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

Farhaan S. Vahidy, MD, PhD; Mohammad H. Rahbar, PhD; Hongjian Zhu, PhD; Paul J. Rowan, PhD; Arvind B. Bambhroliya, MD, MS; Sean I. Savitz, MD

Background and Purpose—Bone marrow–derived mononuclear cells (BMMNCs) offer the promise of augmenting poststroke recovery. There is mounting evidence of safety and efficacy of BMMNCs from preclinical studies of ischemic stroke; however, their pooled effects have not been described.

Methods—Using Preferred Reporting Items for Systematic Review and Meta-Analysis guidelines, we conducted a systematic review of preclinical literature for intravenous use of BMMNCs followed by meta-analyses of histological and behavioral outcomes. Studies were selected based on predefined criteria. Data were abstracted by 2 independent investigators. After quality assessment, the pooled effects were generated using mixed-effect models. Impact of possible biases on estimated effect size was evaluated.

Results—Standardized mean difference and 95% confidence interval for reduction in lesion volume was significantly beneficial for BMMNC treatment (standardized mean difference: −3.3; 95% confidence interval, −4.3 to −2.3). n=113 each for BMMNC and controls. BMMNC-treated animals (n=161) also had improved function measured by cylinder test (standardized mean difference: −2.4; 95% confidence interval, −3.1 to −1.6), as compared with controls (n=205). A trend for benefit was observed for adhesive removal test and neurological deficit score. Study quality score (median: 6; Q1–Q3: 5–7) was correlated with year of publication. There was funnel plot asymmetry; however, the pooled effects were robust to the correction of this bias and remained significant in favor of BMMNC treatment.

Conclusions—BMMNCs demonstrate beneficial effects across histological and behavioral outcomes in animal ischemic stroke models. Although study quality has improved over time, considerable degree of heterogeneity calls for standardization in the conduct and reporting of experimentation. (Stroke. 2016;47:1632-1639. DOI: 10.1161/STROKEAHA.116.012701.)

Key Words: animal experimentation ■ bone marrow cells ■ mesenchymal stem cells ■ monocytes ■ stroke

Stroke imposes tremendous mortality and morbidity burden.1 Despite the established benefit of intravenous (IV) recombinant tissue-type plasminogen activator, it is estimated that only ≈7% of patients with ischemic stroke (IS) receive IV recombinant tissue-type plasminogen activator in the United States;2 and intra-arterial therapy is beneficial in only a selected subset of patients with IS.3 Cellular therapy is an investigative modality that offers considerable hope and promise to promote poststroke recovery.4

Several cell types have been investigated in preclinical studies and in clinical trials. Bone marrow–derived mononuclear cells (BMMNCs) are a heterogeneous group of cells consisting of varying proportions of differentially matured B cells, T cells, monocytes, and a smaller proportion of progenitor cells, such as hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, and very small embryonic-like cells. The relative ease of processing, potential for IV or intra-arterial administration, and opportunity of an autologous harvest make them an attractive option for preclinical testing and clinical applications.

The evidence of beneficial effect of BMMNCs in animal models of IS has been mounting over the past decade. It has been demonstrated that they lead to a reduction in ischemic lesion volume and improvement in behavioral outcomes.5–9 There is evidence that BMMNCs cross the blood–brain barrier,10 exert neuroprotective effects,11,12 and lead to postischemic angiogenesis and neurogenesis.13–15 It has also been demonstrated that IS may lead to activation of BMMNCs, resulting in paracrine-mediated modulation of poststroke inflammatory responses.16

The growing evidence of safety and benefit of BMMNCs in preclinical models of IS has led to initial clinical testing...
of these cells by different investigators. Despite testing in preclinical models and application in the clinical milieu, there are several unanswered questions regarding the use of BMMNCs in patients with IS pertaining to dose, timing, route of administration, and autologous versus allogeneic approach. It is therefore important to study the pooled treatment effects of BMMNCs in relevant preclinical models of IS and explore sources of heterogeneity. We therefore aimed to conduct a systematic review and meta-analysis of BMMNCs in animal models of IS.

**Materials and Methods**

The protocol was developed based on Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. It was approved by all authors and an external member. For detailed protocol, methods, and PRISMA checklist, please see the online-only Data Supplement.

**Study Selection**

Studies were included if they described experiments exclusively on IV administration of autologous, allogeneic, or xenogeneic BMMNCs for preclinical models of focal cerebral ischemia in mice and rats.

**Search Strategy**

We conducted search for literature in MEDLINE, PUBMED, EMBASE, SCOPUS, Cumulative Index of Nursing and Allied Health Literature (CINAHL), and Animal Welfare Information Center (AWIC) databases. Elements of the research question were divided into search components and searched separately followed by combination of search components. The search results were documented at each step to ensure repeatability. The abstracts were reviewed by hand for relevance, and studies were excluded based on predefined criteria.

**Data Extraction and Quality Assessment**

Data were extracted by 2 independent abstractors and entered electronically. One abstractor was blinded to the journal, title, and the authors. For articles reporting data only as figures, quantitative methods were used as described in the protocol (in the online-only Data Supplement). Each selected study was assessed for quality based on published standards.

**Statistical Analysis**

Study characteristics are provided using descriptive analyses. Effect sizes, that is, improvement in outcome for BMMNC-treated animals relative to the control group, were calculated using Hedges’ $G$. Heterogeneity was quantified using the $I^2$ statistic, and weights were assigned using mixed-effect models. Sources of heterogeneity were explored by meta-regression. Publication or selection bias was evaluated using funnel plots, and symmetry was formally tested using the Egger test. Trim and fill approach was used to correct for funnel plot asymmetry. Robustness of estimates to the effect of potentially missed or negative studies was evaluated using Fail-Safe $N$ approach. Alpha of 0.05 was used for statistical testing, and analyses were performed using STATA 13 and Comprehensive Meta-Analysis.

**Results**

**Study Characteristics**

Initial search generated a total of 399 records. Figure 1 illustrates the review process leading to finally selected 22 articles; all published in peer-reviewed journals. An experiment within a study was considered independent if data for a separate control group were available. More than 90% of experiments were done on various species of rats, with 66.3% using allogeneic BMMNCs. The most commonly used doses were 10 and 30 million cells/kg in ≈63% of the studies. In ≈75% of experiments,
BMMNCs were injected within 24 hours of stroke onset. Table 1 summarizes characteristics of the included studies.

### Outcome Measures
A total of 15 outcomes were identified from included studies, and relevant data were abstracted. Five outcomes were measured in 77% of experiments. These major outcomes and number of animals in control or experimental groups for pooled analyses are (1) stroke lesion size absolute reduction (n=113/113) and relative reduction (n=83/66), (2) cylinder
test (n=161/205), (3) adhesive removal by use of paralyzed
limb (n=69/62) and by time to removal (n=67/49), (4) neuro-
ological deficit score (n=74/74), and (5) modified neurological
deficit score (n=48/48). For details on major and other out-
comes, please see the online-only Data Supplement.

Pooled Estimates

The BMMNC-treated animals had significantly reduced
stroke lesion volume and enhanced recovery of sensorimotor
modalities as measured by cylinder test, adhesive removal
test, and neurological deficit score. Standardized mean differ-
ence (SMD) and 95% confidence interval along with number of
animals in the control and intervention group for each of the
5 major outcomes are summarized in Table 2. The correspond-
ing forest plots for lesion size and cylinder test are show in
Figures 2A, 2B, and 3. Forest plots for other major outcomes
are included in the online-only Data Supplement.

Exploration of Heterogeneity and Meta-Regression

The pooled estimates for included experiments in all meta-
alyses exhibited considerable degree of heterogeneity (I^2
values >70% for all analyses). Univariate meta-regression was
conducted to study the effect of dose, timing, and study qual-
ity on observed heterogeneity for lesion volume and cylinder
test. No significant effects were observed.

Study Quality

The median (Q1,Q3) quality score for was 6 (5–7), and the
range was 4 to 10. The experimental quality criteria that were
least adhered to were reporting of power and sample size calcu-
lations, use of animal models with relevant comorbidities,
and reporting of allocation concealment procedures. The coeffi-
cient of meta-regression for study quality with effect size for
lesion volume was 1.44 (P=0.06), and there was a statisti-
cally significant correlation between study quality and year
of publication (P=0.03). Only 6 published articles (27.2%)
directly or indirectly reported details on immunophenotyping
of BMMNCs.

Assessment of Bias and Sensitivity Analysis

Funnel plots for effect size of BMMNCs as measured by
lesion size and cylinder test were asymmetrical (P<0.001
for both). However, the pooled effect size under the random-
effects model remained statistically significant in favor of

Table 2. Pooled Estimates From Meta-Analysis of Major
Outcomes

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Number of Animals (Control/Intervention)</th>
<th>Pooled SMD (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute reduction</td>
<td>113/113</td>
<td>−3.3 (−4.33 to −2.27)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Percent reduction</td>
<td>83/66</td>
<td>−1.6 (−2.47 to −0.73)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cylinder test</td>
<td>161/205</td>
<td>−2.42 (−3.17 to −1.66)*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adhesive removal test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of paralyzed limb</td>
<td>69/62</td>
<td>1.17 (0.51 to 1.84)*</td>
<td>0.001</td>
</tr>
<tr>
<td>Time to adhesive removal</td>
<td>67/49</td>
<td>−1.96 (−4.48 to 0.56)</td>
<td>0.13</td>
</tr>
<tr>
<td>Neurological deficit score</td>
<td>74/74</td>
<td>−1.04 (−1.8 to −0.27)*</td>
<td>0.008</td>
</tr>
<tr>
<td>Modified neurological deficit score</td>
<td>48/48</td>
<td>−1.6 (−3.38 to 0.18)</td>
<td>0.078</td>
</tr>
</tbody>
</table>

BMMNC indicates bone marrow–derived mononuclear cell; CI, confidence
interval; and SMD, standardized mean difference.

*SMD shows significantly favorable effect of BMMNC treatment.

Figure 2. Forrest plot for effect size for intravenous bone marrow–derived mononuclear cells (BMMNCs) on absolute reduction (A) and relative change to the noninfarct side (B). Weights have been calculated using random-effects model. Degree of heterogeneity in the pooled estimates is represented at F statistic. The studies included in meta-analysis of absolute5,6,11,53 and relative14,39,40,44,46,48 decrease in infarct
size are cited. CI indicates confidence interval; and SMD, standardized mean difference.
BMMNCs for lesion volume (SMD: −2.03; 95% confidence interval, −3.48 to −1.06) and cylinder test (SMD: −1.24; 95% confidence interval, −2.09 to −0.39) after the trim and fill procedure (Figure 4A and 4B). The classic Fail-Safe N analysis yielded the lesion volume and cylinder test effect size of BMMNCs to be robust against 748 and 846 potentially missed null studies, respectively. Furthermore, the Orwin Fail-Safe N analysis indicated combined effect sizes to rise above −0.5 if 19 and 12 studies are added, respectively, to lesion volume and cylinder test analyses with SMD of 1.

**Discussion**

In the rapidly evolving field of cellular therapy for IS, there are several unanswered questions with respect to the choice of cell type, timing, route of administration, safe and effective dose, and the purported mechanism of action. As the evidence generated from preclinical studies forms the basis for designing clinical trials, it is important to explore the pooled effects of animal studies and investigate the various sources of heterogeneity. Previous reviews have either pooled results for several neurological disorders or have included multiple different cell types for IS. Other reviews have focused solely on mesenchymal stem cells manufactured from various tissues. Some of these studies did not generate an effect size or analyze study quality, whereas others pooled results by including various routes of delivery. To our knowledge, this is the first systematic review and meta-analysis of BMMNCs in experimental stroke models. The aim was to focus on BMMNCs, administered solely via IV delivery, in a clearly defined disease model of small animal focal cerebral ischemia while examining study quality and pooling estimates of most commonly and homogeneously measured outcomes.

We used a comprehensive search and robust data assimilation procedure. For the 22 studies that were finally selected, 15 different outcomes were analyzed. Many behavioral tests in preclinical models of stroke have been reviewed in the literature. Meta-analyses were only performed for outcomes that were consistent in measurement and reporting. The pooling procedures used and outcome reported were similar to other meta-analyses.

On the basis of arbitrarily defined quantification of effect size, our observed effect sizes for beneficial effect of BMMNCs on histological and behavioral outcomes were very large (between −3.3 and −1.04). All estimates other than modified neurological severity score and time to adhesive removal were statistically significant. The number of animals included for these 2 outcomes in the pooled analyses were small; it is therefore possible that lack of statistical significance for these end points is a function of small sample size. Although methodological differences do not permit a direct comparison with previously conducted meta-analyses, a previous meta-analysis has reported similar favorable effect sizes for mesenchymal stem cells in IS models for modified neurological severity scale and adhesive removal test. Also, another meta-analysis that included multiple cell types reported a comparable SMD for reduction in infarct lesion size in stem cell–treated animals. We therefore think that observing large beneficial effect sizes in preclinical pooled data is not unique to our analysis.
Study quality was assessed using Stroke Therapy Academic Industry Roundtable recommended objective scoring criteria. The importance of assessing study quality has been repeatedly emphasized, and a previous review of mesenchymal stem cells reported a positive correlation between effect size and study quality. Meta-regression yielded a similar trend in our analysis, showing a 44% increase in effect size for 1-point score increase in study quality ($P=0.06$). All included studies were published within the past 10 years (95% and 77% during the past 7 and 4 years, respectively). We also noted a statistically significant correlation between study quality and year of publication. This result may be indicative of better implementation of and adherence to quality standards over time. The quality criteria that were not addressed in most studies were sample size/power calculations, concealment of allocation, and testing of animals with relevant comorbidities. Lack of sample size justification in preclinical experimentation in neuroscience is prevalent, and attention has been drawn to its detrimental influence on overestimation of effect size. Standardization in experimentation and measurement, along with development of data repositories for preclinical disease models, may provide these estimates for investigators. Allocation concealment is necessary to minimize selection bias, and lack thereof is another factor potentially leading to exaggeration of treatment effects. The importance of using disease-specific animal models was emphasized in various Stroke Therapy Academic Industry Roundtable publications and is regarded by some as necessary for any successful translation of a purported new therapy for IS.

We recognize that our results are not immune to publication and small study effect biases. We used funnel plots to examine the possibility of these biases and observed considerable asymmetry resulting from lack of null or negative studies. This asymmetry was also quantified using Egger test that was found to be statistically significant. We made corrections for apparent asymmetry of the funnel plots, using trim and fill approach, and found that our corrected estimates, although reduced in magnitude of effect, remained statistically significant in favor of BMMNC therapy. We further explored the sensitivity of our estimates to the effect of addition of nonsignificant studies and found that a considerably large number of null or negative studies would need to be added to make our estimates statistically nonsignificant. We are also limited by a relatively small number of studies compared with other meta-analyses that fit the specific inclusion criteria. We chose to be specific in our search criteria to describe the effects of a specific type of cell therapy in a relevant preclinical model using an IV delivery. Despite these restrictive selection criteria, a considerable degree of heterogeneity in estimates was observed. We performed univariate meta-regression to study the possible effects of measured variables on effect sizes but did not find any significance. A possible reason could have been a small number of experiments per each outcome. Having fewer studies has also resulted in a relatively small number of animals in experimental and control groups for our pooled analyses. We acknowledge the impact of small sample size on pooled estimates, as has been discussed in literature.

Our results indicate that the IV BMMNCs have significantly beneficial pooled effects on IS lesion size, the cylinder test, the adhesive removal test (as measured by proportional use of the paralytic limb), and neurological deficit score in experimental models of IS. These behavioral tests indicate that BMMNCs carry the potential to improve both modality-specific limb function and overall neurological outcome on a composite score. Estimated effects seem large but are overall robust to potential biases. Compared with other cell therapies, BMMNCs have similar effect sizes and carry the advantage that they can be prepared from patients and readministered intravenously in more acute time windows after stroke. However, there is a considerable degree of unexplained heterogeneity within experiments, despite using restrictive inclusion criteria for study selection. Although the overall study quality has significantly improved over time, standardization of conduct and measurement of preclinical experimentation for various structural and

Figure 4. Funnel plot with standardized mean ($x$ axis) and SE ($y$ axis) for studies included in meta-analysis for absolute reduction in lesion size (A) and cylinder test (B). The bubbles on the left are estimates from actual studies, whereas the bubbles on the right are hypothetical studies included during the trim and fill approach to correct for asymmetry of the funnel plot. The diamonds below the $x$ axis represent actual estimates of effect (left) and correct estimates (right) after trim and fill. Null value is represented by zero on the $x$ axis.
behavioral outcomes of cerebral ischemia may be an important focus area for experts in the field.

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Disclosures

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SUPPLEMENTAL MATERIAL

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

Farhaan S. Vahidy MD PhD¹, Mohammad H. Rahbar PhD², Hongjian Zhu PhD³, Paul J. Rowan PhD⁴, Arvind B. Bambhroliya MD MS¹, Sean I. Savitz MD¹

¹ Department of Neurology, McGovern Medical School, University of Texas Health at Houston
² Department of Epidemiology, Human Genetics and Environmental Sciences, School of Public Health, University of Texas Health at Houston
³ Department of Biostatistics, School of Public Health, University of Texas Health at Houston
⁴ Department of Management, Policy and Community Health, School of Public Health, University of Texas Health at Houston

Corresponding Author:
Farhaan S. Vahidy, MD PhD
Department of Neurology
McGovern Medical School at UT Health
6410 Fannin Street, UTP 11.0025
Houston, TX 77030
Office: 713.500.7928
Fax: 713.500.0643
Email: Farhaan.Vahidy@uth.tmc.edu
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 primates7 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 imaging (DWI / MRI). 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. The finally calculated infarct volume can either be expressed in absolute terms as mm³ 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. 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 EMBASE 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 CINAHL. 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 that have been derived from the criteria agreed upon by a consortium of experts in the field. 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. 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:

$$\delta = \frac{\mu_1 - \mu_2}{\sigma}$$

(1)

Where delta ($\delta$) is the effect size, mu ($\mu$) is the mean, and sigma ($\sigma$) 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. 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:
\[ g = \frac{\mu_1 - \mu_2}{\sigma_{pooled}} \]  

(2)

And

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

(3)

And the 95% confidence interval calculated using

\[ CI = g \pm \text{Critical value at } 0.05 \times SD_g \]  

(4)

Where the SD of \( g \) is given by:

\[ SD_g = \sqrt{\frac{N}{n_1 + n_2} + \frac{g^2}{2N}} \]  

(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:

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

(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.\textsuperscript{25} Egger test has been
proposed as a formal statistical test for assessment of publication bias.\textsuperscript{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.\textsuperscript{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.\textsuperscript{28, 29}

\textit{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.

\textbf{Supplemental Tables:}

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

<table>
<thead>
<tr>
<th>Section/topic</th>
<th>#</th>
<th>Checklist item</th>
<th>Reported on page #</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TITLE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Title</td>
<td>1</td>
<td>Identify the report as a systematic review, meta-analysis, or both.</td>
<td>Title Page (M)*</td>
</tr>
<tr>
<td><strong>ABSTRACT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structured summary</td>
<td>2</td>
<td>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.</td>
<td>1 (M)</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rationale</td>
<td>3</td>
<td>Describe the rationale for the review in the context of what is already known.</td>
<td>2 (M)</td>
</tr>
<tr>
<td>Objectives</td>
<td>4</td>
<td>Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).</td>
<td>2 (M) &amp; (OS)**</td>
</tr>
<tr>
<td><strong>METHODS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protocol and registration</td>
<td>5</td>
<td>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.</td>
<td>Yes (OS)</td>
</tr>
<tr>
<td>Section/topic</td>
<td>#</td>
<td>Checklist item</td>
<td>Reported on page #</td>
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<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Eligibility criteria</td>
<td>6</td>
<td>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.</td>
<td>3 (M) 3 &amp; 4 (OS)</td>
</tr>
<tr>
<td>Information sources</td>
<td>7</td>
<td>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.</td>
<td>3 (M) 4 &amp; 5 (OS)</td>
</tr>
<tr>
<td>Search</td>
<td>8</td>
<td>Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.</td>
<td>5 (OS)</td>
</tr>
<tr>
<td>Study selection</td>
<td>9</td>
<td>State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).</td>
<td>3 (M) 5 (OS) Figure 1</td>
</tr>
<tr>
<td>Data collection process</td>
<td>10</td>
<td>Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.</td>
<td>3 (M) 5 &amp; 6 (OS)</td>
</tr>
<tr>
<td>Data items</td>
<td>11</td>
<td>List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.</td>
<td>6 (OS)</td>
</tr>
<tr>
<td>Risk of bias in individual studies</td>
<td>12</td>
<td>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.</td>
<td>6 (OS) 4 (M)</td>
</tr>
<tr>
<td>Summary measures</td>
<td>13</td>
<td>State the principal summary measures (e.g., risk ratio, difference in means).</td>
<td>4 (M) 6 (OS)</td>
</tr>
<tr>
<td>Synthesis of results</td>
<td>14</td>
<td>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.</td>
<td>4 (M) 6 (OS)</td>
</tr>
<tr>
<td>Risk of bias across studies</td>
<td>15</td>
<td>Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).</td>
<td>4 (M) 6 &amp; 7 (OS)</td>
</tr>
<tr>
<td>Additional analyses</td>
<td>16</td>
<td>Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.</td>
<td>4 (M) 6 &amp; 7 (OS)</td>
</tr>
<tr>
<td>Section/topic</td>
<td>#</td>
<td>Checklist item</td>
<td>Reported on page #</td>
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<tr>
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<tr>
<td><strong>RESULTS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study selection</td>
<td>17</td>
<td>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.</td>
<td>4 (M) Figure 1</td>
</tr>
<tr>
<td>Study characteristics</td>
<td>18</td>
<td>For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.</td>
<td>Table 1</td>
</tr>
<tr>
<td>Risk of bias within studies</td>
<td>19</td>
<td>Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).</td>
<td>Table II (OS)</td>
</tr>
<tr>
<td>Results of individual studies</td>
<td>20</td>
<td>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.</td>
<td>Table II and III (OS)</td>
</tr>
<tr>
<td>Synthesis of results</td>
<td>21</td>
<td>Present results of each meta-analysis done, including confidence intervals and measures of consistency.</td>
<td>5 (M) Figure 2a,2b,3 (M) and Figure IIa,IIb,IIIa,IIIb (OS)</td>
</tr>
<tr>
<td>Risk of bias across studies</td>
<td>22</td>
<td>Present results of any assessment of risk of bias across studies (see Item 15).</td>
<td>6 (M), Figures 4a/4b (M)</td>
</tr>
<tr>
<td>Additional analysis</td>
<td>23</td>
<td>Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).</td>
<td>6 (M)</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summary of evidence</td>
<td>24</td>
<td>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).</td>
<td>6-9 (M)</td>
</tr>
<tr>
<td>Limitations</td>
<td>25</td>
<td>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).</td>
<td>6-9 (M)</td>
</tr>
<tr>
<td>Conclusions</td>
<td>26</td>
<td>Provide a general interpretation of the results in the context of other evidence, and implications for future research.</td>
<td>9 (M)</td>
</tr>
<tr>
<td>Section/topic</td>
<td>#</td>
<td>Checklist item</td>
<td>Reported on page #</td>
</tr>
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<td>---------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>FUNDING</td>
<td>27</td>
<td>Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.</td>
<td>NA</td>
</tr>
</tbody>
</table>

*M: Manuscript  
**OS: Online Supplement
Table II: Major Outcomes of Various Modalities Tested in Experiments Included in Meta-Analyses

<table>
<thead>
<tr>
<th>Outcome &amp; Measurement Scale</th>
<th>Number of experiments – n (%) of 101</th>
<th>Modality Tested</th>
<th>Directional improvement on scale</th>
<th>Estimates Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion Size</td>
<td>28 (27.7)</td>
<td>Histological / Imaging Marker</td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Cubic mm</td>
<td>18</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>% of Contralateral Hemisphere or initial volume mm square</td>
<td>9</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Cylinder Test</td>
<td>18 (17.8)</td>
<td>Motor Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymmetry Score</td>
<td>12</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Symmetry Score</td>
<td>6</td>
<td></td>
<td>Higher</td>
<td>Yes</td>
</tr>
<tr>
<td>Adhesive Removal Test</td>
<td>15 (14.8)</td>
<td>Sensorimotor Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% effort of contralateral limb</td>
<td>8</td>
<td></td>
<td>Higher</td>
<td>Yes</td>
</tr>
<tr>
<td>Time to removal of adhesive</td>
<td>6</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Ratio of limb effort</td>
<td>1</td>
<td></td>
<td>Higher</td>
<td>No</td>
</tr>
<tr>
<td>Neurological Deficit Score</td>
<td>11 (10.9)</td>
<td>Motor Function Composite (Motor, Posture, Locomotion)</td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Score (0 – 5)</td>
<td>6</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Score (0 – 10)</td>
<td>5</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Modified Neurological Deficit Score</td>
<td>6 (5.94)</td>
<td>Composite (Motor, Sensory, Reflex)</td>
<td>Lower</td>
<td>Yes</td>
</tr>
<tr>
<td>Score (0 – 24)</td>
<td>6</td>
<td></td>
<td>Lower</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table III: Outcomes not included in quantitative meta-analyses

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

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.
Supplemental References:

10. Traystman RJ. Animal models of focal and global cerebral ischemia. *ILAR journal*. 2003;44:85-95

20. Mitchell M. Engauge digitizer—digitizing software. 2010


31. de Freitas HT, da Silva VG, Giraldi-Guimaraes A. Comparative study between bone marrow mononuclear fraction and mesenchymal stem cells treatment in sensorimotor recovery after focal cortical ablation in rats. *Behavioral and brain functions : BBF*. 2012;8:58


