Pre-clinical evaluation of therapies to prevent or treat non-union: a systematic review protocol.

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BACKGROUND
Non-union is a condition where fractured bone does not heal. This can result in delayed recovery, re-hospitalisation, unplanned revision surgery and potentially, limb amputation [1, 2]. The rate of non-union can be as high as 30% in the most severe injuries seen in the civilian setting, rising to 50% when seen in injuries caused by military weapons [1-3].

A number of therapies including electromagnetic field stimulation, ultrasound, shockwave intervention, and bone morphogenetic protein therapy have been tested in the clinical setting, aimed at preventing or treating non-union. Moreover, they have been evaluated in meta-analysis of clinical trials [4-7]. However, these meta-analyses reveal that there is limited evidence of the efficacy of these therapies. Pre-clinical and translational work utilising animal models to develop more potent treatments therefore continues.

The clinical problem of bone healing is typically modeled using animals by the creation of a bony defect; i.e. a segment of a long-bone is removed leaving a defect of a size that that would not heal without further intervention. This is known as a ‘critical defect’. It is clearly a distinct situation to non-union in human patients, which is believed to be predominantly due to either a lack of fracture stability or impaired blood supply although is frequently multifactorial. The assumption is that treatments that will promote healing in an animal critical defect will then translate to promote healing in a patient with, or at risk from, non-union.

Animal models eliminate the confounders that beset clinical work, through standardisation of conditions within a model. However there is little standardisation between models used by different researchers with a breadth of methodologies and outcome measures being employed to evaluate models of non-union [8-10]. Current pre-clinical work investigating new therapies for promoting bone healing is prolific,
as identified in a scoping search of primary studies. Novel strategies that have yet to translate to clinical studies include integrating stem cells into a polymer scaffold embedded with hydroxyapatite particles to stimulate bioactivity [11], use of other growth factors such as stromal-cell derived factor and transforming growth factor to augment the effects of bone morphogenetic proteins [12], and novel synthetic bone substitutes [13].

Presently, systematic reviews of pre-clinical studies are not common, but this is an area of methodological research under rapid development [14-17]. A scoping search was performed to identify systematic reviews on bone non-union in animal models. Garcia et al performed a literature review on rodent models of delayed bone healing and non-union formation. However, the absence of a systematic review methodology renders analysis liable to subjectivity and incomplete conclusions [18]. Other reviews have explored potential therapies for non-union, but looking at both animal and human trials rather than animal models alone. Moreover, a systematic review approach was not utilised, and only one subtype of therapy was reviewed [19, 20].

A systematic review of stem cell therapy for non-union in animal models has been performed [21]. However, comparison between all potential therapies for non-union was not possible as the authors focused their review on a single interventional field. Furthermore, restriction of their search criteria to English only texts and large animal models significantly limited the scope of their search for studies of potential stem cell therapies for bone non-union.

To date, there has been no work to systematically examine and compare all the current pre-clinical and translational evidence to prevent and treat non-union, which is yet to be evaluated in clinical trials.

OBJECTIVE

To systematically collect and analyse published and unpublished evidence of therapies for preventing and treating non-union, which are yet to be translated to clinical trials.

METHODS

This protocol was written in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis-Protocols (PRISMA-P) guidelines [22]. Reporting of the full systematic review will take into account both the PRISMA guidelines [23], and guidelines specific for systematic reviews of animal studies [24].

Searches

The following sources will be searched for primary studies:

- Bibliographic databases- Pubmed and Embase via Ovid.
- The Science Citation Index for citation searching.
- The British Library’s Zetoc research database.
- Hand searching citation lists of included studies and relevant reviews.
- Hand searching of relevant conference proceedings i.e. Orthopaedic Research Society, Orthopaedic Trauma Association, Experimental Biology, Tissue Engineering and Regenerative Medicine Society.
- Contact with study authors and researchers of ongoing trials.

A combination of text and index terms relating to the condition (bone non-union) and population (animal model) will be used. Terms relating to intervention (e.g. stem cells, shock waves) will not be included. This is justified as emerging therapies for bone non-union are likely to be so novel that eligible papers may be missed in the search if intervention terms are included. Although this will undoubtedly produce a high yield of studies from the search, the sensitivity of the search (and therefore comprehensiveness of the systematic review) will be improved.

A filter for the retrieval of animal studies as described by de Vries et al. [25, 26] will be utilised. These have been shown to retrieve greater numbers of animal studies than the standard search filters on these engines. Language restrictions will not be applied. A date restriction (2001- present) will be applied. Justification for this decision stems from the likelihood that studies conducted prior to 2001 will either have already translated to clinical research (rendering them ineligible for inclusion in the systematic review), or found to confer insufficient benefit to warrant translation to the human population.

An example search strategy is included in Appendix 1 (to be added). The search will be set up such that it will be updated on a weekly basis. Any new results generated will be emailed to the project team to ensure completeness of the systematic review.

An electronic database (EndNote version X7.1, Thomson Reuters, New York) will be used to store and manage search results, regardless of whether the studies are eventually included or excluded in the systematic review. Titles and abstracts generated from the search will be screened by two reviewers (SKS/PMB) in order to establish if they potentially meet the inclusion criteria. Full articles will be obtained for all papers that meet the inclusion criteria, or where there is uncertainty. Two reviewers (SKS/PMB) will independently screen and evaluate full text reports against the inclusion criteria. Differing of opinions between the two reviewers will be resolved by discussion with a third reviewer (JPB). Where ambiguity remains, contact with the study’s authors will be attempted to clarify issues pertaining to eligibility for the systematic review. Papers in languages other than English, whose abstracts indicate that they may meet the inclusion criteria will be translated in full or in part. A PRISMA flow diagram will be used to document the study selection process, and a record of excluded studies (including reasons for exclusion) will be kept.

**Selection criteria**

**Types of studies**

Only RCTs will be eligible for inclusion. In vitro experiments, cohort studies, case reports, reviews and letters will be excluded. Given the nature and ease of randomisation in animal studies, it is likely that the majority of studies generated from the searches will be randomised controlled trials (RCT). Both unpublished and published work is eligible for inclusion.
**Types of participants**

Eligible studies must use a mammalian model to test an intervention to treat or prevent bony non-union. There will be no restrictions placed on the anatomical site of the bony defect created. Studies with induced co-morbidities (e.g. immunosuppression) in the animal subject will be included.

**Intervention**

The study assesses an intervention in a mammalian animal model intended to

- Prevent non-union
- Treat non-union
- Promote or accelerate healing of a bony defect
- Treat or ameliorate delayed union

The intervention must be administered after formation of a bony defect in an animal model. There may be studies that test an established intervention but delivered to the animal model through a novel vehicle. These will be included as they may represent a more effective treatment modality.

**Comparator**

A group of control animals are described, which receive

- No treatment
- Current standard of care for the insult in question
- An alternative treatment to the intervention being analysed

**Outcome Measures**

The primary outcome of interest is bone formation. A quantifiable measure of bone formation must therefore be assessed (‘morphometrical analysis’). This can be achieved through radiological means and/or histological means.

Secondary outcomes of interest will be related to the structural properties of the newly formed bone (‘biomechanical analysis’), which may include:

- Indentation testing
- Microtensile and microcompressive analysis
- Three- and four-point bending

Papers that perform biomechanical analysis (secondary outcomes) in the absence of morphometrical analysis (primary outcomes) will not be included in the systematic review.

**Exclusion of therapies with evidence of clinical evaluation**

The stated intention of this study is to examine emerging therapies that have not yet been evaluated clinically. In order to filter out studies describing therapies that have translated into clinical studies the following steps will be performed prior to data extraction:

- Pubmed and Embase will be searched for clinical studies that cite each study that otherwise meets the inclusion criteria.
- Pubmed and Embase will be searched using keywords describing the specific therapies for studies describing clinical evaluation.

Studies describing therapies that are subsequently found to have been evaluated clinically will be excluded and the results of this 'clinical filter' will added to the PRISMA flow chart.

**Data extraction**

A proforma detailing the necessary information to be extracted from the eligible articles has been designed, and can be reviewed at Appendix 2. The proforma will be piloted by the two reviewers (SKS/PMB) prior to commencing the data collection formally. This will serve to not only test the efficacy of the proforma itself, but also calibrate the reviewers to ensure consistency. The following information will be required (but not limited to) from each study:

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Authors, year of study, year of publication, geographical location of study, language of paper, funding sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study design and quality</td>
<td>Sample size, p values, methods to randomise, allocation concealment, blinding of outcome assessment.</td>
</tr>
<tr>
<td>Model of non-union</td>
<td>Hypertrophic vs atrophic</td>
</tr>
<tr>
<td>Species of animal</td>
<td>Mouse, rat etc.</td>
</tr>
<tr>
<td>Characteristics of animal</td>
<td>Age, weight, strain, sex, genetic modification, co-morbidities</td>
</tr>
<tr>
<td>Nature of insult</td>
<td>Location of bony insult (tibia, femur etc), fracture model (segmental vs non-segmental), osteotomy technique, size of osteotomy, closed vs open, critical vs non-critical size defect, fixation vs no fixation, internal vs external fixation (if fixation used), isolated or multiple locations,</td>
</tr>
<tr>
<td>Additional insults (other than bony defects)</td>
<td>Infection, contamination, immunosuppression, soft tissue injury, vascular injury.</td>
</tr>
<tr>
<td>Nature of intervention</td>
<td>Type of intervention (pharmacological vs non-pharmacological), time point that intervention was applied to animal model post-fracture creation, frequency of intervention (single vs recurrent), duration of intervention, systemic or local dosing</td>
</tr>
<tr>
<td>Duration of follow up</td>
<td>Single vs multiple time points of follow up</td>
</tr>
<tr>
<td>Outcome Measures</td>
<td>Morphometrical analysis (e.g. histology, radiology), biomechanical analysis (e.g. torsional stiffness)</td>
</tr>
<tr>
<td>Results</td>
<td>Main findings, direction of effect, effect sizes and uncertainty, statistical significance, length and adequacy of follow-up, early animal death</td>
</tr>
<tr>
<td>Additional insults (other than bony defects)</td>
<td>Infection, contamination, immunosuppression, soft tissue injury, vascular injury.</td>
</tr>
</tbody>
</table>
Quality assessment

Potential bias within the pre-clinical studies will be determined by the reviewers prior to analysis. Both the Cochrane Collaboration tool [27] and Systematic Review Centre for Laboratory animal Experimentation’s (SYRCLE) risk of bias tool [28] which is specific for animal studies will be used. Criteria such as sequence generation, allocation concealment, blinded outcome assessment and incomplete outcome data (e.g. animals that died prematurely) will be identified and recorded, and each domain given a rating of ‘high risk’ or ‘low risk’ based on the details provided in the literature. Where information is missing, the domain will be labelled as ‘unclear’. Risk of bias results will be illustrated graphically to identify any trends in bias between studies.

Analysis

Results from this systematic review will be presented in the form of a narrative synthesis. Key study characteristics such as animal species, intervention and results will be tabulated to aid clarity of study findings.

In the instance where homogeneity exists between a number of studies, quantitative synthesis will be attempted using RevMan software. Whilst the generation of pooled effect sizes may not be as meaningful compared to ones provided by clinical trials, meta-analyses of animal studies may still be useful for showing the overall direction of effect and the heterogeneity across studies. Moreover, they may serve to generate hypotheses [15].

Studies are likely to be considered similar enough for meta-analysis where the same animal species was used, radiological OR histological methods were used for morphometrical analysis, and outcomes were assessed at similar time-points. Separate syntheses will be performed, providing sufficient primary studies are available, for: studies considering different types of defect (i.e. critical or not), models of hypertrophic and atrophic non-union, models of delayed versus non-union, and those looking at prevention versus treatment of non-union.

It is however recognised that a large number of sub-group analyses on small numbers of studies may affect the power of the analyses; should this be the case here, any findings from sub-group analyses will have to be interpreted cautiously.

Presentation of (separate sets of) results in Forest plots without the calculation of a summary measure may also be considered in order to highlight any heterogeneity across studies. Outcome data relating to bone formation will predominantly be continuous, for example, bone mass densities (mg m$^{-2}$). In these instances, the mean difference (MD) between formation of bone in the experimental and control groups will likely be presented, and Forest plots can be used to show the pooled mean difference. The standardised mean difference (SMD) may also be used in instances where papers are measuring the same outcome e.g. bone density, but using different modalities to achieve this e.g. micro-CT, photon emission CT, X-ray.

In studies which present dichotomous data, the relative risk may be presented and meta-analysis may enable the calculation of a pooled relative risk. A situation where this could occur is analysis of whether bone formation did or did not occur at a set time point. In studies which measure the time to the
formation of new bone in the in vivo model i.e. time-to-event data, the pooled hazard ratio may be calculable.

Given that residual heterogeneity is anticipated even between studies considered to be similar enough to pool, a random effects model is more likely to be appropriate for meta-analysis. A decision on whether to pool will be made on the basis of an assessment of clinical and methodological heterogeneity. However, statistical heterogeneity will also be reported using the $I^2$ statistic and the Chi$^2$ test. The likelihood of publication bias will be investigated through the construction of funnel plots for each meta-analysis containing ten or more studies.

Due care in data synthesis must also be paid when studies share control groups between experimental groups. For example, a study may have a single control group but three different experimental groups with varying sizes of segmental defects created. When such comparisons are made, animals in the control group are essentially counted three times and the comparisons will receive too much weight in the estimation of summary effect [15]. Techniques such as splitting the control group to equal the number of experimental groups before performing analysis [29], will be used to mitigate against this potential source of bias.

**DISCUSSION**

Given the absence of a widely established, effective treatment for bony non-union, and a lack of systematic reviews of all potentially relevant emerging treatments from the pre-clinical field, a new systematic review is clearly warranted. It is likely that this will be the most comprehensive to date in the area of novel and emerging treatments for bone healing.

Careful consideration and differentiation of the experimental question of studies included in the review is required, in order that accurate comparisons between animal models of non-union can be made. In particular, there are three circumstances that require delineation at this early stage.

Models to prevent non-union are likely to differ significantly in their study design to those that intend to treat non-union. It would be anticipated that that models intending to prevent non-union would instill an intervention or therapy in to an animal model prior to the time point that defines non-union has elapsed. Conversely, those studies that are investigating the treatment of non-union will examine a therapy that has been applied to the animal model after the time point that defines non-union has elapsed.

Secondly, delayed union is a distinct entity and must be differentiated from non-union in the systematic review to ensure transparency of analysis. Delayed union is prolonged bony healing compared to that of a control group, and will often be the pre-cursor to a non-union model. Unlike a model of non-union where the occurrence of such a defect can be objectively assessed at a set time point, delayed union is a continuous process with no defined criteria for assessment. Due to the fundamental differences in the definition of delayed union and non-union, separate syntheses will be performed.

Finally, it is important to consider the difference between atrophic and hypertrophic models of non-union. Atrophic models are characterized by a failure of periosteal and endosteal activity, with minimal callus formation. Hypertrophic non-union is less common, and is typified by high callus formation and
endochondral growth at the fracture sites. Given their contrasting pathophysiological processes, studies with hypertrophic and atrophic models will not be synthesised together.

The nature of animal models means that heterogeneity will likely hamper interpretation of results across all studies. Variations in species, methodology and study characteristics may also limit the number of trials considered similar enough to meta-analyse and sub-group analyses within these sets of studies may also not be possible. However, it is anticipated that by presenting all the available pre-clinical evidence, it will be possible to observe trends for effectiveness for one or more emerging techniques. Pooled effect sizes may not be meaningful in terms of their applicability to human populations, and any findings must be interpreted in the context of study quality and the extent to which they are considered translatable to other settings. However, this systematic review will be useful for hypothesis generating and may be able to aid in the identification of suitable candidate treatments to take forward into clinical trials. Accurate and valid hypotheses can consequently be drawn as to which emerging therapies for bony non-union may translate successfully to the human population.

References


Appendices:
Appendix 1: Sample search strategy for PUBMED
Appendix 2: Data collection form
Appendix 1: Sample search strategy for PUBMED

1. exp Animals/
2. (animal$).mp.
3. Exp Mammals/
4. (mammal$).mp.
5. (murine or mouse or mice).mp.
6. (sheep or ovine).mp.
7. (rat$ or rodent).mp.
8. (dog$ or canine).mp.
9. (cat$ or feline).mp.
10. (pig$ or porcine or swine).mp.
11. (rabbit$).mp.
12. (goat$ or caprine).mp.
13. (monkey$ or chimp$ or ape).mp.
14. (pre-clinical OR preclinical).mp.
15. exp models, animal/
16. exp animal experimentation/
17. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16
18. exp fracture, bone/
19. exp fracture, healing/
20. exp bone regeneration/
21. (Nonunion or non-union).mp.
22. (Bone union).mp.
23. (Bony defect$ or bone$ defect).mp.
24. (Bony fracture or bone$ fracture).mp.
25. (Bony repair or bone$ repair).mp.
26. (Bony injury or bone$ injury).mp.
27. (Critical defect or critical size defect or critical-size defect).mp.
28. (non-healing defect or non healing defect).mp.
29. (Segment$ defect or non-segmental defect or nonsegmental defect).mp.
30. (Bone regeneration or bony regeneration).mp.
31. (Bone healing or bony healing).mp.
32. (Delay$ union).mp.
33. 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32
34. 17 or 33
35. limit 33 to year="2001 -Current"

Exp denotes explode
'S' denotes truncation
.mp. denotes a search of title, original title, abstract, name of substance word and subject heading word.
### METHODS

#### Details of Study

- **Aim of intervention**: (As stated in the trial report. What was the problem that this intervention was designed to address?):

- **Aim of study**: (As stated in the trial report. What was the trial designed to assess?):

- **Model of non-union**:  
  - Hypertrophic □  Atrophic □  
  - Non-union □  Delayed union □  Other (e.g. acceleration of healing): □

**If delayed/non-union, specify prevention or treatment**:  
- Prevention □  Treatment □  Both □

- **Ethical approval**:  
  - Yes □  No □  Unclear □

- **Funding (including source, amount if stated)**:  

Geographical location (*centre and country*):

**Animal characteristics**

Species, age, weight, strain, sex:

Genetic Modification:
Yes □  No □  
*If yes, provide details:*

Induced Co-morbidities
Yes □  No □  
*If yes, provide details:*

**Details of bony insult**

Anatomical location:

Single or multiple defect:
Single □  Multiple □  
*If multiple, specify other locations:*

Fracture model:
- Segmental □  Non-segmental □  
- Closed □  Open □  
- Critical □  Non-critical □  Not specified □

Technique to produce bony defect *e.g. osteotomy:*
### Fixation used?

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
</tr>
</thead>
</table>

### Internal or external fixation *(if applicable)*

<table>
<thead>
<tr>
<th>Internal □</th>
<th>External □</th>
</tr>
</thead>
</table>

*Details of fixation e.g. product/brand name, type of plate and screws:*

### Additional Insults (other than bony defect) *e.g. infection, associated soft tissue injury:*

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
</tr>
</thead>
</table>

*If yes, provide details:*

### Nature of Intervention

*Details of Intervention *(including theoretical basis and references)*:

*Details of Co-interventions *(i.e. interventions separate to the intervention of interest)*:

*Details of control *(e.g. no treatment given, standard treatment given)*:

### Delivery of Intervention *(for each intervention in the study e.g. Intervention A, Intervention B)*

*Local or systemic (to bony insult):*

<table>
<thead>
<tr>
<th>Local □</th>
<th>Systemic □</th>
</tr>
</thead>
</table>

*Timing of application of intervention *(e.g. immediately, 24 hrs post bony defect)*:

*Frequency of intervention:*

| Single □ | Recurrent □ |
If recurrent, specify frequency/number of applications:

Duration of Intervention:

Intervention Quality

Fidelity/Integrity (i.e. was the intervention delivered as intended? Evidence base for the intervention?):

OUTCOMES

Primary Outcome Measure

Histological □  Radiological □  Both □

Modality of primary outcome measure (e.g. CT scan) - if answered ‘both’ above, provide details of each:

Timing of primary outcome measure (including frequency and length of follow up) - if answered ‘both’ above, provide details of each:

Secondary Outcome Measure

Yes □  No □

Modality of secondary outcome measure, if applicable (e.g. three point bending):

Timing of secondary outcome measure, if applicable:

Adverse events (e.g. unexpected death of animal):

RESULTS

Complete on attached Excel spreadsheet
## ASSESSMENT OF RISK OF BIAS

Adapted from ‘SYRCLE risk of bias tool for animal studies’ [28] by Hooijmans et al.

<table>
<thead>
<tr>
<th>DOMAIN</th>
<th>REVIEW AUTHOR’S JUDGEMENT</th>
<th>SUPPORT FOR JUDGEMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence generation</td>
<td>High Risk □</td>
<td>Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups.</td>
</tr>
<tr>
<td></td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>Baseline characteristics</td>
<td>High Risk □</td>
<td>Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment.</td>
</tr>
<tr>
<td></td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>Allocation concealment</td>
<td>High Risk □</td>
<td>Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before and during enrolment.</td>
</tr>
<tr>
<td></td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>Random housing</td>
<td>High Risk □</td>
<td>Describe all measures used, if any, to house the animals randomly within the animal room.</td>
</tr>
<tr>
<td></td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>Blinding (intervention)</td>
<td>High Risk □</td>
<td>Describe all measures used, if any, to blind trial staff from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.</td>
</tr>
<tr>
<td>To what extent will that be</td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td>possible for different</td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>interventions?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random outcome assessment</td>
<td>High Risk □</td>
<td>Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used</td>
</tr>
<tr>
<td>Is it usual not to measure</td>
<td>Unclear □</td>
<td></td>
</tr>
<tr>
<td>outcome in all animals?</td>
<td>Low Risk □</td>
<td></td>
</tr>
<tr>
<td>Domain</td>
<td>High Risk □</td>
<td>Unclear □</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Blinding (outcome)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete data outcome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective outcome reporting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other sources of bias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>What could they be in this context?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTES**

Contact with author attempted:  
Yes □ No □  
If yes, information obtained?:  
Yes □ No □  
Was the study translated from a language other than English?  
Yes □ No □  
If yes, is further translation required (*i.e. of full text*)?  
Yes □ No □