SUPPLEMENTAL MATERIAL

A Systematic Review and Meta-Analysis of Bone Marrow Derived Mononuclear Cells in Animal Models of Ischemic Stroke

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Supplemental Methods:

Proposal for Systematic Review and Meta-Analysis of Bone Marrow Derived Mononuclear Cells for Animal Models of Ischemic Stroke

The aim of this proposal is to evaluate the treatment effect of bone marrow derived mononuclear cells (BM MNCs) on histological and behavioral outcomes in small animal models of ischemic stroke along with assessment of quality of published studies and exploration of sources of heterogeneity within the pooled estimates. This will be achieved via a comprehensive systematic review of up to date literature followed by meta-analysis of selected outcome measures that emulate clinical parameters for determining stroke recovery.

Formulation of the specific research question

There are a few systematic reviews and meta-analyses of preclinical stroke models that have looked at broader questions of efficacy of cellular therapy in neurological diseases and ischemic stroke,[1, 2] or at certain other kinds of cells.[3, 4] However, there have been no systematic reviews published for effect of BM MNCs in pre-clinical models of ischemic stroke. It is recognized that there are several sources of heterogeneity in terms of cellular therapy for stroke and hence it is important to study specific cell types when attempting to pool an interventional effect size. It is therefore the objective of this systematic review to focus in on the use of BM MNCs – it nevertheless remains important to identify the potential sources of heterogeneity within the use of this cell type.

The specific research question for this analysis will therefore be:

Does intravenous transplantation of Bone Marrow derived Mononuclear Cells, without any augmenting agents, confer benefit as measured by stroke lesion volume and other behavioral tests, in small animal models of focal cerebral ischemia?

Selection of animal models

Mice and rats are most commonly used animals to model stroke. However, some larger species like rabbits,[5] canines,[6] and even some non-human primates[7] have been used in some pre-clinical studies. Relatively sparse use of larger animal models may be attributed to considerations of cost and feasibility; however for some species like dogs, there are important anatomical differences in cerebral vasculature that make direct comparisons difficult.[8] For the purpose of this systematic review focus will be on preclinical studies conducted on mice and rats. Like the humans, the Internal Carotid Artery in rats branches off into Anterior and Middle Cerebral Arteries (ACA and MCA). As MCA ischemic stroke is most common in humans,[9] the MCA occlusion (MCAO) in rats and mice is the most commonly used animal model for stroke. A number of methodologies to obtain MCAO in rats and mice have been described; these include electrocoagulation, mechanical occlusion, or a pharmacological intervention following exposure of blood vessel via carniectomy.[10] Other intravascular approaches have also been adopted in which opening of the skull is avoided (hence preventing potential confounding effect of additional trauma on behavioral outcomes), and a thrombus or filament is advanced to block the MCA.[11-13] For the

purpose of this review all models of MCAO will be included. Description of model, along with listing of medications used for anesthesia will be used as a quality index and the possible variance in effects with use of different models will be explored in analysis.

Cell type, source, and administration

As has been stated, this review will focus on use of Bone Marrow derived Mononuclear Cells (BM MNCs). Mononuclear cells (MNCs) are a heterogeneous population of cells that can be isolated from the bone marrow, blood, or even some extra embryonic tissues like umbilical cord. For the purpose of this review only those studies will be included that report utilization of bone marrow BM MNCs. However, BM MNCs obtained from any species i.e. human or animals will be included. The source of BM MNCs will be extracted as a variable for exploration of any potential differences in effects. Furthermore, this review will focus on intravenous administration of BM MNCs. Another factor is time of cell infusion in relation to the induction of stroke in animals. Individual studies have demonstrated that a sub-acute time window may provide better targets for cellular therapy. The time of cell administration will be extracted from studies and will be evaluated for a possible treatment effect.

Outcomes

Most preclinical experiments use histological and functional / behavioral tests for assessment of effect of stroke pharmacotherapy. Infarct volumes can be quantified either by histologically stained brain sections or by using non-invasive imaging modalities like diffusion weighted magnetic resonance imagining (DWI / MRI).[14] While estimating infarct volumes it is important to correct for changes in brain volume consequent to edema. A number of methods for correction of this volume have been described.[15, 16] The finally calculated infarct volume can either be expressed in absolute terms as mm^3 or relative to the volume of contralateral (non-infarcted) brain hemisphere.

Though infarct volume is reported in most pre-clinical studies, behavioral and functional tests performed on animals may have greater clinical relevance for translational purposes. A number of neurological scales, tests for assessment of sensorimotor function, and cognition have been reviewed in literature.[17] The most commonly employed assessments are modified neurological severity score (mNSS), cylinder test, accelerated rotarod, and adhesive removal test.

Study design and Setting

This is a systematic review and meta-analysis for estimating the treatment effect of intravenous infusion of bone marrow derived mono-nuclear cells on infarct volumes and sensorimotor outcomes in rats and mice for focal cerebral ischemia caused by middle cerebral artery occlusion. The search, extraction and storage of data, data analysis, reporting and assimilation of results will be done at the University of Texas Medical School at Texas Medical Center in Houston, TX.

Inclusion / Exclusion Criteria

Studies will be included if they meet all of the following criteria:
1. Pre-clinical models of rat and mice

2. Focal ischemic stroke model caused by middle cerebral artery occlusion
3. Use of bone marrow derived mononuclear cells (BM MNCs)
4. Intravenous administration of BM MNCs
5. All sources of BM MNCs will be included i.e. autologous, allogeneic, or xenogeneic

Studies will be excluded if they have any of the following features
1. Large animal models
2. Global ischemia caused by any other model than MCAO
3. Use of cells other than MNCs, like mesenchymal stromal cells (MSCs) or hematopoietic stem cells (HSCs)
4. Mononuclear cells from sources other than bone marrow like umbilical cord of peripheral blood
5. Models of hemorrhagic stroke
6. Routes of administration other than IV route like stereotactic injection directly into the infarct location / brain
7. Studies in which MSCs are used in conjunction with other mediators of substances that are postulated to enhance availability or activity of cells

Search Strategy: Important aspects of the search strategy to be adopted for this systematic review are detailed below

Identification of databases

The two main databases that will be used for this search are *MEDLINE* and *EMBASE*. The interfaces that will be used to access MEDLINE and EBMASE are PubMed, EMBASE.com, and Ovid. Using PubMed will not only allow access to MEDLINE but also non-indexed citations. Additional sources that will be utilized are Web of Science, Scopus, and CINAHIL. Finally a database for the Animal Welfare Information Center (AWIC) will also be accessed and searched.[18]

Research question component search

The four elements of the specific research question that has been described above are:
Intervention: Bone Marrow Mononuclear Cells
Disease: Focal cerebral ischemia
Population: Rats and Mice
Outcomes: Lesions size and sensorimotor tests

The initial search will comprise of SC 1 and 2, allowing for greater sensitivity. For each SC, a separate string derived from Medical Subject Headings (MeSH) and free text will be used, and later combined using the Boolean operator 'OR'. Finally, the results obtained for separate SC will be combined using the Boolean operator 'AND'.

For each of the SC, a separate search string will be used comprising relevant search terms. These search terms will have two sources; first, Medical Subject Headings (MeSH) will be used to allow for searching all indexed studies on the MEDILINE. And second, free-text terms will be added to the search string to account for non-indexed studies. Both the MeSH and free-text terms

will then be combined with the Boolean Operator 'OR' for each SC. As the aim is to perform a comprehensive search, each search string (for individual SC) will be designed to allow for high sensitivity. It is recognized that this strategy may result in a high rate of 'false positive' results that are potentially irrelevant. The process of search string design will be documented and repeated for each SC. Finally, the results obtained from individuals SC will be combined using the Boolean operator 'AND' from search history. It is important to note that developing and conduct of systematic review with an aim to have high sensitivity in the beginning of the process and selection of only relevant literature by its termination is an iterative process. The outline of this process as described above is schematically represented in Figure I.

Study quality assessment

Each finally selected study based on the methodology described above will be assessed for quality. The scale used for quality assessment will be based on published standards[1] that have been derived from the criteria agreed upon by a consortium of experts in the field.[19] The studies will be assessed on 10 aspects and one point will be ascribed to each criteria fulfilled. The total score therefore will range from $(0 - 10)$ with a higher score indicating higher study quality. These include the following parameters:
1. Publication in a peer-reviewed journal
2. Statements describing control of temperature during experimentation
3. Presence of control group in the experiment
4. Random allocation of animals to the experimental and control arms
5. Allocation concealment
6. Blinded assessment of outcomes
7. Statements describing use or preferably avoidance of drugs / anesthetics – agents which may in themselves have a neuro-protective effect following induced ischemia
8. Use of animals with relevant comorbidities
9. Justification of sample size or power calculations
10. Statement of compliance with animal welfare regulations and conflicts of interest

Data extraction

Data will be extracted from each selected article independently by two investigators independently. The abstracted data will be stored electronically and there is no use of paper case report forms. Two versions of electronic databases will be created in Microsoft Access. These versions will be used by two independent raters. One abstractor will be blinded to the authors, institution, and the journals for the articles. For articles in which only figures are used, the data will be obtained via quantitative methods that employ use of high resolution images and digitizing software.[20] These data will then be compared for consistency and any discrepancies will be adjudicated by a third expert investigator. A difference of less than 5% will be considered acceptable and in such case a mean of two values will be used. Once adjudication of all data elements has been done, the database will be completed for information on the institutions, authors, and journal for all references. In its final shape the database will consists of following variables:

1. Article information on authors, institutions, and journals
2. Intervention data

3. Source of BM MNCs (species)
4. Route of administration (IV or IA)
5. Dose of BM MNCs
6. Timing of administration after stroke induction
7. Type of administration (autologous, allogeneic, xenogeneic)
8. Methods of isolation of BM MNCs
9. Animal Models
10. Types of animals
11. Age / Comorbidities of animals
12. Model used for experimental stroke
13. Use of drugs during experimentation
14. Experimental design data
15. Number of animals per study arm
16. Random allocation and blinded assessments
17. Sample size / Power determination
18. Outcomes data
19. Lesion size
20. Data on sensorimotor outcomes
21. Neurological scales
22. Quality assessment data
23. Statements of ethics and conflict of interest

Data Analysis

A PRISMA flow diagram will be presented to outline the systematic review documentation. Articles with more than one experiment or multiple arms of the same experiment; will be included if data from a control arm and an IV BM MNC treatment arm are exclusively identifiable. Based on their measurement scales, some outcomes (e.g. lesion volume) will be pooled separately whereas inversion of scale will be done for some as has been recommended by the Cochrane Collaboration guidelines. If outcomes are assessed at different time points, then the farthest time point will be selected as that would allow for maximum recovery from experimental stroke in the control group. Descriptive analysis will be done for providing an account of number of studies, animals, and their characteristics included in the final data.

Effect sizes will be defined as the improvement in outcomes for BM MNC treated animals relative to the control group. The standardized difference between means is quantified using the following general formula:

$$\delta = \frac{\mu_1 - \mu_2}{\sigma} \qquad (1)$$

Where delta (δ) is the effect size, mu (μ) is the mean, and sigma (σ) is the standard deviation in the control group. The most commonly employed standardized means differences are Hedges's g and Cohen's d. It has been recommended to use Hedges's g as a pooled estimate for small sample sizes along with a small sample size correction factor.[21] Hedges's g will therefore be used for this analysis and 95% confidence interval will be reported around the estimated pooled effect based on following formulae:

$$g = \frac{\mu_1 - \mu_2}{\sigma_{pooled}} \tag{2}$$

And

$$\sigma_{pooled} = \sqrt{\frac{[(\sigma_1)^2(n_1 - 1)] + [(\sigma_2)^2(n_2 - 1)]}{(n_1 + n_2) - 2}} \tag{3}$$

And the 95% confidence interval calculated using

$$CI = g \pm Critical\ value\ at\ 0.05 \times SD_g \tag{4}$$

Where the SD of g is given by:

$$SD_g = \sqrt{\frac{N}{n_1 + n_2} + \frac{g^2}{2N}} \tag{5}$$

Identification and quantification of heterogeneity

Chi- square test for heterogeneity between the studies will be performed. It has been shown that this test generally has lower power, and therefore a higher significance level (Alpha = 0.1) will be used as has been suggested in literature.[22] Further quantification of heterogeneity will be done using the I^2 statistic. If 'Q' is the chi-square statistic then I^2 is:

$$I^2 = \left[\frac{Q - df}{Q}\right] \times 100 \tag{6}$$

And is arbitrarily interpreted as following overlapping categories[23]
0% - 40%:   Heterogeneity may not be important
30% - 60%:   Moderate heterogeneity
50% - 90%:   Substantial heterogeneity
75% - 100%:   Considerable heterogeneity

It is likely that a significant degree of heterogeneity is observed and an attempt will be made to explore the sources of heterogeneity in terms of important clinical factors like animal species, dose of BM MNCs, site of administration, and time of administration since stroke induction etc, using univariable meta-regression. However feasibility of these analyses rely on number of studies included in the meta-analyses. Furthermore, the quality score for each study will also be assessed for observed heterogeneity. However, with a small number of studies it may not be possible to explain heterogeneity for all sub-groups and random effects models will be used, as has been suggested by the Cochrane collaboration.[24]

Evaluation of publication bias

Publication bias will be evaluated initially using funnel plots. Funnel plots will be constructed as a scatter plot for the effect size of each study against the standard error for the effect size. A

reversed scale will be used, placing larger and more powerful studies towards the top. An overall symmetry in the funnel plot generally satisfies lack of publication bias.[25] Egger test has been proposed as a formal statistical test for assessment of publication bias.[26] This test will also be used to assess for publication bias. In case of obvious asymmetry a Trim and Fill procedure may be used.[27] We will further evaluate the influence of having potential negative or null non-published studies using Fail-Safe N and Orwin Fail-Safe N analyses.[28, 29]

Animal subjects considerations

This study does not involve any animal experimentation. All data to be analyzed is published and publically available. Data analysis and data storage will be on the University of Texas Stroke Server at the University of Texas Medical School.

Supplemental Tables:

Table I: PRISMA checklist for minimum set of items for reporting in systematic reviews and meta-analyses.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Title Page (M)*
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1 (M)
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2 (M)
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2 (M) & (OS)**
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Yes (OS)

Section/topic	#	Checklist item	Reported on page #
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	3 (M) 3 & 4 (OS)
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	3 (M) 4 & 5 (OS)
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5 (OS)
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	3 (M) 5 (OS) Figure 1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	3 (M) 5 & 6 (OS)
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6 (OS)
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6 (OS) 4 (M)
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	4 (M) 6 (OS)
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	4 (M) 6 (OS)
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	4 (M) 6 & 7 (OS)
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	4 (M) 6 & 7 (OS)

Section/topic	#	Checklist item	Reported on page #
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4 (M) Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table II (OS)
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table II and III (OS)
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	5 (M) Figure 2a,2b,3 (M) and Figure IIa,IIb,IIIa,IIIb (OS)
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	6 (M), Figures 4a/4b (M)
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	6 (M)
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	6-9 (M)
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	6-9 (M)
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	9 (M)

Section/topic	#	Checklist item	Reported on page #
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	NA

*M: Manuscript
**OS: Online Supplement

Table II: Major Outcomes of Various Modalities Tested in Experiments Included in Meta-Analyses

Outcome & Measurement Scale	Number of experiments – n (%) of 101	Modality Tested	Directional improvement on scale	Estimates Pooled
Lesion Size	28 (27.7)	Histological / Imaging Marker		
Cubic mm	18		Lower	Yes
% of Contralateral Hemisphere or initial volume	9		Lower	Yes
mm square	1		Lower	No
Cylinder Test	18 (17.8)	Motor Function		
Asymmetry Score	12		Lower	Yes
Symmetry Score	6		Higher	Yes
Adhesive Removal Test	15 (14.8)	Sensorimotor Function		
% effort of contralateral limb	8		Higher	Yes
Time to removal of adhesive	6		Lower	Yes
Ratio of limb effort	1		Higher	No
Neurological Deficit Score	11 (10.9)			
Score (0 – 5)	6	Motor Function	Lower	Yes
Score (0 – 10)	5	Composite (Motor, Posture, Locomotion)	Lower	Yes
Modified Neurological Deficit Score	6 (5.94)	Composite (Motor, Sensory, Reflex)		
Score (0 – 24)	6		Lower	Yes

Table III: Outcomes not included in quantitative meta-analyses

Outcome	Number of experiments – n (%) of 101	Modality Tested	BM MNC Treatment Related Improvement	Data Source
Corner Test	3 (2.9)	Sensorimotor, Postural Asymmetry	Significant improvement	1 Study – 3 Arms
Circling Test / Behavior	2 (1.9)	Motor Function	Significant Improvement with 30 million cells. No difference with 1 million cells	1 Study – 2 Arms
Ladder Rung Test	2 (1.9)	Motor Function / Skilled Walking	No difference for both Young and Aged Rats	1 Study – 2 Arms
Modified Open Field Task	2 (1.9)	Generalized Locomotion	Significant improvement in locomotion	2 Studies
Rota Rod Test	2 (1.9)	Motor Function	No difference in duration sustained on Rota rod	2 Studies
Water Maze	2 (1.9)	Spatial Learning & Memory	Significant improvement in 1 study. No difference in other	2 Studies
Beam Walk	1 (0.9)	Motor Function / Balance	Significant improvement in high difficulty beam. No difference in low and medium difficulty	1 Study
Neurological Score	1 (0.9)	Motor Function	No difference in Menzies Neurological Score	1 Study
Open Field Task (Distance Traveled)	1 (0.9)	Generalized Locomotion	No difference	
Open Field Task (Grooming)	1 (0.9)	Generalized Behavior	No difference	1 Study
Open Field Task (Rearing)	1 (0.9)	Motor Function / Generalized Behavior	No difference	
Open Field Task (Latency Time)	1 (0.9)	Generalized Locomotion	Significant improvement	
Tread Mill	1 (0.9)	Motor Function	Significant improvement	1 Study

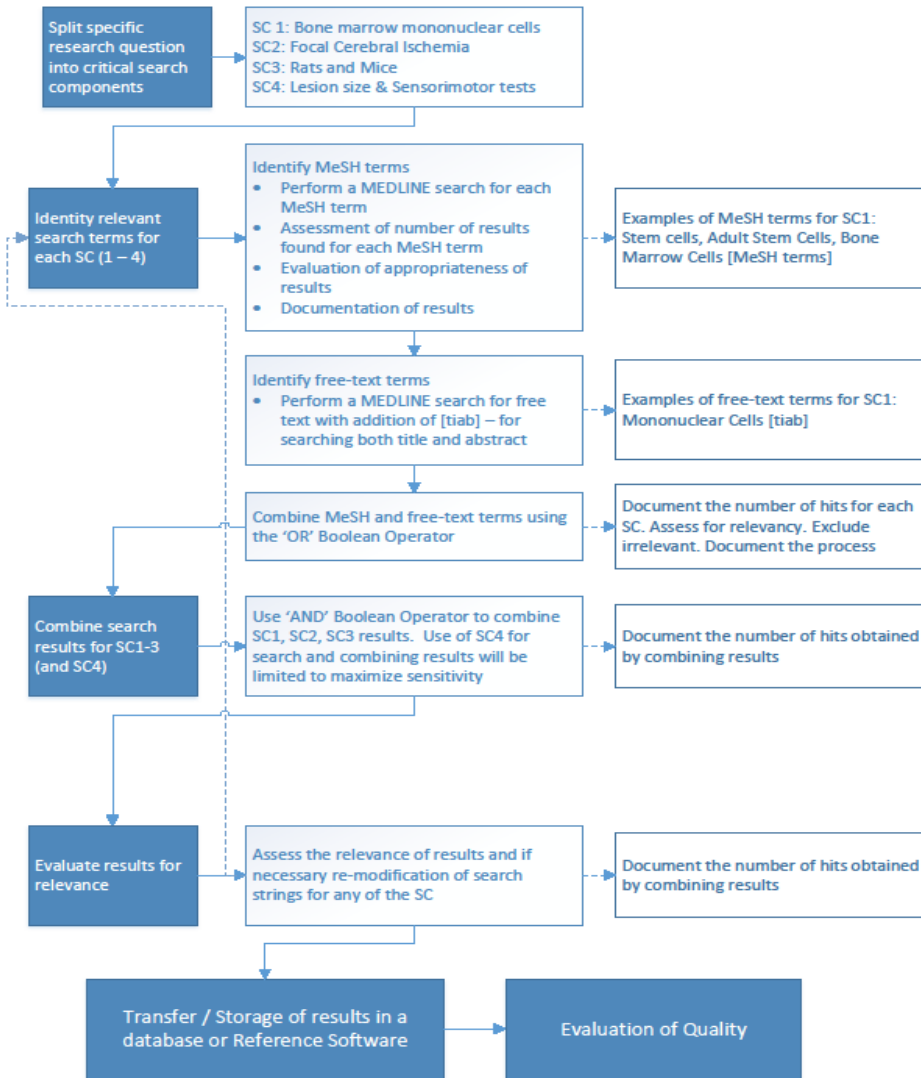**Supplemental Figures and Figure Legends:**

Figure I: Schematic representation of the iterative systematic review procedure and its stepwise documentation. The research question was divided into various Search Components (SC) and individual search was conducted for these components. In latter steps of the search process these search results were combined.
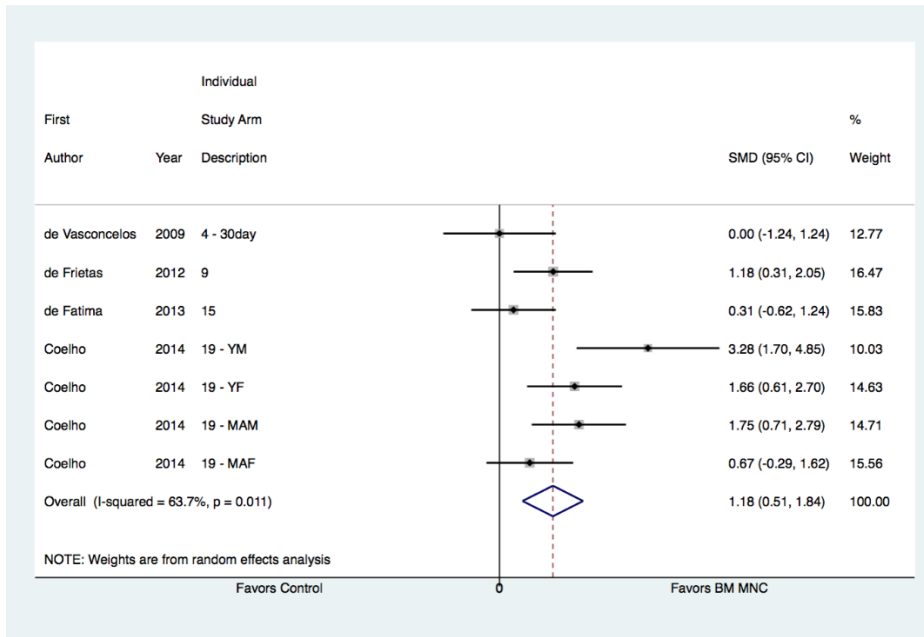
Figure II a: Forrest Plot - Effect Size for IV BM MNC for Adhesive Removal Test as Percent of Paralytic Limb Use in Animal Models. The studies included in this meta-analysis are cited.[30-33]
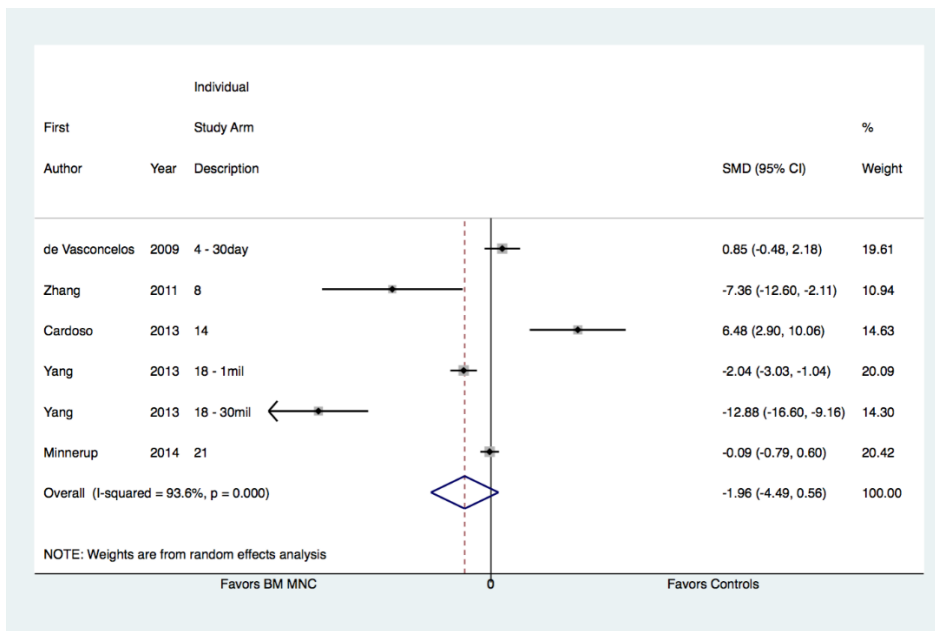

Figure II b: Forrest Plot - Effect Size for IV BM MNC for Adhesive Removal as Time to Removal of Stimulus in Animal Studies. The studies included in this meta-analysis are cited.[30, 34-37]
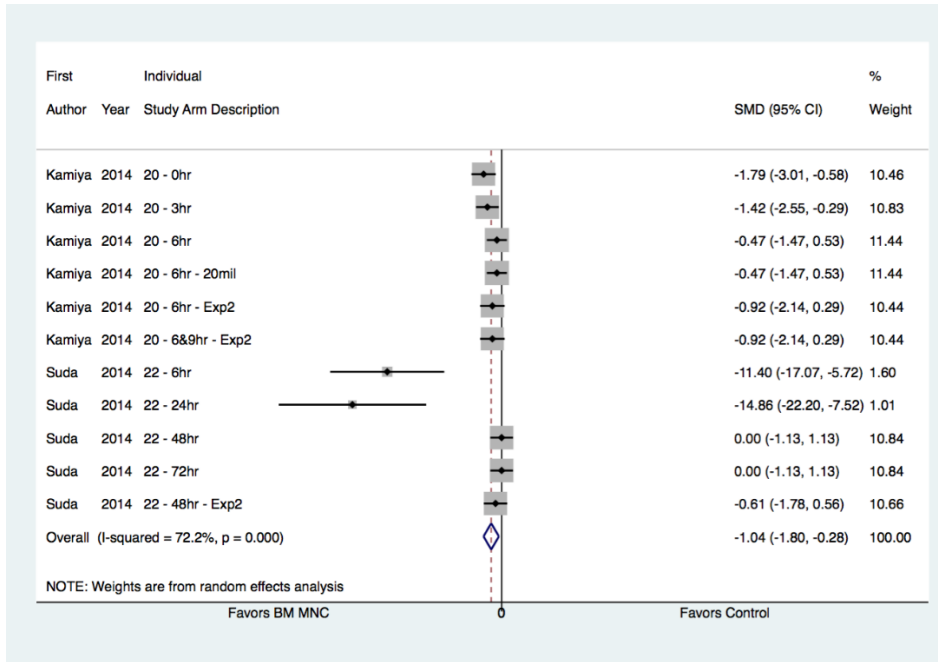
Figure III a: Forrest Plot – Effect Size for IV BM MNC measured by Neurological Deficit Score in Animal Studies. The studies included in this meta-analysis are cited.[38, 39]
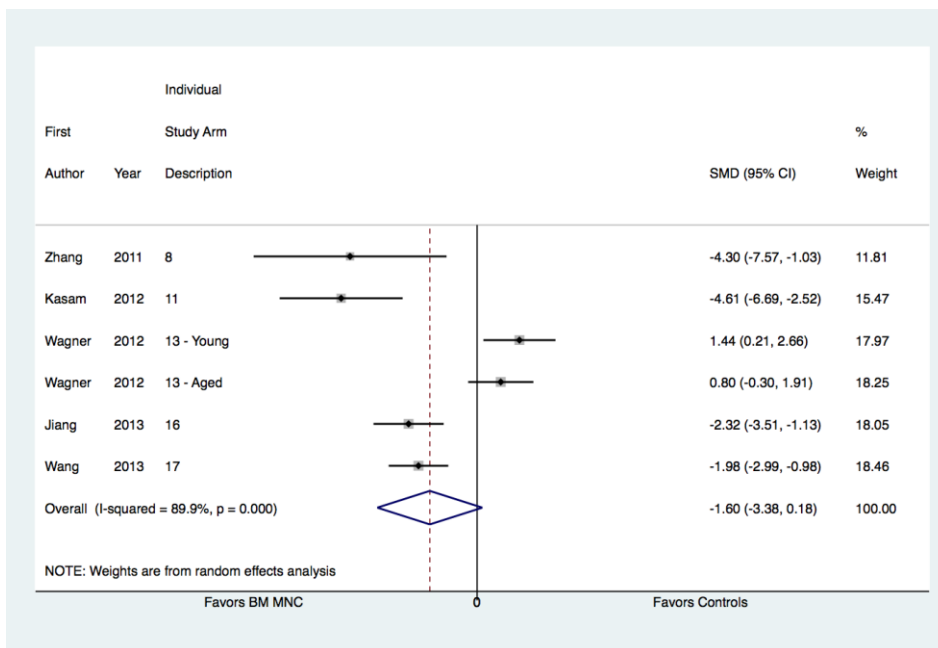

Figure III b: Forrest Plot – Effect Size for IV BM MNC measured by Modified Neurological Deficit Score in Animal Studies. The studies included in this meta-analysis are cited.[34, 40-43]

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