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Systematic review and meta-analysis of the efficacy of melatonin in experimental stroke

Abstract: Melatonin is a candidate neuroprotective drug for ischaemic stroke. Any decision to proceed to clinical trial for such drugs should be based on an unbiased assessment of all available data. Such an assessment should include not only the efficacy of a drug but also the in vivo characteristics and limits – in terms of time window, dose, species and model of ischaemia used – to that efficacy. Here we use a systematic approach to establish the limits to and characteristics of the neuroprotective efficacy of melatonin in experimental stroke. We have used systematic review and meta-analysis to assess the evidence for a protective effect of melatonin in animal models of focal cerebral ischaemia. Fourteen studies were identified describing procedures involving 432 animals. The point estimate for the effect of melatonin was a 42.8% (95% CI 39.3–46.3%) improvement in outcome. Efficacy was greater when ketamine anaesthesia was used, and melatonin was equally effective in permanent or temporary ischaemia. Study quality was generally poor by clinical trial standards, and no evidence was found regarding the efficacy of melatonin in focal cerebral ischaemia in aged, hypertensive or diabetic animals, in species other than rats, or at time windows beyond 2 hr. These findings demonstrate a marked efficacy of melatonin in animal models of focal cerebral ischaemia, identify priority areas for future animal research, and suggest melatonin as a candidate neuroprotective drug for human stroke.

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Introduction

Despite an experimental literature describing the efficacy of more than 700 drugs in experimental stroke, only tissue plasminogen activator has proven efficacy in human studies [1]. This failure of putative neuroprotective drugs in clinical trials represents a major challenge to the doctrine that animals provide a scientifically valid model for human stroke. It has been suggested that for many drugs entering a clinical trial the animal data did not justify the expectations raised, and recently Pound et al. [2] have argued for a much more systematic evaluation of animal data before proceeding to clinical trials. Important information to be gained from animal studies includes not only the demonstration that a drug can have neuroprotective activity under ideal conditions but also some idea of the limits to that efficacy (time to treatment, dose required, efficacy in animals with comorbidities) which are likely to impact on the clinical usefulness of the drug.

The ideal candidate neuroprotective drug is both effective and is sufficiently safe to allow it to be given in a prehospital setting without extensive patient work-up. We have been developing a clinical trial protocol for the prehospital administration of combination neuroprotectants in patients with suspected stroke [the Time Window in Neuroprotection (TWiN) study]. To identify candidate drugs we performed a brief semi-systematic literature review, and then scored drugs according to criteria including the evidence for their efficacy, their safety profile, and their potential ease of use (our unpublished observations). The limits to and determinants of efficacy for drugs thus identified is subsequently examined in more detail using systematic review and meta-analysis.

Systematic review and meta-analysis have contributed greatly to the interpretation and aggregation of data in the clinical sciences. Systematic review uses a methodical approach to minimize the risk of bias in the selection of studies for inclusion, whereas meta-analysis combines results from individual studies to produce a better estimate of treatment effect [3]. Stratified meta-analysis can then be used to explore the impact of particular study characteristics [4]. For instance, we have previously reported a systematic review and meta-analysis of the efficacy of nicotinamide in experimental stroke, in which we showed that aspects of study quality and study design impacted on the estimate of efficacy [5]; additionally, there was evidence to suggest a publication bias in favour of positive results.

Melatonin was identified in our semi-systematic review as a candidate neuroprotective drug for the TWiN study. It has reported efficacy in transient global ischaemia models [6–8] and is held to possess many potentially neuroprotective properties including free radical scavenging [9] and

anti-inflammatory [10] properties. Here we have investigated the neuroprotective properties of melatonin in experimental focal cerebral ischaemia using the techniques of systematic review, meta-analysis and stratified meta-analysis. Specifically, we have calculated a global estimate of the efficacy of melatonin, and we have examined the impact of study quality and various study characteristics on the estimate of effect size.

Methods

Studies of melatonin in animal models of stroke were identified from Pubmed (1974 to Jan 2004), Embase (1980 to Jan 2004) and BIOSIS (1969 to Jan 2004): search strategy [<melatonin>] AND [<stroke> OR <ischemia> OR <ischemia>]; hand-searching abstracts of scientific meetings; reference lists of identified publications; and requests to senior authors of identified publications for references to other studies. We included all controlled studies of the effect of melatonin in animal models of focal cerebral ischaemia where the outcome was measured as a volume of infarction or a neurological score.

We defined a 'comparison' as the assessment of outcome in treatment and control groups following treatment with a given dose of drug or with vehicle, with treatment starting a given time before or after the induction of cerebral ischaemia. For each comparison we extracted data for mean outcome, standard deviation (S.D.), and number of animals per group. Values for data expressed graphically were requested from authors. Where melatonin was given in multiple doses the comparison was grouped according to the first dose at the first time it was given and the dose given was recorded as the total dose in the first 24 hr following ischaemia.

Where neurological tests were performed at different times only the final test was included. Where one group of animals was scored in more than one neurological domain (for instance motor and sensory scores), or where both neurological score and infarct volume was measured, data were combined using meta-analysis (see below) to give an overall estimate of effect size and its standard error. We defined effect size as the proportional reduction in outcome (infarct volume, neurological score or combined score) in treated animals relative to untreated ischaemic controls.

Methodological quality of individual studies was assessed according to previously published criteria [5, 11]. These were peer reviewed publication; statement of control of temperature; random allocation to treatment or control; blinded induction of ischaemia; blinded assessment of outcome; use of anaesthetic without significant intrinsic neuroprotective activity; appropriate animal model (aged, diabetic or hypertensive); sample size calculation; compliance with animal welfare regulations; and statement of potential conflict of interests. Each study was given a quality score out of a possible total of 10 points, and the group median was calculated.

Data were processed as previously described [5]. Briefly, for each comparison, the mean outcome for the treatment group and the S.D.s in treatment and control groups were expressed as a proportion of the outcome in the control group, and the effect size (the difference between the treatment and control groups) and its standard error were calculated. Data were aggregated using a weighted mean difference method with the random effects model of DerSimonian and Laird [12]. This is a generally more conservative technique than fixed effects meta-analysis.

To explore the impact of study characteristics on estimates of effect size we then performed a stratified meta-analysis with experiments grouped according to methodological score; use of aged, diabetic or hypertensive experimental animals; anaesthetic used; whether the data had been published in full or in abstract; permanent or temporary ischaemia; method of occlusion (endovascular or extravascular); outcome measure; route of drug delivery; single or multiple dosing regime; and species and gender of animal used. The significance of differences between groups was assessed by partitioning heterogeneity and using the χ^2 distribution with n − 1 degrees of freedom, where n equals the number of groups.

Results

Electronic searching identified 222 publications, of which 16 (13 papers, three abstracts) described experiments reporting the effect of melatonin in focal cerebral ischaemia where the outcome was expressed as a volume of infarction or a neurological score. Hand searching identified a further four abstracts, none of which met the inclusion criteria. Two abstracts described work also described in full papers [13, 14], and one paper included data later published as part of a larger series [15]. This meta-analysis is therefore based on data from 12 full papers [16–27] and one abstract [28]. Within these 13 publications, 28 comparisons (see Methods for definition) were identified.

Study characteristics are shown in Table 1. No study described a sample size calculation, used aged animals or those with comorbidities, or contained a statement of potential conflict of interest. Random allocation to treatment group and blinded assessment of outcome were described in four studies each, and two studies reported that ischaemia was induced by an investigator blinded to treatment allocation. The median quality score (see Methods) was 4 (range 0–6); eight of 13 studies scored 4, and while classifying the studies by quality score accounted for a significant part of the between-group heterogeneity ($\chi^2 = 9.1$, d.f. = 3, $P < 0.05$) there was no clear association between quality and the estimate of effect size.

The global estimate of the effect of melatonin was 0.428 (95% confidence interval 0.393–0.463, $P < 0.00001$), an improvement in outcome of around 40% (Fig. 1). Overall, there was no significant statistical heterogeneity ($\chi^2 = 25.8$, d.f. = 27, $P = 0.53$) between comparisons. Significant protection was seen with all doses of melatonin, with maximum protection occurring at doses above 5 mg/kg (Fig. 2A). Similarly, significant protection was seen for all time points, but treatments beginning more than 60 min after the onset of ischaemia was less effective than those beginning before this time (Fig. 2B).

Study design characteristics are shown in Table 2. Melatonin was more effective when given in multiple (0.490, 0.443–0.536) rather than single doses (0.350, 0.298–0.403; $\chi^2 = 10.6$, d.f. = 1, $P < 0.01$). There was increased efficacy in studies where ketamine anaesthesia

Table 1. Quality characteristics of included studies

Publication	Year	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Score	Reference
Joo	1998	✔	✔				✔			✔		4	16
Kilic	1999	✔	✔			✔				✔		4	17
Ling	1999	✔	✔	✔	✔	✔	✔					6	18
Peker	2000											0	28
Borlongan	2000	✔	✔	✔			✔			✔		5	19
Sinha	2001	✔	✔				✔			✔		4	20
Pei	2002	✔	✔				✔			✔		4	21
Gupta	2002	✔	✔				✔			✔		4	22
Pei	2002	✔	✔				✔			✔		4	23
Sun	2002	✔	✔	✔	✔	✔	✔					6	24
Pei	2003	✔	✔				✔			✔		4	25
Torii	2004	✔		✔			✔			✔		4	26
Lee	2004	✔	✔			✔	✔			✔		5	27

Studies fulfilling the criteria of (1) peer reviewed publication; (2) control of temperature; (3) random allocation to treatment or control; (4) blinded induction of ischaemia; (5) blinded assessment of outcome; (6) use of anaesthetic without significant intrinsic neuroprotective activity; (7) animal model (aged, diabetic or hypertensive); (8) sample size calculation; (9) compliance with animal welfare regulations and (10) statement of potential conflict of interests.

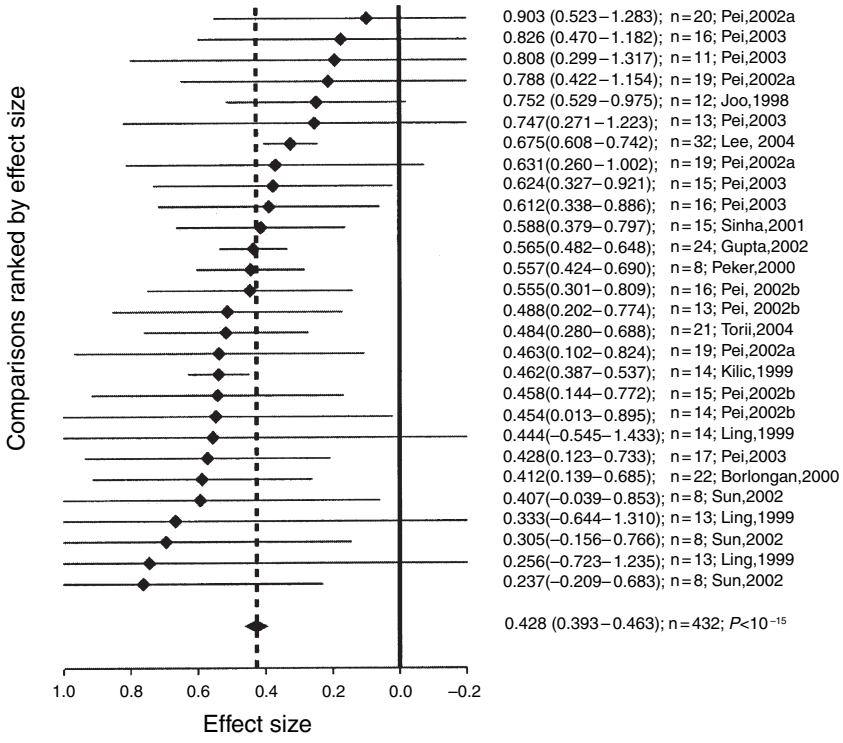

Fig. 1. Point estimate and 95% confidence intervals for global estimate and each of 28 comparisons ranked by effect size. Effect size is the improvement in treated animals expressed as a proportion of the outcome in control animals. The diamond indicates the global estimate and its 95% confidence interval. The solid vertical line marks where treatment and control are equal. The text gives the point estimate and 95% confidence limit for each comparison; the number of animals contributing to that comparison; and the publication from which that comparison was extracted.

was used (0.538, 0.463–0.613 versus 0.397, 0.358–0.437; $\chi^2 = 7.4$, d.f. = 1, $P < 0.01$). Efficacy was higher where the sex of the animal used was not stated (0.514, 0.446–0.582) than where male animals were used (0.393, 0.352–0.434; $\chi^2 = 6.7$, d.f. = 1, $P < 0.01$); no papers reported the use of female animals (Fig. 3).

There were no significant differences in estimates of efficacy between experiments using infarct volume as outcome compared with those using a combined score; between experiments published in full or in abstract; between those using permanent rather than temporary models of focal ischaemia; between those using extravascular (surgical) rather than endovascular (filament) methods for middle cerebral artery occlusion; or between those using intraperitoneal, intravenous, subcutaneous or oral routes of melatonin administration. No experiments described efficacy in animals other than rats, or where outcome was measured as a neurological score alone.

Discussion

Treatment with melatonin led to improved outcome in focal cerebral ischaemia; overall, there was a highly significant effect of more than 40%, and this appeared to be independent

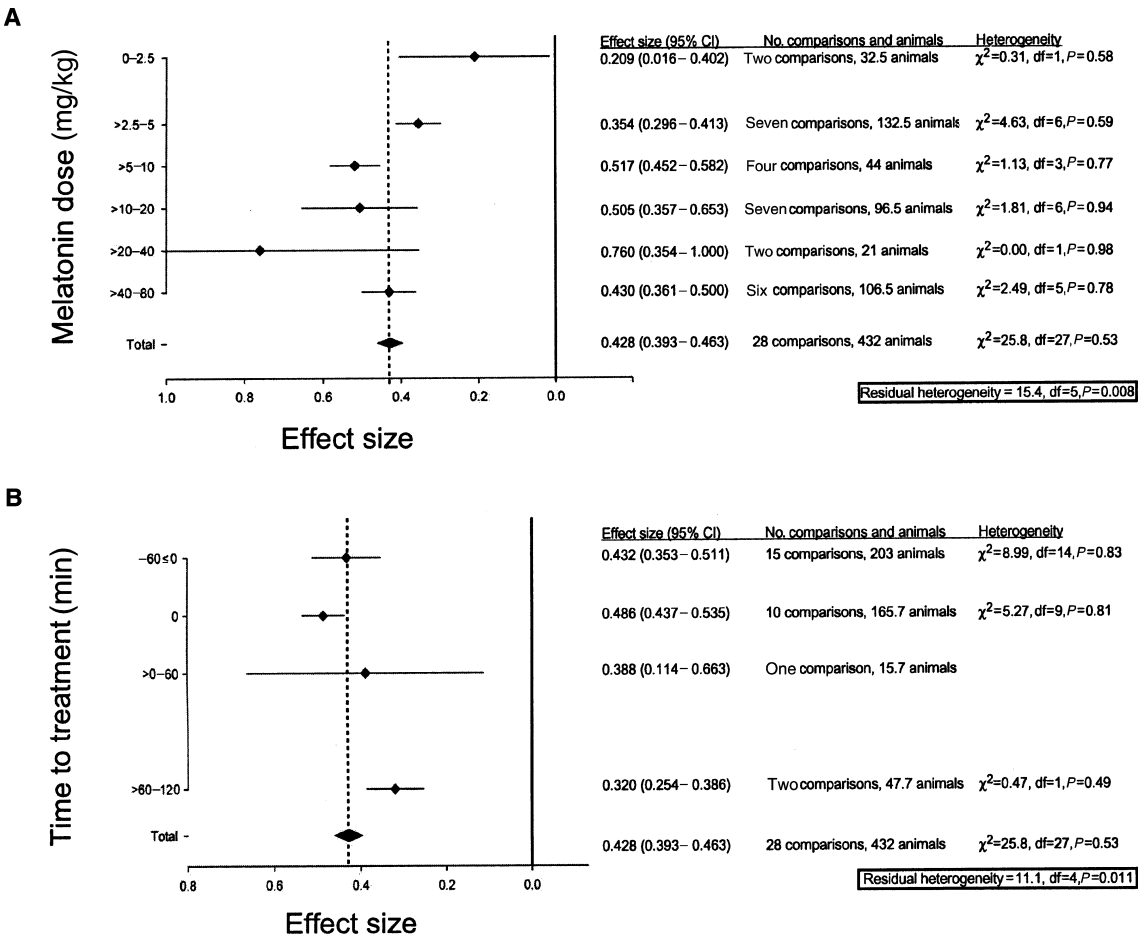

Fig. 2. Point estimates and 95% confidence intervals of effect size for (A) dose and (B) time to treatment. Where the 95% confidence interval does not reach the solid vertical line outcome is significantly different from control ($P < 0.05$). The text indicates effect size and 95% confidence interval; number of comparisons and of animals contributing to each point; χ^2 test and probability of observed heterogeneity. The boxed text describes the residual (between group) heterogeneity and its significance.

Table 2. Design characteristics of included studies

Publication	Gender	n (C)	n (Rx)	Dose range (mg/kg)	Doses in first 24 hr	Time to treatment	Anaesthetic	Permanent or focal ischaemia	Route of drug delivery	Outcome measure
Joo (1998)	Male	6	6	2.5	4	−15 min	Chloral hydrate	Temporary	i.p.	Inf. vol.
Kilic (1999)	Nk	8	6	4	2	0 min	Ketamine	Temporary	Intravenous	Comb
Ling (1999)	Male	9	31	2.5–10	3	−15 min	Chloral hydrate	Temporary	Subcutaneous	Inf. vol.
Peker (2000)	Nk	2	6	2.5	4	−20 min	Not known	Permanent	i.p.	Comb
Borlongan (2000)	Male	11	11	23.2	1	0 min	Halothane	Temporary	Oral	Comb
Sinha (2001)	Male	7	8	20	4	0 min	Chloral hydrate	Temporary	i.p.	Comb
Pei (2002a)	Male	14	61	1.5–50	1	−30 min	Pentobarbital	Temporary	i.p.	Inf. vol.
Gupta (2002)	Male	12	12	20	4	0 min	Chloral hydrate	Temporary	i.p.	Comb
Pei (2002b)	Male	21	23	5–50	1	−30 min	Pentobarbital	Permanent	i.p.	Inf. vol.
Sun (2002)	Male	6	18	2.5–10	3	−15 min	Chloral hydrate	Temporary	i.p.	Inf. vol.
Pei (2003)	Male	44	57	5–15	1–3	0–120 min	Pentobarbital	Temporary	i.p.	Inf. vol.
Torii (2004)	Male	11	10	5	1	0 min	Halothane	Temporary	Oral	Inf. vol.
Lee (2004)	Male	16	16	5	1	90 min	Halothane	Temporary	Intravenous	Comb

Number of animals in control group [n (C)]; number of animals in experimental group [n (Rx)]; dose range; number of doses given in first 24 hr; interval from onset of ischaemia to start of treatment; anaesthetic used; and outcome measure used; Nk, not known; i.p., intraperitoneal.

of the outcome measure used. Improved outcome was seen for every dose of melatonin used and at each time point studied, but maximum efficacy was seen at doses of more than 5 mg/kg given within 60 min of the onset of ischaemia, and there was no proximal limit to the time window for protection. The dose of melatonin at which full efficacy is

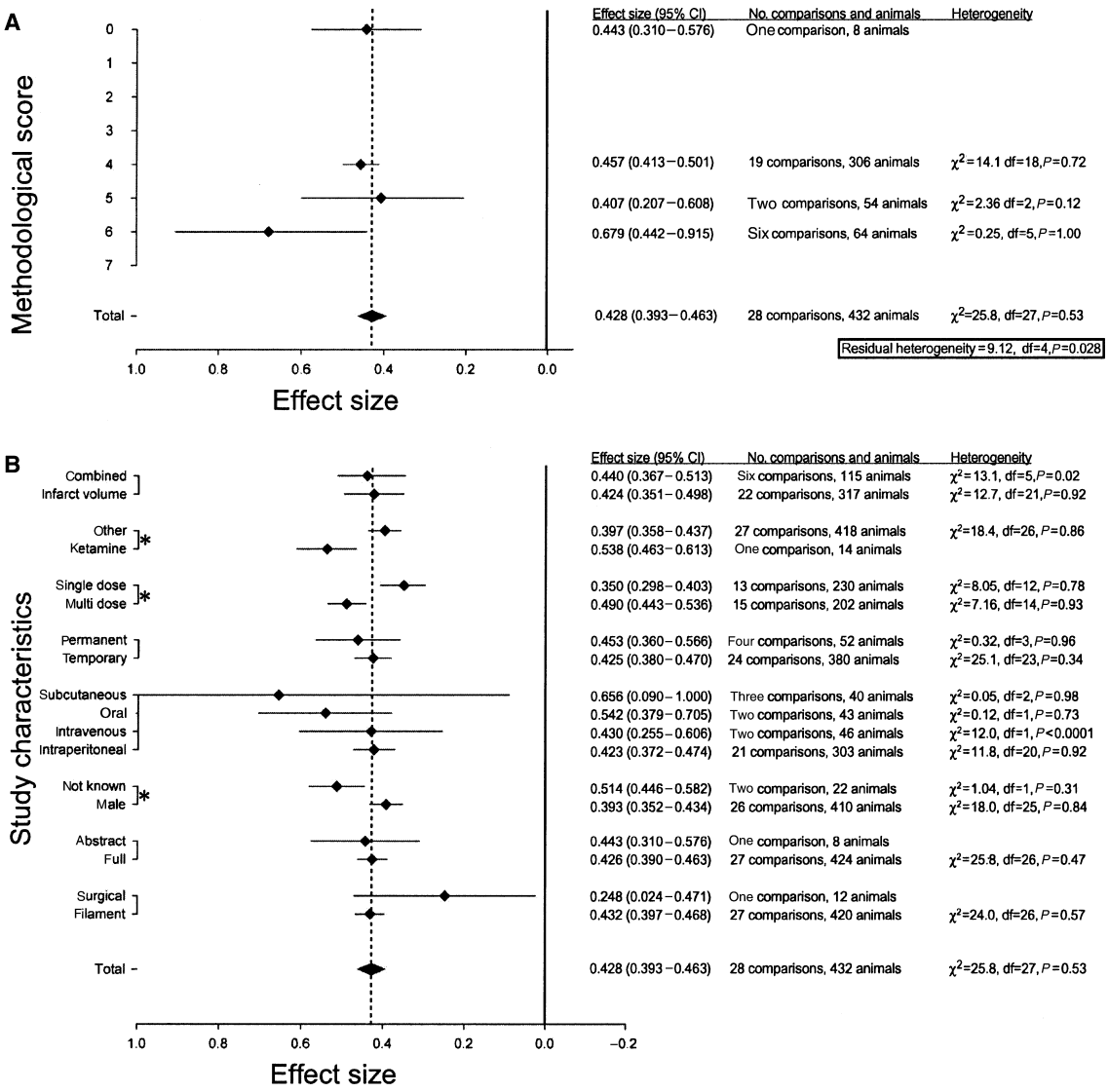

Fig. 3. Point estimate of effect size and 95% confidence intervals for a stratified meta-analysis according to (A) methodological score or (B) study characteristics. For details see Fig. 2 legend. In (B) $*P < 0.01$ by partitioning of heterogeneity.

seen is substantially higher than that used in man for the prevention of jet lag (around 0.05 mg/kg) [29], although humans have received doses approaching 100 mg/kg/day without apparent ill effect [30]. Interestingly, and in contrast to our findings for nicotinamide [5], melatonin was equally effective in permanent and in temporary ischaemia. Since our search was performed, two further publication meeting our inclusion criteria have been published [31, 32], and both give estimates for the efficacy of melatonin consistent with those from our meta-analysis.

The stratified meta-analysis suggested that the use of ketamine anaesthesia leads to an absolute over-estimation of effect size of around 15%. This is in keeping with its known actions as a noncompetitive NMDA antagonist [33], with reports of synergistic activity with putative neuroprotective drugs [34], and with our findings for nicotinamide [5]. There was an absolute increase in effect size of around 15% where melatonin was given in multiple doses rather than a single dose. The elimination half-life of melatonin following oral administration in rats is <20 min [35], so it may be that increased efficacy following multiple dosage reflects a short duration of action following single dosage. Alternatively, it may reflect drug dose; 14 of 15 comparisons using a multiple dosage regime gave more than 5 mg/kg in the first 24 hr, compared with five of 13 comparisons using single dose.

The global estimate of the efficacy of melatonin is higher than that reported for nicotinamide, the only other neuroprotective drug to have been analysed in this way. While melatonin may indeed be more effective than nicotinamide, there are a number of other possible explanations for this observation. First, evidence for nicotinamide came from experiments involving mice as well as those involving rats, and from experiments using animals with co-morbidities (hypertension and diabetes) as well as the healthy young adult rats used in the melatonin experiments analysed here. Secondly, there were differences in experimental design; the efficacy of nicotinamide was tested up to 12 hr following stroke, compared with only 2 hr for melatonin. A higher

proportion of the melatonin comparisons (13 of 28) gave repeated doses of drug than was the case for nicotinamide (three of 72), and repeated melatonin dosage was significantly more effective than once-only treatment. Finally, the melatonin and the nicotinamide experiments were carried out by different research groups, and it may be that different groups have slightly different experimental protocols which lead to over- or under-estimates of true effect size.

Grouping studies by quality accounted for a degree of the heterogeneity between studies, but there was no clear relationship between increasing quality and the estimate of effect size. Study quality was similar to that for nicotinamide [5] but no study met published criteria for reporting clinical trials [36]. To what extent deficiencies in study design such as failure to randomize between groups and unblinded assessment of outcome may have lead to the overestimation of the effect of melatonin is not known. However, these deficiencies apply to most if not all of the animal literature, and do not invalidate comparison with other putative neuroprotective drugs.

True study quality is often higher than the score allocated from details available in the publication, particularly for publications available only in abstract. Where the length of an abstract is limited it is clearly difficult to convey experimental detail, and even in full publication constraints of space may militate against a complete description of the steps taken to minimize bias. We therefore support the development of an agreed quality standard for experiments in focal cerebral ischaemia, compliance with which could be stated in full publication or abstract at minimal cost in space.

There are a number of potential difficulties with the application of meta-analysis in this context. First, the analysis can only take into account published or otherwise identifiable data. If there is a tendency – as has been demonstrated in clinical medicine – for negative studies to remain unpublished, then any attempt at meta-analysis will overstate efficacy. Furthermore, if there is bias in the results of individual studies, perhaps because of nonrandomization or unblinded assessment of outcome, then this bias will be reflected in the meta-analysis. Secondly, it has been argued that the important human outcome following stroke is in a broad sense 'behavioural' rather volumetric and therefore, it is argued, only studies with a behavioural outcome should be included. While this approach is empirically attractive there is no evidence that behavioural outcomes in an experimental animal provide any better indication of efficacy in humans than measurement of infarct volume. Thirdly, stratified meta-analysis remains a form of subgroup analysis; although the stratifications reported here were prespecified and all such analyses have been reported, the results may have arisen by chance and should be interpreted with caution; they should be viewed as hypothesis generating only.

Finally, there is a conflict in the selection of studies for inclusion; on one hand, one would wish only to include studies of the highest quality which are likely to give the most precise estimate of effect size. On the other hand, if there is publication bias then restricting inclusion in this way might have the effect of increasing such bias, for instance because of the exclusion of data presented in abstract only as discussed above. However, as the weighting given to each study is based on its standard deviation then some account is given to study quality, as poor studies are likely to give less precise estimates of effect size with higher standard deviations and thus be given less weight in the meta-analysis.

On the basis of our findings we suggest that the priorities for research regarding the neuroprotective properties of melatonin in focal cerebral ischaemia include (i) efficacy beyond 2 hr; (ii) efficacy in animals with diabetes and hypertension, and in aged animals; (iii) efficacy in animals other than rats and (iv) head-to-head comparison of efficacy with and without ketamine anaesthesia, in permanent versus temporary ischaemia, in single versus multiple dosage and in males versus females. Accumulation of such evidence would allow a more complete assessment of melatonin's neuroprotective properties.

These findings also have broader implications for the use of systematic review and meta-analysis in the assessment of putative neuroprotective drugs. This technique has now been applied to two drugs, and in both cases has provided novel insights into drug properties in in vivo models of ischaemia. We have now accumulated data for 1333 animals from 91 individual comparisons. As the number of drugs analysed in this way increases, multiple regression modelling should allow identification of those factors which have greatest impact on estimates of effect size. In turn, this may allow identification of a subset of variables which are sufficient to describe the properties of a given drug, potentially reducing the number of experiments needed to characterise that drug. Where drugs or groups of drugs depart from the derived model, this may reflect distinct in vivo properties of those drugs, and this could provide the basis for a system of drug classification based on in vivo rather than in vitro or ex vivo characteristics.

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