent models.

### Sodium nitroprusside (SNP)

SNP reduced total infarct volume after permanent and transient stroke, although it did not appear to change cortical CBF.

### Organic nitrates

Glyceryl trinitrate (GTN) did not alter cortical CBF after permanent ischaemia, although this finding was based on one study. There were no studies examining the effect of GTN on infarct volume.

### NONOates

The NONOates significantly reduced total infarct volume in transient, but not permanent, models. There was a non-significant trend to increased cortical CBF following reperfusion in transient models.

### Sydnominines

The sydnominines were effective in both permanent and transient stroke models. In permanent models they reduced total infarct volume and increased cortical CBF. In transient models they significantly reduced total infarct volume.

### Hybrid donors

Total infarct volume was reduced in one study [19]. There were no data available for CBF outcomes.

Table 1  
Included studies

Drug	Studies	Species	STAIR rating	Total N	Model P/T	Occlusion G/F	1st dose timing (min)	Route	Measures	
									Infarct vol.	CBF
L-Arginine	Bednar [28]	NZR	2	27	P	F	+30	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
	Buisson [44]	SDR	2	31	P	F	-30	i.p	mm <sup>3</sup>	
	Dalkara [34]	SHR	2	28	P	F	+5, +15, +30	i.v		%
	Escott [40]	SDR	6	55	T	F	+5	i.p.	mm <sup>3</sup>	
	He [17]	SHR	2	43	P	F	-20	i.p	mm <sup>3</sup>	ml min <sup>-1</sup> g <sup>-1</sup>
	Humphreys [35]	SDR	2	63	T	G	-30	i.v		%
	Iadecola [33]	SHR	2	53	P	F	+1440	i.p	mm <sup>3</sup>	
	Morikawa [56]	SHR	2	60	P	F	-960	i.p	mm <sup>3</sup>	
	Morikawa [36]	SHR	2	36	P	F	+5	i.v		%
	Morikawa [32]	SHR, SDR	3	53	P	F	+5	i.v	mm <sup>3</sup>	%
	Prado [39]	SHR	3	31	P	F	-1080	i.p	mm <sup>3</sup>	ml min <sup>-1</sup> g <sup>-1</sup>
	Sadoshima [18]	SHR	1	34	T	G	+60	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
	Zhang [43]	SDR	1	71	T	F	+1440	i.p	mm <sup>3</sup>	
Zhao [37]	WR	2	12	T	G	-30	i.p		%	
GTN	Bednar [28]	NZR	3	27	P	F	+30, +150	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
SNP	Bednar [28]	NZR	3	27	P	F	+30, +150	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
	Chi [38]	LER	1	28	P	F	+40	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
	Salom [31]	WR	3	56	T	F	0	i.v	%	%
	Zhang [29]	SDR	2	31	P	F	+3	i.a	mm <sup>3</sup>	%
	Zhang [30]	SHR, SDR	3	74	P	F	+3	i.a	mm <sup>3</sup>	%
Sydnominimes	Chi [38]	LER	1	28	P	F	+40	i.v		ml min <sup>-1</sup> g <sup>-1</sup>
	Coert [41]	WR	3	92	T	F	-30	i.v	mm <sup>3</sup>	
	Coert [42]	WR	4	60	T	F	-30	i.v	mm <sup>3</sup>	
	Zhang [30]	SHR, SDR	3	74	P	F	+3	i.a	mm <sup>3</sup>	%
	Zhang [9]	SHR	2	39	P	F	+3, 15, 30, 60, 120	i.a	mm <sup>3</sup>	%
NONO-ates	Coert [41]	WR	3	92	T	F	-30	i.v	mm <sup>3</sup>	
	Mason [13]	WR	2	12	T	G	+5	i.v	mm <sup>3</sup>	
	Pluta [14]	SDR, NZR	2	41	T	F	+60	i.a	mm <sup>3</sup>	%
	Salom [31]	WR	3	56	T	F	0	i.v		%
	Zhang [20]	WR	7	?	P	F	+1440, 2880	i.p	%	
Hybrid donors	Fredduzzi [19]	SHR	2	40	P	F	+10	i.p	mm <sup>3</sup>	

Abbreviations: SHR, spontaneously hypertensive rat; SDR, Sprague–Dawley rat; WR, Wistar rat; LER, Long-Evans rat; NZR, New-Zealand rabbits; M, male; F, female; P, permanent; T, transient; G, global; F, focal; i.v., intra-venous; i.a., intra-arterial; i.p., intra-peritoneal; infarct vol., infarct volume; CBF, cerebral blood flow.

Table 2

Effect of NO donors on lesion volume and cerebral blood flow by brain region; data are standardised mean difference (95% confidence intervals)

Outcome	Permanent models			Transient models		
	Total	Cortical	Sub-cortical	Total	Cortical	Sub-cortical
Lesion volume	-1.21*	-1.40*	-0.55*	-0.78*	+2.35*	+0.54
	(-1.69, -0.73)	(-1.94, -0.86)	(-0.86, -0.24)	(-1.21, -0.35)	(0.97, 3.72)	(-0.49, 1.58)
	S = 10, n = 235	S = 6, n = 181	S = 5, n = 131	S = 7, n = 228	S = 1, n = 16	S = 1, n = 16
Cerebral blood flow	+0.66	+0.86*	+0.48	No data	+0.34	+0.64
	(-0.02, 1.35)	(0.52, 1.21)	(-0.18, 1.14)		(-0.02, 0.70)	(-0.18, 1.47)
	S = 1, n = 24	S = 8, n = 220	S = 3, n = 42		S = 4, n = 108	S = 1, n = 24

Abbreviations: S, number of studies; n, number of animals.

\* p < 0.05.

### Timing of treatment

Pre-treatment with NO from any source reduced infarct volume in transient but not permanent models of ischaemia. Early administration of NO

from 0 to 1 h of onset was equally effective in both transient and permanent stroke. Later treatment with NO had no overall beneficial effect on infarct volume in either transient or permanent stroke.

Table 3

Effect of different nitric oxide donors infarct volume and cerebral blood flow; data are standardised mean difference (95% confidence intervals)

	Permanent		Transient	
	Total infarct volume	Cortical CBF	Total infarct volume	Cortical CBF
L-Arginine	-0.59, (-1.33, 0.16) <i>S</i> = 5, <i>n</i> = 118	+0.82*, (0.42, 1.23) <i>S</i> = 4, <i>n</i> = 101	+0.96, (-2.15, 4.07) <i>S</i> = 2, <i>n</i> = 36	+0.52, (-0.16, 1.20) <i>S</i> = 2, <i>n</i> = 35
Sodium nitroprusside	-2.23*, (-3.27, -1.20) <i>S</i> = 2, <i>n</i> = 41	+0.60, (-0.44, 1.64) <i>S</i> = 4, <i>n</i> = 45	-1.14*, (-2.08, -0.20) <i>S</i> = 1, <i>n</i> = 25	-0.04, (-0.76, 0.67) <i>S</i> = 1, <i>n</i> = 22
Glyceryl trinitrate	No data	-0.35, (-1.60, 0.91) <i>S</i> = 1, <i>n</i> = 10	No data	No data
NONOates	-0.42, (-1.24, 0.40) <i>S</i> = 1, <i>n</i> = 16	No data	-0.79*, (-1.39, -0.20) <i>S</i> = 4, <i>n</i> = 83	+0.43, (-0.09, 1.28) <i>S</i> = 2, <i>n</i> = 51
Sydnonimines	-2.06*, (-3.06, -1.07) <i>S</i> = 2, <i>n</i> = 50	+1.26*, (0.56, 1.95) <i>S</i> = 3, <i>n</i> = 64	-1.03*, (-1.54, -0.52) <i>S</i> = 2, <i>n</i> = 84	No data
Hybrid donors	-1.09*, (-2.04, -0.14) <i>S</i> = 1, <i>n</i> = 20	No data	No data	No data

Abbreviations: *S*, number of studies; *n*, number of animals.\* *p* < 0.05.

Table 4

Effect of timing of administration (within 1 h versus &gt;1 h) on total infarct volume; data are standardised mean difference (95% confidence intervals)

	Timing		
	Pre-treatment	Early treatment	Late treatment
Permanent models	-0.08 (-1.78, 1.61) <i>S</i> = 2, <i>n</i> = 35	-1.77* (-2.23, -1.30) <i>S</i> = 6, <i>n</i> = 158	-0.17 (-0.73, 0.38) <i>S</i> = 3, <i>n</i> = 46
Transient models	-0.75* (-1.21, -0.29) <i>S</i> = 2, <i>n</i> = 102	-1.15* (-1.58, -0.73) <i>S</i> = 4, <i>n</i> = 86	+2.60* (1.15, 4.05) <i>S</i> = 1, <i>n</i> = 16

Abbreviations: *S*, number of studies; *n*, number of animals.\* *p* < 0.05.

## Discussion

This systematic review has assessed the effect of NO donors on lesion size and cerebral blood flow in experimental stroke models. With one exception [43] most of the individual studies were positive or neutral; when combined, NO significantly reduced infarct volume and improved CBF after permanent stroke. NO similarly reduced lesion volume in transient stroke. One article noted that NO-induced changes in CBF preceded electroencephalographic brain recovery [34]. Others found concomitant increases in pial vessel diameter [32,36,44]. Taken together these findings suggest that vascular effects of NO are important for limiting neuronal damage, perhaps because they lead to improved collateral blood flow in areas of compromised cerebral perfusion.

### Source of NO

In general, the NO donors (SNP, Sydnonimines, NONOates, and hybrid donors) were all effective at reducing lesion volume in transient stroke, permanent stroke, or both. An exception was L-arginine, which

increased cortical CBF after permanent occlusion but was otherwise ineffective. In addition, L-arginine significantly increased infarct volume in one article [43]. Several factors may account for these findings: L-arginine stimulates hormones such as insulin, glucagon, growth hormone, and catecholamines [45] and can be converted to toxic by-products such as polyamines [46] or agmatine [47]. Alternatively, L-arginine may enhance the synthesis of potentially detrimental NO from iNOS since it countered the neuroprotective effects of iNOS inhibitor in two studies [33,43]. Hence, L-arginine is probably not an agent of first choice for testing in clinical stroke.

### Timing of treatment

The timing of drug administration varied considerably from 18 h prior to 24 h after onset of ischaemia. Early treatment within 1 h of ischaemia was effective in both transient and permanent models of stroke whereas later treatment was ineffective. This biphasic response is can be explained by NO having neuroprotective activity. Further experimental studies with delayed NO administration are required in order to assess when the optimum time window closes.

### Types of models

Reductions in lesion volume were present in both permanent and transient models of stroke. No direct comparisons of efficacy in these different models have been published. However, indirect assessment using the data in this review suggest that NO donors may be less effective after transient ischaemia. Reductions in total lesion volume for transient models were about half those seen for permanent ischaemia. Also, whilst there was evidence of benefit on infarct volume in permanent cortical and sub-cortical stroke there was none in transient stroke. It is difficult to interpret the findings in transient models since focal and global models of transient ischaemia differ neuro-pathologically [48], and it was not possible to examine them separately because of lack of studies. Nevertheless, the most likely explanation is that since transient ischaemia was associated with less cerebral damage than seen with permanent models, there was a smaller lesion for NO donors to modify. Alternatively, the apparent differences may reflect chance, paucity of data and varied protocol design (e.g., timing of drug administration). Additionally, NO might induce reperfusion injury after transient ischaemia [49] although NO has been proposed as a treatment for reducing reperfusion injury after thrombolysis [14].

### Limitations

Although, this review has demonstrated an association between NO administration and reduction in infarct volume and improved CBF in experimental stroke, the findings are limited by several factors. First, there were considerable variations in animal species, physiological parameters (e.g., blood pressure), drug administration (timing, dosage, and route), surgical methodology, and duration of ischaemia between the studies, as highlighted above. Unfortunately, it is not possible to judge if the relationships we observed are independent of these factors. Inevitably, the variety of protocols will have made the results more heterogeneous and we allowed for this by using a random effects statistical model. Second, since Egger's asymmetry test suggested publication bias was likely, it is possible that our extensive search strategy did not identify all studies. Publication bias could have resulted from both the lack of reporting of some neutral or negative studies, and through commercial pressures not to publish positive studies involving patented drugs in development. Consequently, the benefits of NO on infarct volume and CBF might have been either over or underestimated.

Third, when numerical data were not available the information on volume and blood flow was extracted from published figures. This can be imprecise, although we enlarged graphs and two independent authors

extracted the data. Fourth, a few articles administered phenylephrine in order to counter hypotension induced by high doses of SNP and sydnonimines [9,30,50]. These studies are complex to assess, in part, because phenylephrine could itself be beneficial after stroke [51]. Fifth, the studies came from a relatively small number of research groups and involved a limited number of animals. Both of these factors could have influenced the outcome of the analysis. The extraction of multiple pieces of information from a limited number of sources further risks introducing bias into the review. Finally, it is worth noting that the median STAIR rating was only 2 out of a maximum of 8. Several key concerns exist when considering study quality: few studies stated that they used randomisation to treatment; only one study reported using a blinded observer to assess outcome [14]; and functional outcomes were only assessed in two studies [20,40]. Future studies evaluating neuroprotective agents in experimental stroke models should follow published recommendations on preclinical drug development [24].

In summary, administration of NO in experimental stroke is associated with a reduction in infarct size. This effect may be mediated, at least in part, through beneficial effects on CBF in areas of compromised perfusion. NO administration was also effective in multiple brain regions in permanent and, to a lesser extent, transient stroke. This would support the continued development of NO sources for treatment of human stroke, as has been started in phase II clinical trials [52,53]. However, the potentially narrow time window for efficacy with NO donors might be a limiting factor. The potential of NO administration in acute stroke will remain uncertain until the results of a large ongoing randomised controlled trial is available [54].

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