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Methodological quality of animal studies on neuroprotection in focal cerebral ischaemia

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Abstract *Background* The recurrent failure of apparently promising neuroprotective drugs to improve outcome in trials of patients with acute ischaemic stroke may partially be explained by overoptimistic conclusions about efficacy as a result of methodological shortcomings in preclinical studies. We assessed the methodological quality of animal studies of five different neuroprotective agents that have been tested in 21 clinical trials including a total of more than 12,000 patients with acute ischaemic stroke. *Methods* We performed a literature search restricted to full publications on the effects of clomethiazole, gavestinel, lubeluzole, selfotel, or tirilazad mesylate on infarct volume or functional outcome in animal models of acute focal cerebral ischaemia. We used a rating scale to assess the methodological quality of the included studies. One point was attributed to each of 10 items. A score of 4 to 6 points was considered "medium" and a score above 7 "high." *Results* A total of 45 articles were included. The median score on the methodological quality index was 3; 18 studies had a medium score and one a high score. Randomised treatment allocation was mentioned in 19 studies (42 %), blinded administration of study medication in 10 (22 %), and blinded outcome assessment in 18 (40 %). The study drug was administered at a median of 10 min (range, –60 to 360 min) after the onset of ischaemia. *Conclusion* The evidence for neuroprotective efficacy that formed the basis for initiating the 21 trials was obtained in animal studies with a methodological quality that would, in retrospect, not justify such a decision. More rigorous preclinical study methodology may lead to more reliable and reproducible results.

Key words animal models · cerebral ischaemia · neuroprotection · review · methodological quality

Introduction

Although numerous compounds have proved to be effective in animal models of focal cerebral ischaemia, only recombinant-tissue plasminogen activator (rt-PA) and aspirin have convincingly demonstrated efficacy in clinical trials of acute ischaemic stroke [4, 17, 34]. Factors that may explain the disparity between the results of animal models and clinical trials have been given exhaustive attention in reviews and comments [6, 11–13, 21, 24, 31, 32, 36], and include differences in time windows for drug administration, selection of outcome measures, timing of outcome measurements, and characteristics of the study population other than species. However, the majority of these reviews were based on

the authors' impressions of the characteristics of animal studies rather than on systematic evaluation.

We hypothesised that the recurrent failure of apparently promising neuroprotective drugs to improve functional outcome in patients with acute ischaemic stroke might in part have been caused by inadequate data and overoptimistic conclusions about efficacy as a result of methodological flaws in preclinical studies. This has been suggested before by Wardlaw et al. after the publication of the negative results of the Glycine Antagonist In Neuroprotection (GAIN) trials [36]. To answer this question, we evaluated the methodological quality of animal studies of five different compounds from different classes of alleged neuroprotective agents that have recently been tested in 21 clinical trials including a total of more than 12,000 patients with acute ischaemic stroke [2, 10, 15, 27].

Methods

Search Strategy

The literature search for this review was restricted to full publications on the effects of the compounds clomethiazole (a GABA agonist), gavestinel (a glycine site antagonist), lubeluzole (several mechanisms), selfotel (an NMDA antagonist), or tirilazad mesylate (a radical scavenger) on infarct volume or functional outcome in animal models of acute focal cerebral ischaemia. These drugs were chosen because each compound had been tested in at least two phase III clinical trials that included the largest number of patients in their drug category. Publications were identified independently by the first and third author searching Medline (1966 to 2002) and Embase (1980 to 2002) using the names and synonyms of the above compounds, and the key terms [< stroke > OR < ischaemia > OR < ischemia >], as described by Macleod et al. [25]. Of all studies thus found, including those in which the results of the clinical trials were presented, reference lists were checked for additional studies. This method of cross-checking was continued until no further studies were found.

Eligibility

Criteria for inclusion of studies in this review were (1) assessment of the effect of one or more of the above compounds on infarct volume (or a derivative) and/or functional outcome after focal cerebral ischaemia, (2) description of a control group, (3) journal publication in full. Studies were excluded if the effect of a compound was tested only in combination with another potentially neuroprotective strategy or agent, except for thrombolysis.

Data extraction

The first three authors independently extracted data from eligible studies by means of a standardised data extraction form. In case of disagreement, the observers reviewed the article in question together. Data in the following categories were extracted: (1) animal (species, weight, age, gender, and the presence of hypertension or diabetes), (2) model (permanent or temporary ischaemia, and measurements of blood pressure and brain and body temperature), (3) methodology (power calculations, numbers of animals in actively treated and control groups, numbers of excluded animals and reasons for exclusion, method of treatment allocation, blinding of drug administration and outcome evaluation), (4) drug (name and start time), (5) outcome (type, time of evaluation), and (6) study funding. If outcome was assessed at different time points following the onset of ischaemia in the same animal, only the last assessment was included in the analysis.

Methodological quality

A rating scale was used to assess the methodological quality of the included studies (Table 1), based on the "recommendations for standards regarding preclinical and restorative drug development" by the Stroke Therapy Academic Industry Roundtable (STAIR) [31]. This scale resembles that used by Horn et al. [16] in a systematic review of nimodipine in experimental focal cerebral ischaemia. A "clinically relevant time window for start of treatment" was defined as treatment started more than 60 minutes after the onset of ischaemia, as treatment of patients between 60 and 90 minutes after the onset of symptoms has been shown to be feasible in a small group of patients [34]. One point was attributed for each of the 10 items if mentioned in the article. A total score of 0 to 3 was considered "low", a total score of 4 to 6 "medium", and a total score above 7 "high".

Statistical analysis

The data were entered on paper review sheets by the individual assessors and entered in a Microsoft Access database after resolution of possible discrepancies between reviewers. Descriptive data are expressed as frequencies, means, or medians and range as appropriate. For two-group comparisons the Mann-Whitney U-test was used.

Results

Electronic searching identified 673 publications, of which 47 (7%) fulfilled the inclusion criteria. Handsearching identified one additional publication. Two duplicate publications on selfotel were not included. One publication giving an overview of the preclinical development of gavestinel was excluded because the data on the focal cerebral ischaemia experiments were deemed too scanty. Therefore, a total of 45 publications were included in the present study: 9 on the effect of clomethiazole, 4 on gavestinel, 6 on lubeluzole, 10 on selfotel, and 18 on tirilazad mesylate (see appendix). One study tested the effect of both clomethiazole,

Table 1 Methodological quality index (one point for each of the following study attributes)

- Monitoring of physiological parameters
- Group size based on a-priori power calculation
- Treatment allocation via randomisation
- Blinded drug administration
- Blinded outcome evaluation
- Use of aged, diabetic, or hypertensive animals
- Clinically relevant time window for start of treatment
- Assessment of both infarct volume and functional outcome
- Outcome assessment in the acute phase (1 to 6 days)
- Outcome assessment in the chronic phase (7 to 30 days)

gavestinel, and selfotel. Characteristics of the included articles are presented in Table 2. Thirty nine studies (87%) were performed in rodents (29 in rats, 2 in gerbils, 4 in mice, and 5 in rabbits; one study used both rats and mice); we found 3 studies performed in primates and 3 performed in cats. Except in two studies performed in marmosets, the age of the animals was not mentioned. The weight of the rats was almost invariably 250–400 g, indicating an age of less than one-sixth of their normal life expectancy. In 38 studies (84 %) only male animals were used, in one (2 %) only females, in two (4%) both males and females, and in four (9 %) gender was not mentioned. Three studies (7 %) were performed in animals with hypertension, and none in animals with diabetes.

In only one study (post-hoc) were power calculations performed. Randomised treatment allocation was mentioned in 19 studies (42%), blinded administration of study medication in 10 (22%), and blinded outcome assessment in 18 (40 %). In two articles double blinding for treatment allocation was reported. Exclusion of animals, mainly because of mortality, was mentioned in 12 articles (27%). None of studies used an intention-to-treat analysis. The median score on the methodological quality index was 3 (range, 1 to 7); 26 studies (58 %) had a low score, 18 (40 %) had a medium score, and one (2 %) had a high score.

We derived and analysed a total of 129 individual pairwise comparisons (study drug versus control) from the 45 publications (Table 2). The median group size of actively treated animals was 9, that of control animals 10 (range, 4 to 23 and 5 to 50, respectively). The study drug was administered at a median of 10 min (range, –60 to 360 min) after the onset of ischaemia, and outcome was assessed at a median of 24 h (range, 4h – 20 weeks) after the onset of ischaemia. In 34 of the 129 comparisons (26 %) the time interval between onset of ischaemia and start of treatment was more than one hour. In 29 studies (64 %) the manufacturer of the compound under study was involved either financially or in person. There was no relation between involvement of the manufacturer of the compound and methodological quality of the study (P = 0.95).

Discussion

The present study confirms previously expressed concerns about the disparity between animal models of focal cerebral ischaemia and clinical trials of acute ischaemic stroke [6, 11–13, 21, 24, 31, 32, 36]. In contrast to the clinical trials of the compounds under study, the animal studies that formed the justification for these trials were often characterised by a short and clinically unattainable time window for start of treatment, very early assessments of outcome, and an emphasis on infarct volume rather than functional outcome as a primary outcome measure. In addition, the characteristics of the experimental animals used did not reflect the population of patients with acute ischaemic stroke. Almost invariably, the animals were young, and were neither hyperglycaemic or hypertensive, conditions that are present in about half and the majority of the patients with acute stroke, respectively [35].

As suggested after the publication of the negative results of the Glycine Antagonist In Neuroprotection (GAIN) International trial [22], methodological flaws observed in the present review may be a fundamental source of bias in the preclinical evaluation of neuroprotective agents [36]. Random treatment allocation was reported in only 42 % of the studies reviewed, blinded administration of the study agent in 22 %, and blinded

Table 2 Characteristics of the studies

	total	clomethiazole	gavestinel	lubeluzole	selfotel	tirilazad
articles (n)[a]	45	9	4	6	10	18
pair-wise comparisons (n)	129	15	17	49	16	32
total actively treated animals (n)	1265	135	132	555	125	318
total controls (n)	881	97	54	343	122	265
group size active treatment (median (range))	9 (4–23)	9 (4–10)	9 (5–10)	10 (6–23)	8 (5–12)	10 (5–19)
group size controls (median (range))	10 (5–50)	10 (5–10)	9 (7–10)	10 (6–50)	10.5 (5–19)	10 (5–23)
score methodological QI (median (range))	3 (1–7)	2 (1–4)	2 (1–4)	4 (1–5)	3 (1–4)	4 (2–7)
start treatment[b] (minutes, median (range))	10 (–60–360)	60 (–60–180)	60 (–30–360)	30 (0–360)	0 (–30–75)	2.5 (–30–240)
time outcome assessment[c] (h, median (range))	24 (4–3360)	24 (24–3360)	24 (24–144)	24 (4–336)	36 (4–144)	25.5 (4–336)
functional outcome (n (%))[d]	12 (27)	2 (22)	0 (0)	1 (17)	1 (10)	8 (44)
funding by manufacturer (n (%))	29 (64)	8 (89)	3 (75)	4 (67)	5 (50)	9 (50)

QI indicates quality index; *IQR* interquartile range

[a] numbers do not add up to 45 because one study tested both clomethiazole, gavestinel, and selfotel; [b] in minutes after onset of ischaemia; [c] in hours after onset of ischaemia; [d] number of studies in which functional outcome was tested

assessment of outcome in 40 %. Previous evaluations of clinical trials and animal studies have suggested that both non-random and inadequately concealed treatment allocation may lead to overestimation of treatment effects [3, 18, 29]. Negative preclinical studies are much more likely to remain unpublished than negative large clinical trials [7]. In a systematic review of experimental stroke studies describing the efficacy of nicotinamide, comparisons published only in abstract form gave a significantly lower estimate of effect size than those published in full, demonstrating publication bias [25]. It is therefore conceivable that the career of a preclinical investigator is more dependent on obtaining positive results than that of a clinical trialist. For this reason, randomisation and blinding, which are considered essential precautions against bias in clinical trials [26, 30], should be valued equally in animal studies.

Because of their complexity, stroke models are inherently vulnerable to complications that may affect outcome, such as failure to obtain sufficient ischaemia, or perioperative hypotension or even hypoxaemia, when the airway is not secured and the animal is not ventilated with blood gas control. Given the explanatory character of preclinical studies, it appears justifiable to exclude animals with complications from the analyses of treatment effects, provided that the exclusion criteria are predefined and not determined on a *post-hoc* basis, the latter also because of the open character of most experiments. In view of the above, it is not surprising that in none of the studies an intention-to-treat analysis was used. However, only one study mentioned predefined in- and exclusion criteria, and in 12 articles (27%) exclusion of animals from analysis was mentioned and substantiated.

The above factors contributed to the low median score on the employed methodological quality scale. The scale we used differed in several aspects from those used by Horn et al. [16] and Macleod et al. [25]. Horn et al. did not include the items of sample size calculation and blinded administration of the study agent, which we consider essential in the preclinical evaluation of neuroprotectants. Omitting a sample size calculation may lead to a lack of power and thereby to an inability to detect a clinically relevant effect [8]. As discussed above, open administration of the study agent may give rise to a bias in the severity of the induced infarct.

In their 10-point scale, Macleod et al. also attributed points for the following items: peer-reviewed publication; use of an anaesthetic without significant neuroprotective activity (i. e. no ketamine); compliance with animal welfare regulations; and statement of potential conflict of interest [25]. Because of our search strategy, in which abstracts were purposely excluded, we did not attribute points for peer review of the publication. We did not include an item on the anaesthetic used, as not only ketamine [5], but also other frequently used anaesthetics such as halothane and isoflurane are reported to have intrinsic neuroprotective properties [14, 19]. Most journals now require a statement of compliance with animal welfare regulations. Although we believe such compliance is a prerequisite for doing animal research, we are not aware of evidence that this improves the methodological quality of experimental studies.

We agree with Macleod et al. that financial interests of authors or sponsors may lead to biased data interpretation. However, as stated above, obtaining positive rather than negative results may not only favour financial interests but also career opportunities. For this reason, we put an emphasis on randomisation and blinding in our quality scale, as means to prevent biased data collection and interpretation.

The last four items of our methodological quality scale, which were all based on the STAIR recommendations [31], were also incorporated in Horn's scale but not in Macleod's. In clinical trials targeting acute ischaemic stroke published between 1995 and 1999, the median time to start of treatment was 14 hours [20]. Despite increasing public awareness of acute stroke treatment, the vast majority of stroke patients do not reach the hospital within 3 hours after the onset of symptoms [1]. Although time windows for effective stroke treatment in rats may not be comparable to those in man, we think it is essential that putative neuroprotective treatment strategies are tested at clinically relevant time points after the onset of ischaemia. In our scale, a "clinically relevant time window for start of treatment" was defined as treatment started more than 60 minutes after the onset of ischaemia, as treatment of patients between 60 and 90 minutes after the onset of symptoms has been shown feasible in a small group of patients [34].

Functional outcome is indisputedly the primary measure of efficacy in clinical trials, whereas animal studies usually rely on infarct volume. Unfortunately, infarct volume does not tell us whether surviving neurons are functional, dysfunctional, or destined for death in a delayed fashion [11]. In addition, several studies have suggested that in patients the relationship between infarct volume and functional outcome is moderate at best [28, 33]. For these reasons, we suggest that in animal studies testing putative neuroprotective compounds both infarct volume and functional outcome should be assessed. We included the items on timing of outcome assessment in our scale because several studies have suggested that some neuroprotective treatment strategies only delay but do not prevent cell death [11]. However, compared with the requirements of clinical trials, our scale is still rather crude and turns a blind eye to several items that are considered desirable in clinical trials. We therefore support the call for the development of more sophisticated quality scores, perhaps with weighting of different components [25].

The aim of our study was to evaluate the method-

ological quality of animal studies testing neuroprotective compounds in focal cerebral ischaemia. We purposely only included full publications and no abstracts. Owing to space constraints, study quality reported in abstracts only is likely to under-represent true study quality. We decided not to perform a meta-analysis of the efficacy results of the animal experiments under study, as this should have included results published only in an abstract.

For the above reason, a drawback of the present study is the fact that we have likely reviewed only a subset of all animal experiments actually performed with the compounds under study. We have limited this review to studies published as full papers and have not included unpublished work, for example preliminary studies performed by the manufacturer in its research laboratories. As there is no registry of animal studies of neuroprotectants in acute ischaemic stroke and unpublished experiments may have been performed in laboratories unknown to us, we did not attempt to obtain unpublished material. Nonetheless, we consider it unlikely that the results of the present review would have been more positive if unpublished studies had been included. We also acknowledge that a decision to start a clinical trial of a specific compound is not only based on published animal work, but also on circumstantial evidence of efficacy from *in vitro* studies and evidence from 'non-stroke' models such as global ischemia and traumatic brain injury. However, the gap between ischaemic neurons in culture and the patient with ischaemic stroke should be bridged by methodologically sound animal studies of focal cerebral ischaemia.

It is obvious that the failure of neuroprotective stroke trials cannot only be attributed to flaws in preclinical studies, but also to shortcomings of clinical trials. These include, amongst others, long time windows for start of treatment, insufficient statistical power, and patient heterogeneity [11, 13]. Several recent publications have provided valuable tools to improve the identification of neuroprotective treatments in clinical trials [9, 23, 32, 37].

In conclusion, the evidence that formed the basis for the decisions that initiated 21 randomised clinical trials including more than 12,000 patients with acute ischaemic stroke was thin and obtained in animal studies with a methodological quality that would not justify a decision to perform these trials. More rigorous preclinical study methodology may lead to more reliable and reproducible results.

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Appendix

Included publications on clomethiazole [4, 11, 18, 19, 35–39], gavestinel [6, 11, 14, 30], lubeluzole [2, 3, 8, 10, 12, 13], selfotel [11, 17, 21, 22, 24, 28, 29, 31, 34, 41], and tirilazad mesylate [1, 5, 7, 9, 15, 16, 20, 23, 25–27, 32, 33, 40, 42–45].

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