Nitric oxide synthase inhibitors in experimental stroke and their effects on infarct size and cerebral blood flow; a systematic review

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ABSTRACT

Gene knock-out studies in acute experimental stroke suggest that nitric oxide (NO) produced by the neuronal or inducible isoforms of nitric oxide synthase (nNOS, iNOS) is detrimental, whilst that derived from the endothelial isoform (eNOS) is beneficial. However, experimental studies with nitric oxide synthase inhibitors (NOS inhibitors) have given conflicting results.

Published controlled studies of NOS inhibitors in experimental stroke were identified from EMBASE, PubMed and reference lists. Data on the effect of NOS inhibition on lesion volume (mm^3, %) and cerebral blood flow (CBF, %, $ml.min^{-1}.g^{-1}$) were extracted and analysed using the Cochrane Review Manager software.

72 studies were identified. NOS inhibitors reduced total infarct volume in permanent (standardised mean difference, SMD -0.51, 95% confidence intervals, 95% CI -0.82, -0.20) and transient (SMD -1.01, 95% CI –1.29, -0.73) experimental stroke. Similar reductions in lesion volume were present in cortical and sub-cortical areas in both models. Cortical CBF was reduced in permanent (SMD -0.80, 95% CI –1.34, -0.27) but not transient stroke. When assessed by type of inhibitor, total lesion volume was reduced in permanent models by nNOS (SMD -1.36, 95% CI -0.67, -2.05) and iNOS (SMD -0.92, 95% CI –1.16, -0.69) inhibitors, but not by non-selective inhibitors. All types of NOS inhibitors reduced infarct volume in transient models.

Selective inhibitors of nNOS and iNOS appear to reduce lesion volume in both permanent and transient models of cerebral ischaemia. The lack of effect on lesion size by non-selective inhibitors could reflect co-inhibition of eNOS. NOS inhibition may have negative effects on CBF but further studies are required. Selective nNOS and iNOS inhibitors are candidate treatments for acute ischaemic stroke.

Key Words: Nitric oxide, nitric oxide synthase inhibitor, nitric oxide synthase, stroke, animal

Running title: Nitric oxide synthase inhibitors in stroke models

INTRODUCTION

Nitric oxide (NO) is synthesised from its precursor L-arginine by the action of nitric oxide synthase (NOS). NO is produced in the brain following the onset of cerebral ischaemia (Malinski et al 1993), although its precise role in the pathophysiology of stroke is unclear. Gene knockout studies have determined that NO derived from the endothelial isoform of NOS (eNOS) is beneficial in acute stroke (Huang et al. 1996). This may be due, in part, to antiplatelet effects (Welch and Loscalzo 1994) and preservation of cerebral blood flow (Iadecola et al. 1994). In contrast, NO produced by the neuronal and inducible isoforms of NOS (nNOS, iNOS) can be neurotoxic (Huang et al. 1994; Zhao et al. 2000). This probably occurs through NO induced formation of peroxynitrite (Beckman et al. 1990) and toxic free radicals leading to damage by lipid peroxidation (Radi et al. 1991). NO further potentiates damage by inhibiting enzymes needed for mitochondrial respiration (respiratory chain complex 1 and 2), glycolysis (GAPDH) and DNA replication (ribonucleotide reductase) (Dawson et al. 1992b; Dimmeler and Brune 1993; Garthwaite 1991; Nathan 1992). Moreover, NO has been reported to stimulate the release of the neurotransmitter glutamate and could contribute to excitotoxicity (Montague et al. 1994; Sorkin 1993). Consequently, inhibition of NO production has been considered to be a candidate treatment for acute stroke.

The first NOS inhibitors were the guanidino aminoacids, many of which act competitively at the NOS active site. Examples include, N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME, a methyl ester pro-drug that is activated to become L-NNA) and N^G-monomethyl-L-arginine (L-NMMA). Both L-NAME and L-NNA exhibit greater *in-vitro* potency than L-NMMA in inhibiting nNOS and eNOS versus iNOS. However, none of the guanidino aminoacids discriminate sufficiently to enable them to be used to target a single NOS isoform. By contrast, some inhibitors possess higher affinity against one isoform and are commonly referred to as 'selective', although this term is used rather indiscriminately (Alderton et al. 2001). Agents that are used to target iNOS include: aminoguanidine, N^G-iminoethyl-L-lysine (L-NIL), N^G-iminoethyl-L-ornithine (L-NIO), the bis-isothioureas (PBITU) (Garvey et al. 1994), 1400W (N-[3-(aminomethyl)benzyl]acetamidine), GW273629 and GW274150 (Young et al. 2000). Other agents are used to target nNOS and include: 7-nitroindinazole (7-NI), tri(fluoromethylphenyl)imidazole (TRIM) (Handy et al. 1995), ARL 17477, AR-R18512 (Reif et al. 2000), BN 80933 (Chabrier et al. 1999), S-ethyl and S-methyl thiocitrulline and vinyl L-NIO. Recent *in-vitro* studies have suggested that in some cases the distinction between selective iNOS and selective nNOS inhibitor may not be straightforward. For example, aminoguanidine is only mildly selective against nNOS *in-vitro* (~5 fold) and probably affects other molecular targets (Alderton et al. 2001). Similarly, 7-NI has been found to be an equipotent inhibitor of all three isoforms of NOS at the isolated enzyme level (Alderton et al. 2001; Escott et al. 1998) although it has more selectivity for nNOS *in vivo*, possibly a consequence of cell specific effects (neuronal verses endothelial) (Alderton et al. 2001).

Pre-clinical studies of all of these agents have given variable results for effects on lesion size and cerebral blood flow (CBF) in animal models of cerebral ischaemia (Willmot and Bath 2003). Hence, the aim of the present investigation was to determine systematically what effect NOS inhibitors have on these parameters.

METHODS

Study identification

Experimental studies assessing the effects of NOS inhibitors on stroke lesion volume and cerebral blood flow (CBF) in ischaemic stroke models (transient or permanent, global or focal, any species) were identified. Searches were made of 'EMBASE' and 'Pubmed' by MW for articles published from 1980 - 2002. For the EMBASE search four primary keywords (nitric oxide, brain, ischaemia, non-human) were chosen combined with a fifth chosen from a list of NOS inhibitors. Different primary keywords were used in the Pubmed search (nitric oxide, cerebro*, ischaemia) which was then limited to animal studies. Other publications were found from reference lists and review articles by CG, SM and PB. Abstracts were then used to select relevant articles for an examination of the full publication by MW. Final decisions on inclusion or exclusion were made by MW and PB. Articles were excluded for several reasons, namely: not a stroke model, NOS inhibitor not administered, outcomes other than infarct volume or CBF, no control group, insufficient other data given, or duplicate publication.

Data Extraction

Infarct volume data (mm^3 or % of normal brain) and CBF data ($ml.min^{-1}.g^{-1}$ or % of baseline readings or baseline control) were extracted for analysis. Infarct volume measurements from the longest period of follow-up were used. CBF measurements after one hour of occlusion or reperfusion were used in permanent and transient stroke respectively. Where possible, regional infarct volume and CBF data were obtained separately for total brain, cortex and sub-cortex. In cases where region was not specified then the measurements were classified as total brain. If an article investigated dose response relationships or optimal timing of administration then data from each individual experimental condition were included separately. In cases where the number of animals in each experiment was given as a range then it was assumed to be the lowest figure. Where numerical values were not available, data were estimated directly from enlarged graphs using a ruler. All data extraction was done by two independent authors (MW, CG); discrepancies were resolved by PB. Finally, the methodological quality of the included articles was assessed on an 8 point scale (Horn et al. 2001) based on published recommendations for investigating new agents in experimental stroke (Stroke Therapy Academic Industry Roundtable (STAIR) 1999); one point was given for written evidence of each of the following factors; (i) 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 day 1-3; (vii) assessment of outcome at day 7-30 and; (viii) combined measurement of infarct volume and functional outcome.

Analysis

Study data were grouped by protocol prior to analysis: (i) experimental model - permanent or transient; (ii) outcome location - total brain, cortex, sub-cortex; (iii) outcome measure - infarct volume (mm^3, %), and CBF ($ml.min^{-1}.g^{-1}$, %). Data from each of these groups were analysed as forest plots using the Cochrane Collaboration Review Manager (RevMan version 4.1) software. Publication bias was assessed using Egger's asymmetry test (Egger et al. 1997) (Stata function 'metabias'). Results are given as standardised mean difference (SMD, reported in units of standard deviation), which allows data measured on different scales to be merged, and 95% confidence intervals (CI). A random effects model was used since statistical heterogeneity, assessed with a χ^2 test, was expected in view of the wide range of protocols. Sensitivity analyses were performed to look at likely sources of heterogeneity, including NOS inhibitor type (grouped as non-selective inhibitors, iNOS inhibitors, nNOS inhibitors), animal species and timing of administration. For the latter, articles were divided into those that administered NOS inhibitors before (pre-treated), 0 to 1 hour after (early treatment) or >1 hour after (late treatment) onset. Meta-regression (STATA, version 7.0) was used to analyse the relationship between timing of administration and the effect on

total infarct volume (SMD) in order to try and determine the size of the therapeutic window. Studies were weighted by sample size and only those that administered NOS inhibitors after onset of ischaemia were included. Significance was set at $P<0.05$.

RESULTS

Design of studies

Altogether 455 articles were found in the literature search (figure 1). Most of these did not qualify for the review, leaving 72 studies (s). Table 1 summarises the characteristics of the included articles. The most commonly used agents were non-selective inhibitors such as L-NAME (s=37) and L-NNA (s=15). In addition, several compounds were used to target nNOS, including: 7-NI (s=9), AR-R 17477 (s=4), BN 80933, PPBP and TRIM. In contrast, there were only two types of iNOS inhibitor represented: aminoguanidine (s=12) and 1400W. NOS inhibitors were administered to 23 permanent / focal models and to one permanent / global model. More articles assessed the effect of NOS inhibition in transient stroke (29 focal, 13 global and 2 using both). In addition, 2 studies were included which compared a combination of permanent and transient models (Buchan et al. 1994; Dawson et al. 1994). Rats were the animal models used in the majority of studies (24 Sprague Dawley, 21 Wistar, 7 spontaneously hypertensive, 1 Long Evans, 1 Lewis, 1 Fischer). Other studies used diverse species, including; rabbits (Anderson and Meyer 1996; Anderson and Meyer 2000), cats (Clavier et al. 1994; Nishikawa et al. 1993; Nishikawa et al. 1994), mice (Carreau et al. 1994; Ding-Zhou et al. 2002; Goyagi et al. 2001; Gursoy-Ozdemir et al. 2000; Kamii et al. 1996; Nowicki et al. 1991; Sugimoto and Iadecola 2002; Zhu et al. 2002), gerbils (Chabrier et al. 1999; O'Neill et al. 2000; Spatz et al. 1995), pigs (Greenberg et al. 1995; Hiramatsu et al. 1996; Schleien et al. 1998; Segawa et al. 1998), and lambs (Dorrepaal et al. 1997).

Variable methods of drug administration were utilised, e.g. different routes (oral, intra-venous, intra-ventricular, intra-arterial, intra-peritoneal), different timings (1^{st} dose ranging from 6 weeks before to 48 hours after induction of ischaemia) and different dosage regimens (e.g. total administered dose of L-NNA 0.06 – 40.0 mg/kg, L-NAME 0.1mg/kg – 4.2 g/kg, aminoguanidine 100 – 800 mg/kg, ARL-17477 1.0 – 10.0 mg/kg and 7-NI 0.1 – 100 mg/kg). Study design was more consistent for outcome measures. Infarct volumes were assessed by histological staining techniques and image analysis of sequential coronal brain sections in nearly all of the studies. An exception to this were 3 articles that used serial MRI techniques to monitor lesion progression (Cash et al. 2001; Quast et al. 1995; Wei and Quast 1998). Regional CBF after stroke onset was commonly measured as percentage (of baseline or control values) using laser doppler flowmetry (Batteur-Parmentier et al. 2000; Buchan et al. 1994; Goyagi et al. 2001; Greenberg et al. 1995; Gursoy-Ozdemir et al. 2000; Hashimoto et al. 1999; Humphreys and Koss 1998; Iadecola et al. 1996; Iadecola et al. 1995; Jiang et al. 1999; Prado et al. 1993; Sakashita et al. 1994; Santizo et al. 2000; Sugimura et al. 1998; Zhang and Iadecola 1993; Zhao et al. 1999). In addition, one article indirectly assessed total CBF using an ultrasonic flow transducer (Dorrepaal et al. 1997). Alternatively, several studies analysed CBF as $ml.min^{-1}.g^{-1}$ using ^{14}C-iodoantipyrine (Ashwal et al. 1994; Stagliano et al. 1997; Wei et al. 1994), hydrogen clearance (Matsui et al. 1997; Sadoshima et al. 1997; Segawa et al. 1998; Uetsuka et al. 2002), radiolabelled microsphere (Clavier et al. 1994; Hiramatsu et al. 1996; Nishikawa et al. 1993; Nishikawa et al. 1994; Schleien et al. 1998) or umbelliferone fluorescence (Anderson and Meyer 1996; Anderson and Meyer 2000) techniques.

The median quality rating for the included articles was 3 points (range 1-6/8). Treatment was allocated by randomisation in only 16 articles whilst 26 studies assessed dose response; just 13 studies assessed the optimal timing of administration. Most studies examined outcome measures between days 1-3; only 9 did this blinded to treatment whilst 11 looked at functional outcome as well as infarct volume.

Infarct volume and CBF

Collectively, NOS inhibitors caused a significant reduction in total, cortical and sub-cortical infarct volume of magnitude 0.5 to 1.2 standard deviations (table 2). Paradoxically, detrimental effects on CBF were observed in the cerebral cortex of permanent stroke models. Most CBF data came from

studies administering non-selective inhibitors. There was no evidence of publication bias for articles reporting the effect of NOS inhibition on total lesion volume in permanent (Egger's test p=0.33), or transient (Egger's test p=0.65) models. Since heterogeneity was observed in several of the analyses, infarct volume and CBF were further examined by type of NOS inhibitor (table 3), in different animal models (table 4) and by different timings of administration (table 5).

Type of NOS inhibitor

Non-selective inhibitors

In permanent stroke models non-selective inhibitors did not significantly alter infarct volume but did reduce cortical CBF (table 3). By contrast, after transient ischaemia non-selective inhibitors reduced total infarct volume and did not affect CBF (table 3).

nNOS inhibitors

nNOS inhibitors significantly reduced infarct volume in both permanent and transient stroke models (table 3). At 1 hour of reperfusion there was no overall effect on cortical CBF in transient models. However, reduced cortical CBF was seen in one small (n=8) study involving permanent ischaemia.

iNOS inhibitors

Aminoguanidine and 1400W significantly reduced infarct volume in both permanent and transient stroke models. No studies of selective iNOS inhibitors on CBF were identified.

Timing of treatment

Treatment before stroke onset was effective at reducing infarct volume in transient models (table 4) whilst early administration of NOS inhibitors (within 1 hour of onset) was effective in permanent stroke. Later treatment after 1 hour of onset had a beneficial effect on infarct volume in both types of stroke model. Meta-regression analysis found no evidence of a relationship between total infarct volume (SMD) and timing of administration in permanent (p=0.18) or transient (p=0.20) models (figure 3).

Animal Model

Overall, NOS inhibitors appeared to reduce total brain lesion size in agyrencephalic species (table 5) but not in rabbits or cats. Limited data were available on the effects of NOS inhibitors in higher animals.

DISCUSSION

We have examined systematically the effects of NOS-inhibitors on infarct size in experimental stroke models. Apart from 7 studies (Hamada et al. 1995; Kamii et al. 1996; Kuluz et al. 1993; Nakashima et al. 1999; Xu et al. 2000; Yamamoto et al. 1992; Zhang and Iadecola 1993), most of the individual articles were either positive or neutral for this outcome. However, when considered together there was an overall beneficial effect, such that NOS-inhibitors decreased lesion size by about 0.5 to 1.2 standard deviations. Mechanisms that may explain these findings include; reduced formation of peroxynitrite and reactive oxygen species (Caldwell et al. 1995; Gumuslu et al. 1997; Seif-el-Nasr and Fahim 2001; Solenski and Kwan 2000), inhibition of brain oedema (Peeters-Scholte et al. 2002; Quast et al. 1995), reduced vascular damage (Ding-Zhou et al. 2002; Gursoy-Ozdemir et al. 2000), and inhibition of apoptosis and necrosis (Charriaut-Marlangue et al. 1996; Peeters-Scholte et al. 2002). Additional work confirms that NOS inhibitors can increase hippocampal neuronal survival in experimental stroke (Caldwell et al. 1994; Chabrier et al. 1999; Jones et al. 1998; Kohno et al. 1995; Nanri et al. 1998; O'Neill et al. 1996; O'Neill et al. 1997; O'Neill et al. 2000) and improve functional outcome (Chabrier et al. 1999; Ding-Zhou et al. 2002; Gursoy-Ozdemir et al. 2000; Nagayama et al. 1998; Parmentier et al. 1999). However, not all published experimental studies agree with these findings, possibly because of differences in drug pharmacology, dosage, route of administration, timing of treatment, animal model and type of ischaemia. Some of these factors were examined further in this systematic review.

NOS inhibitor type

Non-selective inhibitors did not alter infarct volume in permanent ischaemia, whereas the selective nNOS and iNOS inhibitors reduced lesion size regardless of experimental model. It is likely that the beneficial effects of non-selective inhibitors were limited because they inhibit eNOS to a similar degree as nNOS or iNOS. Consequently, they may aggravate brain ischaemia by increasing platelet aggregation and white cell activity, raising blood pressure, and by restricting penumbral blood supply. Evidence of reduced CBF after administration of non-selective inhibitors to permanent stroke models is consistent with this hypothesis. Hence, the non-selective inhibitors are not agents of first choice for testing in clinical stroke.

Type of experimental model

Indirect assessment of the pooled data in table 2 suggests that NOS inhibitors were equally effective in transient and permanent ischaemia. However, sub-group analysis revealed that non-selective inhibitors did not work in permanent stroke but did in transient stroke (table 3). This discrepancy could be attributed to the presence of additional beneficial effects after transient ischaemia, such as limitation of reperfusion injury caused by eNOS-derived NO (Gursoy-Ozdemir et al. 2000). Alternatively, it could be due to the absence of detrimental effects of non-selective inhibitors in transient models. Consistent with this second theory is the observation that non-specific inhibitors had no detrimental effects on CBF at 1 hour of reperfusion (table 3).

Timing of treatment

The administration of 1st dose varied considerably from 6 weeks prior to 24 hours after onset of ischaemia. Treatment with NOS-inhibitors was effective prior to onset of transient ischaemia, within 1 hour of permanent ischaemia, and even beyond 1 hour of onset in both transient and permanent ischaemia models. The neutral findings seen with pre-treatment of permanent models and early treatment of transient models probably represents the use of non-selective agents and paucity of data rather than lack of response to NOS inhibitors. More important is the confirmation that these agents reduce infarct volume even when administered beyond 1 hour after stroke onset. If NOS-inhibitors are effective beyond the normal neuroprotective window of 2-4 hours, then it is likely that they work through other mechanisms, perhaps enhancing neurogenesis. Also, beneficial activity several hours after stroke suggests that the NOS inhibitors might be useful in clinical

stroke. Unfortunately, meta-regression analysis of timing of administration did not find any evidence of a therapeutic window. More experimental studies with delayed administration are required to assess when the optimum time window closes.

Animal model

Significant infarct reductions after either transient or permanent ischaemia were seen in all rat strains apart from Fischer rats. Only spontaneously hypertensive rats responded to NOS inhibitors in both transient and permanent models. This discrepancy could arise because normotensive strains (SDR, WR, Fischer) suffer smaller and more variable infarcts than SHR (Ginsberg and Busto 1989). In mice, NOS inhibitors worked in permanent but not transient stroke. This inconsistency is most likely due to lack of data, although these studies are complex to assess because different transgenic mouse strains were used. Unfortunately, lack of data prevents any definitive statements about the role of NOS inhibitors in rabbits and cats. More studies need to be performed in lissencephalic and/or gyrencephalic species before clinical trials are commenced, as per the STAIR recommendations (Stroke Therapy Academic Industry Roundtable (STAIR) 1999).

Limitations

Although this study has demonstrated an association between administration of NOS inhibitors and reduced infarct volume, the findings are limited by several factors. First, there were differences between study protocols in terms of animal species, physiological parameters (e.g. blood pressure), drug administration (dosage, route), surgical methodology, and duration of ischaemia. Unfortunately, it is not possible to judge whether the relationships we observed were independent of these factors. In addition, protocol variations can lead to statistical heterogeneity and make the analysis less reliable. To take account of this we used a random effects model and performed sensitivity analyses to identify sources of heterogeneity. Second, some relevant articles may not have been identified for inclusion in the review. Publication bias can contribute to this, either through of lack of reporting of neutral or negative studies or through suppression of positive studies for commercial reasons, e.g. intellectual property rights. This could mean the benefits of NO on infarct volume and CBF have been either over or underestimated. Statistical assessment using Egger's asymmetry test did not suggest the presence of publication bias but the possibility that some relevant data was omitted cannot be ruled out. Third, there were several instances when numerical data were not readily available and we had to derive these directly from published figures. This can be imprecise, although we enlarged graphs and used two authors to extract data. Fourth, some NOS inhibitors work though additional neuroprotective mechanisms that do not only involve inhibition of NO synthesis. Moreover, some of the 'selective' inhibitors only discriminate moderately between iNOS and nNOS (Alderton et al. 2001). Unfortunately, it is not possible to ascertain whether the relationships we have observed in each sub-group are independent of this. Fifth, the technique of extracting multiple pieces of information from single publications has a potential to introduce bias into the review since the results would have been generated by the same investigators / laboratories. Finally, since the median quality rating of the studies was only 3 out of a total of 8, there are likely to be methodological weaknesses in the included studies. Two key areas of concern are that studies are not reporting that they randomised animals to active and control treatment, and performed outcome assessment blinded to treatment assignment. All studies evaluating agents in experimental stroke models should follow published recommendations on preclinical drug development (Stroke Therapy Academic Industry Roundtable (STAIR) 1999).

Accepting these limitations, this systematic review brings together data from all published studies and suggests that NOS inhibitors, especially if 'selective' to nNOS or iNOS, reduce infarct volume in experimental stroke. Non-selective inhibitors may be less effective, probably because they compromise CBF. Consequently, selective NOS inhibitors are candidate treatments for testing in clinical stroke.

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TABLE 1

Included studies

Drug	Studies	Species	Quality	Total N	Model P/T	Occlusion G/F	1st dose timing (min)	route	Measures	
									infarct vol	CBF
Non specific inhibitors										
L-NAME	(Anderson and Meyer 1996)	R	3	42	T	F	-20	i.v.		$ml.min^{-1}.g^{-1}$
	(Anderson and Meyer 2000)	R	3	15	P	F	-30	i.v.	%	$ml.min^{-1}.g^{-1}$
	(Ashwal et al. 1993)	SHR	2	14	T	F	-60	i.v.	mm^3	
	(Ashwal et al. 1994)	SHR	3	?	T	F	+0, +120, +150	i.v.	mm^3, %	$ml.min^{-1}.g^{-1}$, %
	(Ashwal et al. 1995)	SHR	3	14	T	F	-60	i.p.	% , mm^3	
	(Batteur-Parmentier et al. 2000)	SDR	3	110	T	F	+5	i.p.	mm^3	%
	(Buisson et al. 1992)	SDR	2	?	P	F	+5	i.p.	mm^3	
	(Buisson et al. 1993)	SDR	2	50	P	F	-30	i.p.	mm^3	
	(Charriaut-Marlangue et al. 1996)	SDR	1	42	T	F	+5	i.p.	mm^3, %	
	(Clavier et al. 1994)	C	3	24	T	G	+180	i.v.		$ml.min^{-1}.g^{-1}$
	(Coert et al. 1999)	WR	3	92	T	F	-30	i.v.	mm^3	
	(Dawson et al. 1992a)	SDR	3	18	P	F	-30	s.c	mm^3	
	(Dawson et al. 1994)	SDR	3	67	P, T	F	-30	i.p.	mm^3	
	(Ding-Zhou et al. 2002)	M	4	?	T	F	+180	i.p.	mm^3	
	(Greenberg et al. 1995)	P	4	20	T	G	-60	i.v.		$ml.min^{-1}.g^{-1}$
	(Hamada et al. 1995)	WR	4	77	P	F	-20	i.c.v.	mm^3	
	(Hiramatsu et al. 1996)	P	2	40	T	G	-?	i.v.		$ml.min^{-1}.g^{-1}$
	(Humphreys and Koss 1998)	SDR	3	63	T	G	-30	i.v.		%
	(Iuliano et al. 1995)	WR	4	78	T	F	-30	i.v.	mm^3	
	(Kamii et al. 1996)	M	2	?	T	F	+5	i.p.	mm^3	
	(Kuluz et al. 1993)	WR	4	24	T	F	-15	i.v.	mm^3	
	(Margaill et al. 1997)	SDR	4	?	T	F	+5, 180, 360, 540,720	i.p.	mm^3	
	(Nishikawa et al. 1993)	C	4	20	P	F	-30	i.v.	% , mm^3	$ml.min^{-1}.g^{-1}$
	(Nishikawa et al. 1994)	C	2	49	T	F	-60, +45	i.v.	%	$ml.min^{-1}.g^{-1}$
	(Prado et al. 1993)	WR	2	14	T	G	-5	i.v.		%
	(Puisieux et al. 2000)	WR	2	52	P	F	-4320	i.p.	mm^3	
	(Quast et al. 1995)	SDR	2	36	T	F	-1	i.v.	mm^3	
	(Schleien et al. 1998)	P	3	18	T	G	-?	i.v.		$ml.min^{-1}.g^{-1}$

	(Segawa et al. 1998)	P	4	12	T	G	+90	i.v.		$ml.min^{-1}.g^{-1}$
	(Sercombe et al. 2001)	SDR	1	37	P	F	-20160, -60480	p.o.	mm^3	
	(Stagliano et al. 1997)	WR	3	29	P	F	+5	i.v.		$ml.min^{-1}.g^{-1}$
	(Sugimura et al. 1998)	WR	3	18	T	G	-?	?		%
	(Uetsuka et al. 2002)	WR	3	40	T	G	-120	i.v.		$ml.min^{-1}.g^{-1}$
	(Wei et al. 1994)	LER	2	28	P	F	+15	i.v.		$ml.min^{-1}.g^{-1}$
	(Wei and Quast 1998)	SDR	2	22	T	F	-1	i.p.	mm^3	
	(Zhang and Iadecola 1993)	SDR	2	31	P	F	+3	i.a.	mm^3	%
	(Zhao et al. 1999)	WR	2	36	T	G	-30	i.p.		%
L-NNA	(Buchan et al. 1994)	WR, SHR	6	?	T, P	G, F	-30 , +5	i.p.	mm^3	%
	(Carreau et al. 1994)	M	3	?	P	F	+5	i.p.	mm^3, %	
	(Dorrepaal et al. 1997)	L	3	18	T	G	+35	i.v. .		%
	(Gursoy-Ozdemir et al. 2000)	M	4	65	T	F	+105	i.p.	mm^3	
	(Hashimoto et al. 1999)	WR	2	?	T	F	-10	i.p., i.a..	%	%
	(Matsui et al. 1997)	SDR	4	148	T	F	-5, +5	i.p.	mm^3	$ml.min^{-1}.g^{-1}$
	(Nakashima et al. 1999)	WR, FR	1	?	T	F	+120	i.p., i.v. .	mm^3	
	(Nowicki et al. 1991)	M	1	31	P	F	+5	i.p.	mm^3	
	(Sadoshima et al. 1997)	SHR	2	34	T	G	+60	i.v.		$ml.min^{-1}.g^{-1}$, %
	(Santizo et al. 2000)	SDR	2	26	P	F	-45	i.v.		%
	(Spatz et al. 1995)	MG	2	?	T	G	-240	i.p.		%
	(Spinnewyn et al. 1999)	SDR	3	88	T	F	+240	i.v.	mm^3	
	(Xu et al. 2000)	SDR	1	24	P	F	-30	i.p.	mm^3	
	(Yamamoto et al. 1992)	WR	2	37	P	F	+5	i.v.	mm^3	
	(Zhang et al. 1996b)	WR	3	73	T	F	+120	i.v.	%	%
L-NMMA	(Sakashita et al. 1994)	WR	1	30	T	G	-5	i.p.		%
nNOS inhibitors										
7NI	(Coert et al. 1999)	WR	3	92	T	F	-60	i.p.	mm^3	
	(Escott et al. 1998)	SDR	6	55	T	F	+5, +90	i.p.	%, mm^3	
	(Goyagi et al. 2001)	WR, M	5	115	T	F	-30	i.p.	%	
	(Gursoy-Ozdemir et al. 2000)	M	4	65	T	F	-30, +90	i.p.	mm^3	
	(Humphreys and Koss 1998)	SDR	3	63	T	G	-30	i.v. .		%
	(Jiang et al. 1999)	WR	3	18	T	G	-20	i.p.		%

	(Kamii et al. 1996)	M	2	?	T	F	+5	i.p.	mm^3	
	(Uetsuka et al. 2002)	WR	3	40	T	G	-60	i.p.		$ml.min^{-1}.g^{-1}$
	(Yoshida et al. 1994)	SDR	3	55	P	F	+5	i.p.	mm^3	
AR-R 17477	(Harukuni et al. 1999)	WR	5	53	P	F	-30, +60	i.v. .	mm^3	
	(O'Neill et al. 2000)	MG, WR	4	?	T	F, G	0, +30, +120	i.v.	mm^3	
	(Santizo et al. 2000)	SDR	2	26	P	F	-45	i.v.		%
	(Zhang et al. 1996b)	WR	3	48	T	F	+120	i.v.	%	%
BN 80933	(Chabrier et al. 1999)	SDR, MG	6	?	T	F, G	+5, 240, 360, 480, 1440	i.v.	mm^3	
PPBP	(Goyagi et al. 2001)	WR, M	5	115	T	F	-30	i.p. .i.v.	%	
TRIM	(Escott et al. 1998)	SDR	6	55	T	F	+5 or 90	i.p.	%, mm^3	
iNOS inhibitors										
1400W	(Parmentier et al. 1999)	SDR	3	?	T	F	+1080	s.c.	mm^3	
Aminoguanidine	(Cash et al. 2001)	SDR	3	?	T	F	+360	i.p.	mm^3	
	(Cockroft et al. 1996)	LR	4	?	P	F	+15, 60, 120, 180	i.p.	%	
	(Han et al. 2002)	SDR	3	?	T	F	+0	i.p.	%	
	(Iadecola et al. 1995)	SHR	2	53	P	F	+1440	i.p.	mm^3	%
	(Iadecola et al. 1996)	SDR	2	79	T	F	+360	i.p.	mm^3	
	(Nagayama et al. 1998)	SHR	4	60	P	F	+1440	i.p.	mm^3, %	
	(Sugimoto and Iadecola 2002)	M	3	58	P	F	+1080	i.p.	mm^3	
	(Tsuji et al. 2000)	WR	3	31	P	G	-60	i.p.	%	
	(Xu et al. 2000)	SDR	1	24	P	F	-30	i.p.	mm^3	
	(Zhang et al. 1996a)	SDR	1	71	T	F	+1440	i.p.	mm^3, %	
	(Zhang and Iadecola 1998)	SHR	3	47	P	F	+0	i.p.	mm^3	
	(Zhu et al. 2002)	M	2	75	T	F	+360	i.p.	%	

Abbreviations: Mice (M); Mongolian Gerbil (MG); Piglets (P); Lambs (L); Cats (C); Spontaneously Hypertensive rat (SHR); Sprague-Dawley rat (SDR); Wistar rat (WR); Long-Evans rat (LER); Lewis Rats (LR); Fischer Rats (FR); Rabbits (R); male (M); female (F); permanent (P); transient (T); global (G); focal (F); intra-venous (i.v.); intra-arterial (i.a.); intra-peritoneal (i.p.); infarct volume (infarct vol.); cerebral blood flow (CBF)

TABLE 2

Effect of NOS inhibitors on lesion volume and cerebral blood flow (SMD, 95% CI) by brain region in permanent and transient ischaemia

Outcome	Permanent			Transient		
	Total	Cortical	Sub-cortical	Total	Cortical	Sub-cortical
Lesion volume	-0.51* (-0.82, -0.20) S=19, n=532	-1.13* (-1.57, -0.70) S=15, n=303	-0.57* (-0.92, -0.23) S=13, n=318	-1.01* (-1.29, -0.73) S=28, n=919	-0.69* (-0.94, -0.43) S=12, n=488	-0.40* (-0.74, -0.06) S=11, n=363
CBF	No data	-0.80* (-1.34, -0.27) S=6, n=87	-0.73 (-1.83, 0.36) S=1, n=14	-0.57 (-1.26, 0.11) S=8, n=120	-0.22 (-0.52, 0.07) S=15, n=320	-0.38 (-1.03, 0.26) S=3, n=66

Abbreviations: Standardised mean difference (SMD); cerebral blood flow (CBF); 95% confidence intervals (95% CI); number of studies (S); number of animals (n); *p<0.05

TABLE 3

Effect of selective and non-selective nitric oxide synthase inhibitors on infarct volume and cerebral blood flow (SMD, 95%CI) in stroke models

	Permanent		Transient	
Inhibitor	Total Volume	Cortical CBF	Total Volume	Cortical CBF
Non-selective	0.11 (-0.43, 0.65) S=13, n=258	-0.68* (-1.17, -0.19) S=6, n=83	-0.90* (-1.34, -0.47) S=18, n=455	-0.33 (-0.68, 0.02) S=14, n=266
iNOS	-0.92* (-1.16, -0.69) S=6, n=256	No data	-1.98* (-2.80, -1.15) S=6, n=91	No data
nNOS	-1.36* (-2.05, -0.67) S=1, n=30	-2.62* (-4.89, -0.35) S=1, n=8	-0.90* (-1.28, -0.54) S=7, n=373	+0.12 (-0.38, 0.62) S=4, n=66

Abbreviations: Inducible nitric oxide synthase (iNOS); neuronal nitric oxide synthase (nNOS); standardised mean difference (SMD); cerebral blood flow (CBF); 95% confidence intervals (95% CI); number of studies (S); number of animals (n); *p<0.05

TABLE 4

Effect of timing of administration on total infarct volume (SMD, 95%CI)

Model	Timing		
	Pre-treatment	Early	Late
Permanent	+0.40	-0.83*	-1.20*
	(-0.01, 0.81)	(-1.25, -0.41)	(-1.55, -0.86)
	S=8, n=170	S=8, n=249	S=4, n=141
Transient	-1.55*	-0.16	-0.87*
	(-2.11, -0.99)	(-0.98, 0.67)	(-1.24, -0.50)
	S=9, n=191	S=3, n=140	S=11, n=454

Abbreviations: Standardised mean difference (SMD); 95% confidence intervals (95% CI); number of studies (S); number of animals (n); *p<0.05

TABLE 5

Effect of nitric oxide synthase inhibitors by animal model on total infarct volume (SMD, 95%CI)

Model	Spontaneously hypertensive rat	Lewis rat	Sprague Dawley rat	Wistar rat	Fischer rat	Mouse	Rabbit	Cat
Permanent	-0.99* (-1.36, -0.61) S=3, n=135	-0.89* (-1.28, -0.50) S=1, n=80	-0.18 (-0.74, 0.39) S=7, n=152	+1.05 (-0.05, 2.15) S=3, n=55	No data	-1.64* (-2.22, -1.07) S=2, n=68	+1.31* (0.20, 2.42) S=1, n=15	+0.20 (-0.68, 1.08) S=1, n=20
Transient	-2.10* (-2.81, -1.39) S=1, n=51	No Data	-1.15* (-1.56, -0.74) S=9, n=239	-0.61* (-1.10, -0.12) S=5, n=236	+0.69 (-0.06, 1.43) S=1, n=34	-0.91 (-1.90, 0.08) S=3, n=156		-0.05 (-0.81, 0.71) S=1, n=30

Abbreviations: Standardised mean difference (SMD); 95% confidence intervals (95% CI); number of studies (S);

FIGURE 1

Search process showing reasons for exclusion of studies

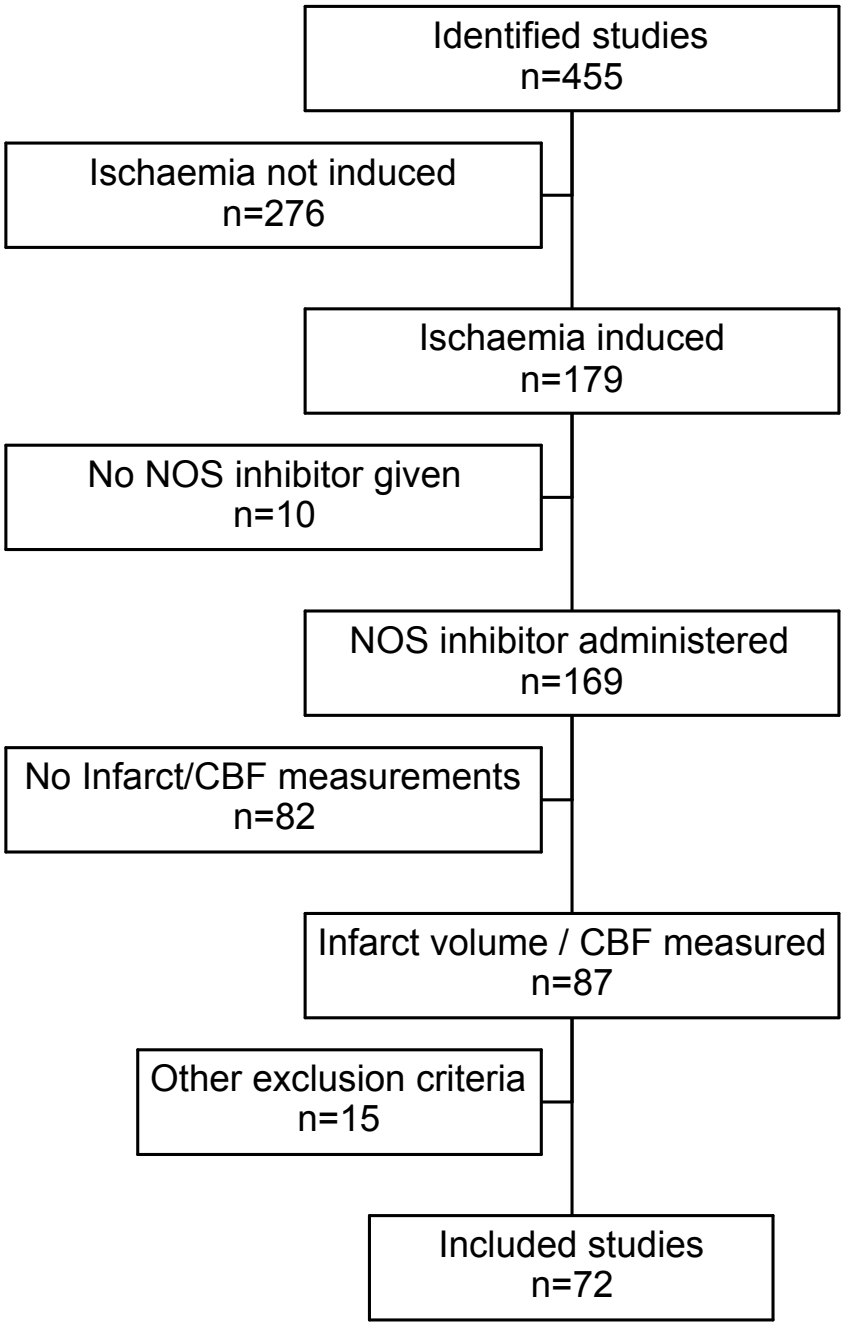


FIGURE 2a

Total lesion volume by different NOS inhibitor types for permanent models of ischaemia

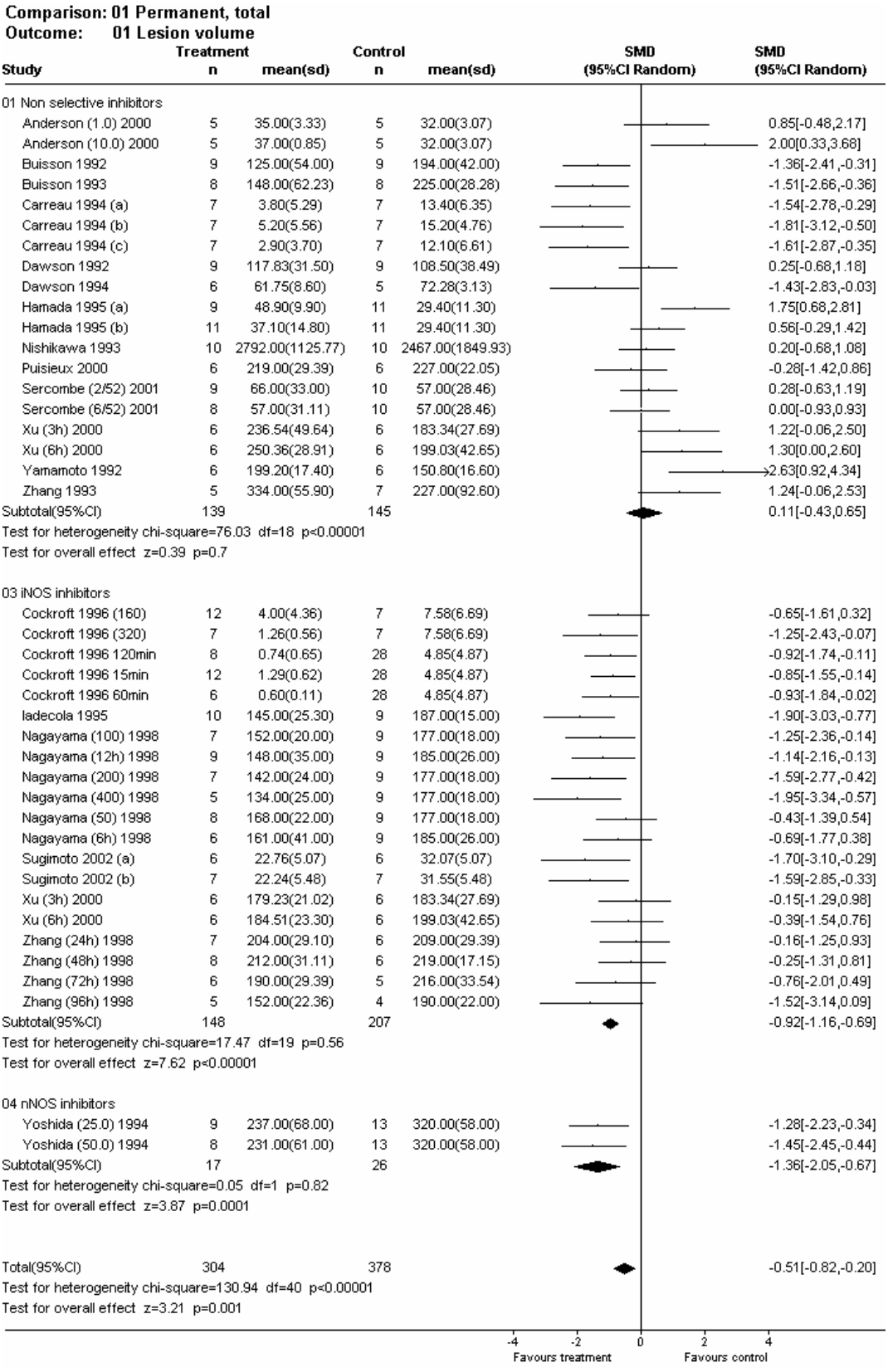


FIGURE 2b

Cortical CBF by different NOS inhibitor types for transient models of ischaemia

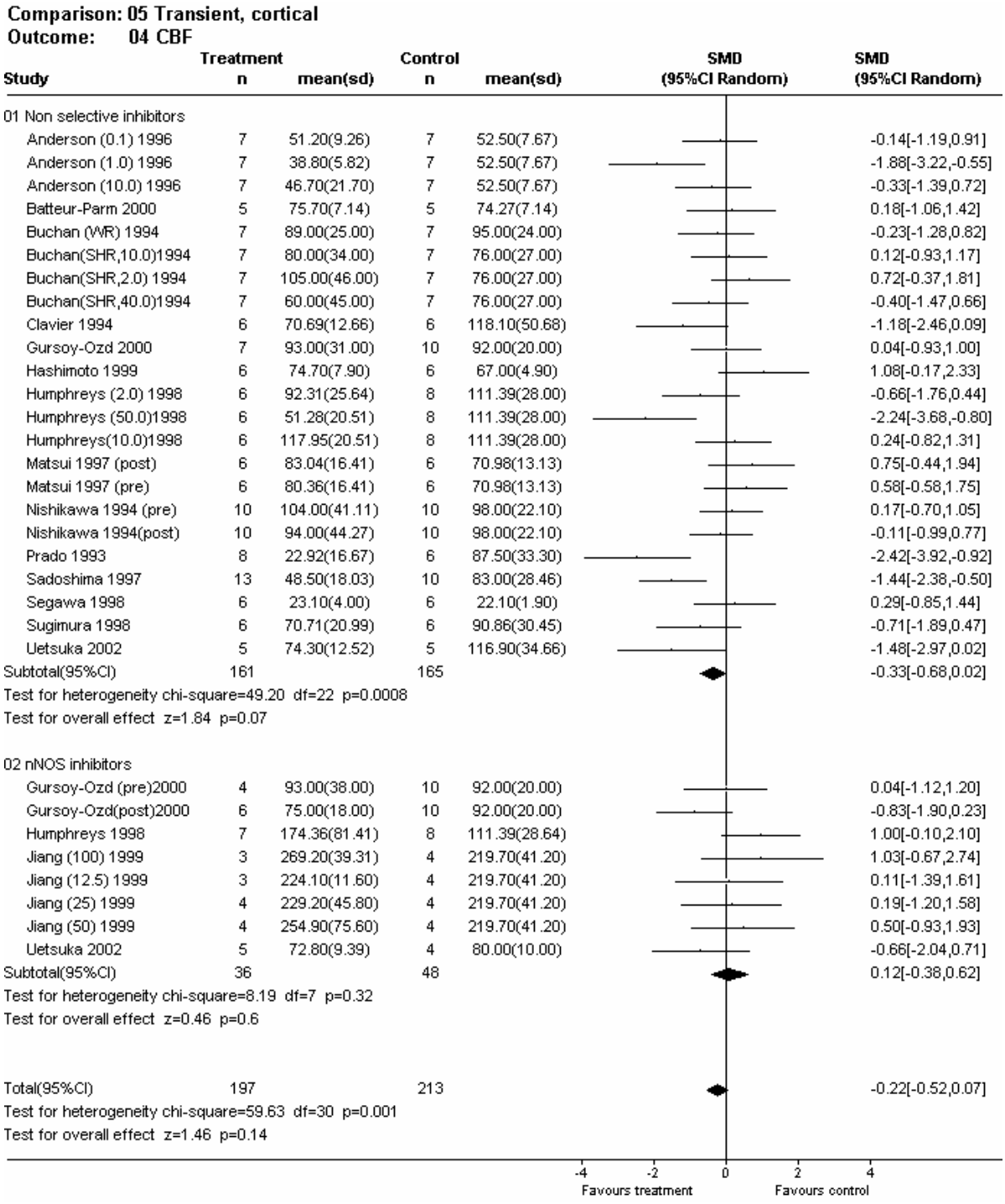
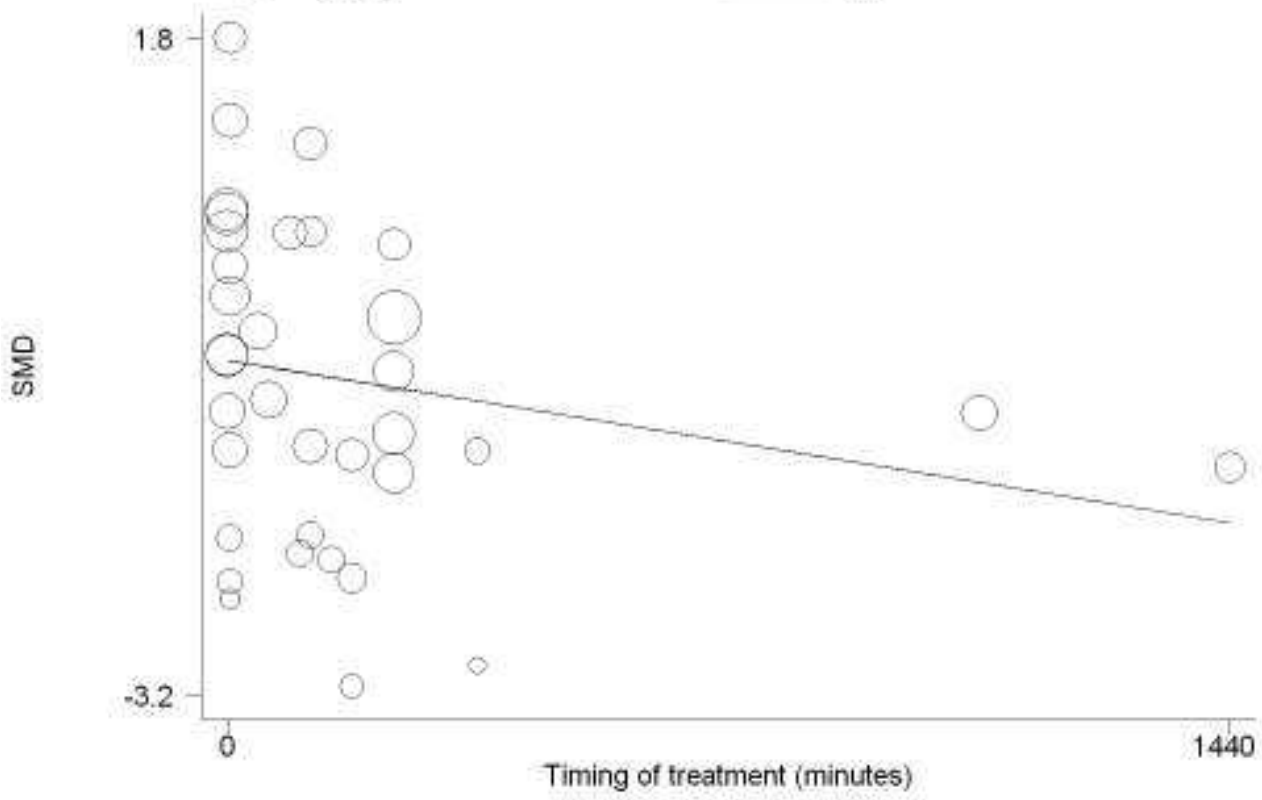


Figure 3. Effect of delay until first dose (minutes) on total infarct volume (SMD) in transient ischaemia. Size of circle proportional to size of study.



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