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Research Paper

# Investigation of S-Nitrosoglutathione in stroke: A systematic review and meta-analysis of literature in pre-clinical and clinical research

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ABSTRACT

*Background:* S-Nitrosoglutathione (GSNO) is a nitric oxide donor that has been investigated for neuroprotective and neuro-recovery effect. We aimed to conduct a systematic review on the published literatures using GSNO in both pre-clinical and clinical stroke studies.

*Methods:* We searched PubMed up to June 30, 2019, using the following keywords: S-Nitrosoglutathione, GSNO, stroke, cerebrovascular, carotid arteries, middle cerebral artery, and middle cerebral artery occlusion. Only studies published in the English language providing efficacy results of GSNO on ischemic stroke were included. Stroke Therapy Academic Industry Roundtable (STAIR) score was used to assess the quality of pre-clinical studies and PEDro score for clinical trials. A meta-analysis was conducted to compare the effect size.

*Results:* Of 39 articles identified, 10 (6 for pre-clinical and 4 for clinical studies) met the eligibility criteria and were included. The median STAIR score across the pre-clinical studies was 5.5 (range: 4–7), and the median PEDro score for the 4 clinical trials was 10 (ranged: 6 to 10). Among the 6 pre-clinical studies, GSNO reduced infarct size in 6 studies and improved neurological behavior scales in 5 studies compared to placebo. Inverse-variance weighted linear meta-analysis of standardized mean difference (Hedge's $g$) on 4 human studies revealed a big effect size (Hedge's $g = -0.82$, 95% CI: $[-1.26, -0.38]$, $P = .0003$) favoring the GSNO group in term of reducing embolic signals. $I^2$ value was 0 across the included clinical studies in the meta-analysis.

*Conclusions:* Pre-clinical studies showed positive benefit of GSNO in animal stroke models. The meta-analysis of clinical studies demonstrated that GSNO is effective in reducing embolic signals in patients with symptomatic internal carotid artery stenosis undergoing carotid endarterectomy or stenting. Further investigation of this molecule is warranted.

## 1. Introduction

Stroke is one of the leading causes of morbidity and mortality globally (Benjamin et al., 2018). Early recanalization therapy through intravenous thrombolysis and/or mechanical thrombectomy is the crucial part in the early management of ischemic stroke (Powers et al., 2018). Despite the high rate of successful recanalization, there is only approximately 50% rate of functional independence at 90 days post-stroke (Davalos et al., 2017). Seeking effective neuroprotectant remains critical in the acute ischemic stroke treatment. While pre-clinical data

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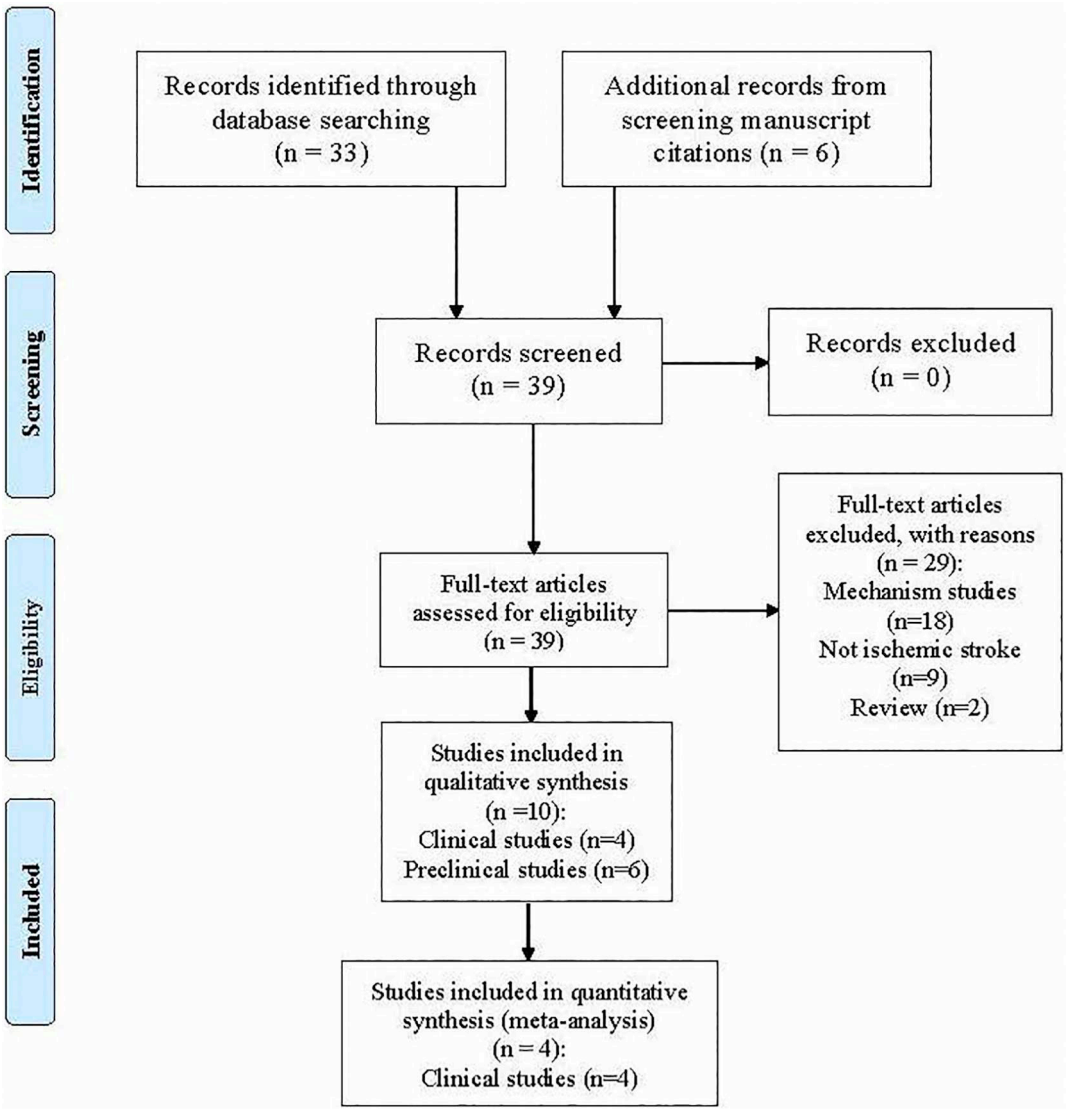


**Fig. 1.** Data flow of study selection.
Of 39 articles identified, 10 (6 for pre-clinical and 4 for clinical studies) met eligibility criteria and were included; and the other 29 articles were excluded for the following reasons: 18 studies were basic science studies without providing efficacy data, 9 studies did not investigate the ischemic stroke conditions, and 2 studies were reviews.

showed the promising efficacy of numerous neuroprotective molecules in ischemic stroke, clinical trials have not been successful in translating these agents into bedside.

S-nitrosoglutathione (GSNO),[1] an endogenous low molecular weight S-nitrosothiol formed by nitrosation of reduced glutathione, is a natural component of the human body existing in the brain and other organs (Kluge et al., 1997; Singh et al., 1996). GSNO is involved in storing and transporting of nitric oxide (NO)[2] (Khan et al., 2015a), which is a key signaling molecule in regulating cerebral blood flow, and the NO derived from endothelial nitric oxide synthase were shown to have neuroprotective effects (Garry et al., 2015). GSNO protects the ischemia/reperfusion injury against inflammation and neuron cell death by modulating the NO system (Khan et al., 2005). GSNO has the effect of systemic vasorelaxation (Rassaf et al., 2002), which may be related with the inhibition of platelet functions (de Belder et al., 1994; Gordge and Xiao, 2010; Salas et al., 1998). GSNO also showed the protective effect of blood-brain barrier and epithelia permeability (Khan et al., 2009; Savidge et al., 2007). Exogenous administration of GSNO may have the potential of stimulating neuroregeneration via the stabilization of the HIF-1α/VEGF pathway in the chronic phase of stroke disease (Khan et al., 2015a). Prior studies indicated GSNO was effective in improving neurological functional recovery in animal models of ischemia (Sakakima et al., 2012), traumatic brain injury (Khan et al., 2009; Khan et al., 2011) and subarachnoid hemorrhage (Sehba et al., 1999). GSNO has been applied in human both in healthy condition and vascular disease conditions including stroke.

Before planning costly clinical trial using GSNO, it is an important step to conduct a critical appraisal of the published literatures in both pre-clinical and clinical studies on this topic to identify potential gaps and issues. Therefore, we aimed to conduct both qualitative (systematic review) and quantitative (meta-analysis) review on the investigation GSNO in stroke.

[1] [1]GSNO: S-nitrosoglutathione.
[2] NO: nitric oxide.

**Table 1**
Summary of the included pre-clinical studies.

| Study | Study animal | Study model | GSNO treatment descriptions | | | | Key outcome(s) vs. control | |
|---|---|---|---|---|---|---|---|---|
| | | | Route | Dose | Time | N | Behavioral outcomes (P)* | Surrogate infarct volume (P)* |
| (Parent et al., 2015) | Wistar rats | MCAO by blood clots | Sub | 7.5 mg/kg unloaded | 2 h after MCAO | 8 | − | − |
| | | | Sub | 30 mg/kg in situ implant | 2 h after MCAO | 8 | − | + |
| | | | Sub | 30 mg/kg microparticled | 2 h after MCAO | 6 | + (15 sensori-motor items) | + |
| | | | Sub | Vehicle | 2 h after MCAO | 8 | N/A | N/A |
| (Khan et al., 2015c) | SD rats | 60-min MCAO | IV | 0.25 mg/kg GSNO slowly infused | 1 h after reperfusion | 5 | + (4-point scale) | + |
| | | | | 7-nitroindazole slowly infused | 1 h after reperfusion | 5 | − | + |
| | | | | Vehicle slowly infused | 1 h after reperfusion | 5 | N/A | N/A |
| | | | | Sham MCAO | 1 h after reperfusion | 5 | N/A | N/A |
| (Khan et al., 2015b) | SD rats | 60-min MCAO | IV | 0.25 mg/kg GSNO slowly infused | 1 h after reperfusion | 7 | + (4-point scale) | + |
| | | | | 0.25 mg/kg GSNO + 5 mg/kg 2ME slowly infused | 1 h after reperfusion | 7 | − | − |
| | | | | Vehicle slowly infused | 1 h after reperfusion | 7 | N/A | N/A |
| | | | | Sham MCAO | 1 h after reperfusion | 7 | N/A | N/A |
| (Sakakima et al., 2012) | SD rats | 60-min MCAO | IV + gavage fed | 0.25 mg/kg GSNO slowly infused + gavage fed | At reperfusion | 8 | + (4-point scale) | + |
| | | | | EX | At reperfusion | 8 | − | − |
| | | | | 0.25 mg/kg GSNO slowly infused + gavage fed + EX | At reperfusion | 8 | + (4-point scale) | + |
| | | | | Vehicle | At reperfusion | 8 | N/A | N/A |
| (Khan et al., 2006) | SD rats | 20-mim MCAO | IV | 0.7 mg/Kg and 1.0 mg/kg GSNO infused in 10-20 min | At reperfusion | 14 | - (4-point scale) at dose of 1.0 mg/kg | + |
| | | | | Vehicle | At reperfusion | 14 | N/A | N/A |
| | | | | Sham | At reperfusion | 7 | N/A | N/A |
| (Khan et al., 2005) | SD rats | 20-mim MCAO | IV | 1 mg/kg GSNO slowly infused | At reperfusion | 7 | + | + |
| | | | | Vehicle slowly infused | At reperfusion | 7 | N/A | N/A |
| | | | | Sham | At reperfusion | 7 | N/A | N/A |

*P*(+) = Positive; P*(−) = Neutral. Abbreviations: SD: Sprague-Dawley; MCAO: middle cerebral artery occlusion; IV: intravenous; Sub: subcutaneous; GSNO, S-nitrosoglutathione; 2ME: HIF-1αinhibitor 2-methoxyestradiol; EX: exercise.

**Table 2**
Summary of the included clinical studies.

| Study | Study population | Location | Sample size | GSNO descriptors | Key prognostic indicator(s) at baseline | Key outcome(s) |
|---|---|---|---|---|---|---|
| (Kaposzta et al., 2002b) | Patients with active embolization with 50% symptomatic ICAS | 1 site in UK | GSNO: n = 10<br>Placebo: $n$ = 10 | An intravenous infusion at a rate of 1.5 g/kg/min for 90 min | Mean (range) TCD ESs per hr: GSNO: 6.9 [3, 13]; Placebo: 7.3 [4, 12] | Mean (SD) TCD ES intensity: GSNO: 13.07 (3.15) vs. Placebo: 16.69 (3.58); $P < .001$ |
| (Kaposzta et al., 2002a) | Patients undergoing carotid angioplasty and stenting for symptomatic ≥70% ICAS | 2 sites in UK | GSNO: n = 8<br>Placebo: $n$ = 8 | An intravenous infusion at a rate of 1.5μg/kg/min for 90 min | Mean (range) TCD ESs per hr: GSNO: 3.9 [0, 27]; Placebo: 1.9 [0, 14] | Mean (SD) TCD ES intensity: GSNO: 12.41 (3.27) vs. Placebo: 15.94 (3.89); $P < .001$ |
| (Kaposzta et al., 2001) | Patients undergoing carotid endarterectomy for symptomatic > 70% ICAS | 1 site in UK | L –arginine: n = 14<br>GSNO: $n$ = 14<br>Placebo: n = 14 | An intravenous infusion at a rate of 1.5μg/kg/min for 90 min | ES: L –arginine: 2; GSNO: 4; Placebo: 3 | Mean (SD) TCD ES intensity: GSNO: 12.12 (2.38) vs. Placebo: 16.07 (4.94); $P < .0001$ |
| (Molloy et al., 1998) | Patients undergoing carotid endarterectomy for symptomatic > 70% ICAS | 1 site in UK | GSNO: $n$ = 12<br>Control: n = 12 | An intravenous infusion at a rate of 1.5μg/kg/min until 2 h after skin closure | N/A | Mean (SD) TCD ES intensity: GSNO: 12.30 (4.30) vs. Control: 14.27 (4.71); $P < .0001$ |

Abbreviations: GSNO, S-Nitrosoglutathione; ICAS, internal carotid artery stenosis; TCD, Transcranial Doppler; ES, embolic signals; SD, standard deviation.

## 2. Materials and methods

This systematic review and meta-analysis was in accordance with the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis: The PRISMA Statement (Moher et al., 2009).

### 2.1. Study search

We searched PubMed up to June 30, 2019, using the following keywords: S-Nitrosoglutathione, GSNO, stroke, cerebrovascular event, carotid arteries, middle cerebral artery (MCA), and middle cerebral artery occlusion. To get fully understanding of the application of GSNO in other vascular disease conditions, we also searched Pubmed and included clinical studies with either healthy subjects or patients with vascular diseases other than stroke (Supplemental Table).

### 2.2. Study selection

We read the full-text articles to assess eligibility. Inclusion criteria were: (1) published on peer-reviewed journals in English language; (2) randomized controlled methods (for human clinical studies only) are listed in the manuscript; (3) contained efficacy outcomes of GSNO. Exclusion criteria were: (1) basic science studies investigated mechanisms of GSNO only without outcome data; (2) study protocols; (3) review articles; (4) studies focused on disease conditions other than ischemic stroke.

### 2.3. Data extraction and quality assessment

We extracted data based on the studies' objectives to test the efficacy of GSNO on ischemic stroke. Two investigators collected the data independently and discrepancies were resolved by discussion.

Stroke Therapy Academic Industry Roundtable (STAIR) score was used for the quality assessment of pre-clinical studies, including 1) publication in a peer-reviewed journal; 2) statement confirming compliance with animal welfare requirements; 3) avoided neuroprotective anesthetics; 4) statements describing control of temperature; 5) random treatment assignment; 6) allocation concealment; 7) blinded outcome assessment; 8) inclusion of a sample-size calculation; 9) use of animals with relevant comorbidities; and 10) inclusion of a statement declaring presence or absence of any conflicts of interest (Fisher et al., 2009). One point was given for each criterion reported. Potential score ranges from 0 to 10 with higher scores indicating greater methodological rigor.

PEDro score was used to rate randomized clinical controlled trials (Blobaum, 2006) The rating scale is a checklist of “yes or no” answers to each criteria, including 1) eligibility criteria were specified; 2) subjects were randomly allocated to groups; 3) allocation was concealed; 4) the groups were similar at baseline regarding the most important prognostic indicators; 5) there was blinding of all subjects; 6) there was blinding of all therapists who administered the therapy; 7) there was blinding of all assessors who measured at least one key outcome; 8) measures of at least one key outcome were obtained from more than 85% of the subjects initially allocated to groups; 9) all subjects for whom outcome measures were available received the treatment or control condition as allocated or, where this was not the case, data for at least one key outcome was analyzed by “intention to treat”; 10) the results of between-group statistical comparisons are reported for at least one key outcome, and 11) the study provides both point measures and measures of variability for at least one key outcome. The score ranges from 0 to 11 with higher score meaning better quality.

**Table 3**
Quality check of pre-clinical studies using STAIR guideline.

| | Scoring item | (Parent et al., 2015) | (Khan et al., 2015c) | (Khan et al., 2015b) | (Sakakima et al., 2012) | (Khan et al., 2006) | (Khan et al., 2005) |
|---|---|---|---|---|---|---|---|
| 1 | Published in peer-reviewed journal | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | Statement confirming Compliance w/ animal welfare requirements | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | Avoided neuroprotective anesthetics | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Control of temperature | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | Random treatment assignment | 1 | 1 | 1 | 1 | 0 | 0 |
| 6 | Allocation concealment | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Conflict of interest statement | 1 | 1 | 1 | 0 | 0 | 0 |
| 8 | Blinded outcomes | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | Animals with comorbidities | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | Sample size calculation | 1 | 0 | 0 | 0 | 0 | 0 |
| | Total Score | 7 | 6 | 6 | 5 | 4 | 4 |

The median STAIR score across the pre-clinical studies was 5.5 (range: 4–7).

### 2.4. Data analyses

We used Review Manager 5.3 (Cochrane IKMD – Copenhagen, Denmark; Freiburg, Germany; London, UK; USA). We performed an inverse-variance weighted random effects linear model meta-analysis of standardized mean difference (Hedge's *g*) to measure the effect of GSNO (after and before GSNO interventions) in reducing embolic signals detected by Transcranial Doppler (TCD). Hedge's *g* value of $< 0.2$ was considered a mild effect, ~0.5 and $> 0.8$ were considered moderate and strong effect, respectively. $I^2$ value was calculated to test heterogeneity of included studies in the meta-analysis, and we consider $I^2$ value of $> 25\%$ as presence of heterogeneity. Effects were compared using z-tests. A *P*-value of $< 0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Description of studies

The data flow diagram of study search and selection is shown in Fig. 1. Of 39 articles identified, 10 (6 for pre-clinical and 4 for clinical studies) met the eligibility criteria and were included. Studies included for the systematic review were described in Table 1 and Table 2.

### 3.2. Risk of bias assessment

The median STAIR score across the pre-clinical studies was 5.5 (range: 4–7) (Table 3). Among the 4 clinical studies, the PEDro score ranged from 6 to 10, with median of 10 (Table 4).

### 3.3. Pre-clinical studies

We included 6 pre-clinical studies with 180 rats in total (Table 1). GSNO was administered by various routes: subcutaneously (1 study), orally (1 study) and intravenously (5 studies). The dose of GSNO ranged from 0.25 mg/Kg to 30 mg/Kg and the timing of giving medicine ranged from at reperfusion to 2 h after reperfusion. Among the included 6 pre-clinical studies, 6 studies found that GSNO reduced infarct size with multiple doses or routes compared to placebo, and 5 studies demonstrated GSNO improved neurological behavior measured by 15 sensori-motor items or 4-point scale (Table 1).

### 3.4. Clinical studies

Among the 4 included clinical trials with 88 subjects (44 patients received GSNO and 44 patients were allocated to the control/placebo groups) (Table 2). The included study subjects all had symptomatic internal carotid artery stenosis (i.e. patients suffered stroke or transient ischemic attack including amaurosis fugax) and underwent carotid endarterectomy or stenting. GSNO were all administered in the same way by an intravenous infusion at a rate of 1.5 g/Kg/min or 1.5μg/Kg/min. Inverse-variance weighted linear meta-analysis of standardized mean difference (Hedge's *g*) on 4 human studies revealed a big effect size (Hedge's $g = -0.82$, 95% CI: $[-1.26, -0.38]$, $P < .0003$) favoring the GSNO group in term of reducing embolic signals (Fig. 2).

## 4. Discussion

We systematically reviewed the existing pre-clinical and clinical studies investigating the potential effect of GSNO in the treatment of ischemic stroke. Among the included 6 pre-clinical studies, GSNO reduced infarct size in 6/6 studies and improved neurological behavior in 5/6 studies. Among the included 4 clinical studies, meta-analysis revealed a big effect size (Hedge's $g = -0.82$, 95% CI: $[-1.26, -0.38]$, $P = .0003$) favoring the GSNO group in reducing embolic signals.

We consider the studies having low risks of bias via quality assessment. Based on the STAIR score, all the included studies were published in the peer-reviewed journals and followed the animal welfare requirements, with controlled experiment temperature and blinded outcomes. Random treatment assignments were applied to 4/6 studies. All the studies failed to avoid neuroprotective anesthetics, but anesthetic medications were unavoidable in building middle cerebral artery occlusion models. None of the included pre-clinical studies used animals with comorbidities. We also noticed that allocation concealment was lacking in all 6 pre-clinical studies; and the sample size calculation was conducted only in one study (Parent et al., 2015).

Pre-clinical studies found the protective effect of GSNO in ischemia/reperfusion, which might be related with modulating NO system (Khan

**Table 4**
Quality check of human studies using PEDro score.

| | Scoring item | (Kaposzta et al., 2002b) | (Kaposzta et al., 2002a) | (Kaposzta et al., 2001) | (Molloy et al., 1998) |
|---|---|---|---|---|---|
| 1 | Eligibility criteria were specified | 1 | 1 | 1 | 1 |
| 2 | Subjects were randomly allocated to groups (in a crossover study, subjects were randomly allocated an order in which treatments were received) | 1 | 1 | 1 | 1 |
| 3 | Allocation was concealed | 0 | 0 | 0 | 0 |
| 4 | The groups were similar at baseline regarding the most important prognostic indicators | 1 | 1 | 1 | 0 |
| 5 | There was blinding of all subjects | 1 | 1 | 1 | 1 |
| 6 | There was blinding of all therapists who administered the therapy | 1 | 1 | 1 | 0 |
| 7 | There was blinding of all assessors who measured at least one key outcome | 1 | 1 | 1 | 1 |
| 8 | Measures of at least one key outcome were obtained from more than 85% of the subjects initially allocated to groups | 1 | 1 | 1 | 0 |
| 9 | All subjects for whom outcome measures were available received the data for at least one key outcome was analyzed by "intention to treat" | 1 | 1 | 1 | 0 |
| 10 | The results of between-group statistical comparisons are reported for at least one key outcome | 1 | 1 | 1 | 1 |
| 11 | The study provides both point measures and measures of variability for at key outcome | 1 | 1 | 1 | 1 |
| | Total Score | 10 | 10 | 10 | 6 |

The median PEDro Score across the clinical studies was 10 (range: 6–10).

et al., 2005), Akt pathway (Sakakima et al., 2012), HIF-1α/VEGF pathway (Khan et al., 2015b), inhibiting nNOS/peroxynitrite/AMP Kinase cycle (Khan et al., 2015c) and reducing oxidative stress (Khan et al., 2006). GSNO exerts its cellular actions through both NO- and Snitrosation-dependent mechanisms (Broniowska et al., 2013).

With the potential of regulating platelet functions and hemodynamics, the effects of GSNO were also investigated in other vascular disease studies besides ischemic stroke (Supplemental Table). Early-phase clinical studies found GSNO increased left ventricular function by tonic releasing of NO (Rassaf et al., 2006), improved systemic vasodilation and hemodynamics (Rassaf et al., 2002), reduced maternal mean arterial pressure, platelet activation, and uterine artery resistance for preeclampsia (Everett et al., 2014; Lees et al., 1996), increased the clitoral blood flow in healthy subjects (Souto et al., 2011), and prevented platelet activity after percutaneous transluminal coronary angioplasty (Langford et al., 1994). We found it is hard to do meta-analysis of the infarct size with the preclinical studies, due the heterogeneity of results expression: 2/6 studies only provided qualitative results (Khan et al., 2015b; Khan et al., 2015c), 2/6 studies used infarct volume (Khan et al., 2006; Khan et al., 2005), 2/6 study expressed corrected percentage of infarct (Sakakima et al., 2012; Parent et al., 2015).

Although the meta-analysis of the four human studies demonstrated a large effect size, the results have several caveats. First, despite the fact that the heterogeneity was low with $I^2 = 0$, we have to point out that all four clinical trials were conducted by the same groups and all focused on one disease condition - patients with symptomatic internal carotid stenosis, i.e., patients suffered stroke or transient ischemic attack including amaurosis fugax. Similarly, 5 out of 6 pre-clinical manuscripts are from one group. Second, although PEDro scores are good, none of the four studies concealed group allocation which significant bias could be introduced. Third, the embolic signals assessed by TCD examinations were used as the surrogate outcomes in clinical trials. While GSNO intravenously appears to be effective (with a large effect size) reducing these emboli during procedures (most of them are likely asymptomatically), whether this surrogate outcome can be translated to better clinical outcomes were not evaluated in these 4 studies. Future studies should consider to use clinical outcomes in addition to the surrogate outcomes. Fourth, safety profile at various dose of GSNO appeared to be reasonable, but blood pressure drop (a drop in mean arterial pressure ≥ 10 mmHg) were reported in 2 subjects (Molloy et al., 1998). Safety profile needs to be systematically monitored in future studies. Lastly, the dose of GSNO varied significantly across different clinical studies. It is a challenge to decide the optimal safe dose of GSNO, and it is possible that the optimal dose could differ in various disease conditions.

## 5. Conclusions

There are positive data about GSNO use in both animal and human stroke studies. This meta-analysis demonstrated that GSNO is effective in reducing embolic signals during carotid endarterectomy or stenting procedure in patients with symptomatic internal carotid stenosis. The safety profile of this molecule appeared to be reasonable but needs to be continuously monitored. It is a logical step to plan for a phase II study to systematically investigate the neuroprotective effect of GSNO in patients with acute ischemic stroke or cerebrovascular disease.

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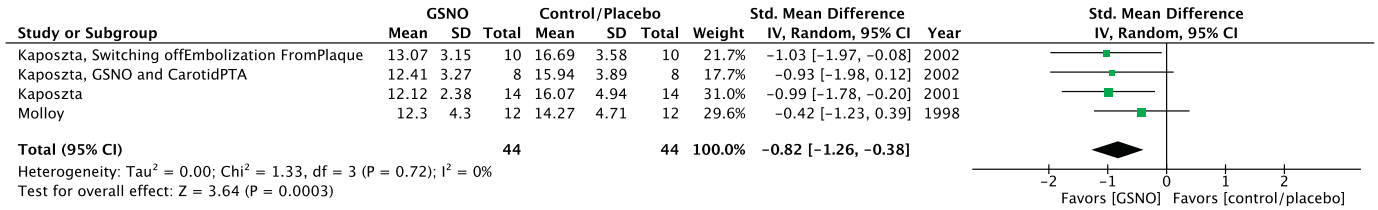


**Fig. 2.** Forrest plot of the mean density of embolic signals under Transcranial Doppler examination.
Inverse-variance weighted linear meta-analysis of standardized mean difference (Hedge's g) on 4 human studies revealed a big effect size (Hedge's $g = -0.82$, 95% CI: $[-1.26, -0.38]$, $P = .0003$) favoring the GSNO group in term of reducing embolic signals. $I^2$ value was 0 across the included studies.

## Declaration of Competing Interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2020.113262.

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