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BMSCs with tissue-engineered scaffolds for large bone segmental defects. A systematic review

Nicolò Rossi[1,2], Henrique Hadad[3], Maria Bejar-Chapa[2], Giuseppe M. Peretti[4], Mark A. Randolph[2], Robert W. Redmond[1], Fernando P.S. Guastaldi[3]

[1]Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

[2]Division of Plastic and Reconstructive Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

[3]Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Harvard School of Dental Medicine, Boston, MA.

[4]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy.

nic.ross91@gmail.com

henriquehadad@gmail.com

mbejarchapa@mgh.harvard.edu

giuseppe.peretti@unimi.it

marandolph@mgh.harvard.edu

redmond@helix.mgh.harvard.edu

fguastaldi@mgh.harvard.edu

Nicolò Rossi, Henrique Hadad, Maria Bejar-Chapa, and Mark A. Randolph

55 Fruit Street

Massachusetts General Hospital

Boston, MA 02114

617-726-8857

Robert W. Redmond

55 Fruit Street

Massachusetts General Hospital, Wellman Center for Photomedicine

Boston, MA, USA

617-726-8857

Fernando P.S. Guastaldi

50 Blossom St, Thier Research Building, 513A

Massachusetts General Hospital

Boston, MA 02114

857-265-8690

Giuseppe M. Peretti

University of Milan, Department of Biomedical Sciences for Health

Via Festa del Perdono, 7, 20122

Milano, Lombardia, IT

+39 339-661-0282

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Corresponding author

Fernando Pozzi Semeghini Guastaldi, DDS, MSc, PhD

Skeletal Biology Research Center

Department of Oral and Maxillofacial Surgery

Massachusetts General Hospital, Harvard School of Dental Medicine

50 Blossom St, Thier Research Building, 513A

Boston, MA 02114

Tel: +1 617-726-5205

Email: fguastaldi@mgh.harvard.edu

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Abstract

Critical-sized bone defects (CSBD) represent a significant clinical challenge, stimulating researchers to seek new methods for successful bone reconstruction. The aim of this systematic review is to assess whether bone marrow stem cells (BMSCs) combined with tissue-engineered scaffolds have demonstrated improved bone regeneration in the treatment of CSBD in large preclinical animal models. A search of electronic databases (PubMed, Embase, Web of Science, and Cochrane Library) focused on *in vivo* large animal studies identified ten articles according to the following inclusion criteria: 1. *in vivo* large animal models with segmental bone defects; 2. treatment with tissue-engineered scaffolds combined with BMSCs; 3. the presence of a control group; 4. a minimum of a histological analysis outcome. ARRIVE guidelines were used for quality assessment and SYRCLE's risk of bias tool was used to define internal validity. The results demonstrated that tissue-engineered scaffolds, either from autografts or allografts, when combined with BMSCs provide improved bone mineralization and bone formation, including a critical role in the remodeling phase of bone healing. BMSCs-seeded scaffolds showed improved biomechanical properties and microarchitecture properties of the regenerated bone when compared to untreated and scaffold-alone groups. This review highlights the efficacy of tissue engineering strategies for the repair of extensive bone defects in pre-clinical large-animal models. In particular, the use of mesenchymal stem cells, combined with bio-scaffolds seems to be a successful method in comparison to cell-free scaffolds.

Keywords: Critical-sized bone defects; Bioengineering; Large animal study; Bone regeneration; Grafts.

Impact statement

The combination of BMSCs with tissue-engineered scaffolds is an innovation that can bridge the gaps that still exist in treating CSBD, creating a more favorable environment for tissue healing. This systematic review gives insight into the current evidence for this innovation in improving bone regeneration of critical-sized bone segmental defects in large animal models. Moreover, challenges and future directions for translational implication are discussed.

Introduction

Critical-sized bone defects (CSBD) caused by congenital defects, oncological resections, necrosis, osteomyelitis, and high-energy trauma can cause consequential bone loss [1] and represent significant surgical and clinical challenges [2]. Even though bone tissue has unique intrinsic healing ability, large bone defects have limited capacity for spontaneous repair. The poor healing potential in CSBDs can lead to prolonged hospitalization and often multiple revision surgeries that negatively impact patients' quality of life. It has been estimated that there are more than 150 million new fractures globally each year [3]. In the United States alone, approximately 100.000 fractures result in non-union annually. The yearly cost of health care for the treatment of CSBD is approximately $ 2.5 billion every year in the US, creating a significant socioeconomic burden [4-6].

Over recent decades, research has predominantly focused on enhancing surgical techniques and providing new biomaterials, with varying results. Bone autograft and allograft, either used alone or with biological augmentation, have been considered the gold standard for many years as an osteoinductive substrate leading to good outcomes regarding mechanical stability and osteointegration [7-10]. Vascularized bone grafts introduced a half-century ago revolutionized the treatment of large segmental bone defects [11,12]. This strategy bridged defects with viable living bone that increased the osteointegration of the reconstructed bone [11] and decreased complications and reintervention rates [13]. Nevertheless, vascularized fibular grafts continue to be a challenging surgical approach reliant on highly technical graft harvest and microsurgical skills [14] The bone-transport technique with distraction osteogenesis has represented a keystone in treating significant segmental bone defects with higher rates of primary union [15]. This technique introduced by Ilizarov [16] also is not exempt from complications and downsides [17-19].

Metals, polymers, ceramics, and natural materials are commonly used as bone-like biomimetic substrates [20]. A recent review underlined the pivotal role of bone tissue engineering and bone repair scaffolds in bone defect repair as they can provide a suitable

adhesion and proliferation environment for osseous cells [21,22]. However, the long-term *in situ* problems of osteolysis or the necessity of secondary surgeries to remove non-absorbable implants have raised concerns [23-27].

An ideal treatment for a critical-sized bone defect would be one that provides mechanical stability, osteointegration, good functional outcomes, no need for reintervention, and no complications. Currently, there is no such treatment for these types of defects. Large animal models provide a stepping-stone for the translation of bone tissue engineering materials to the clinical setting. Hence, this systematic review assesses whether BMSCs combined with tissue-engineered scaffolds can improve bone regeneration in the treatment of CSBD in large preclinical animal models.

Materials and Methods

Protocol and registration

This systematic review is based on the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) Figure 1 [28] checklist structure and followed the recommendations of the Enhancing the Quality and Transparency Of health Research (EQUATOR Network). Moreover, this systematic review was registered in the international prospective register of systematic review (CRD42022338257).

Focused question

This systematic review was conducted to answer the following question: “Does the combination of BMSCs with tissue-engineered scaffolds improve bone regeneration in large bone segmental defects?”

Search strategy

A literature search was performed among 4 databases (PubMed, Embase, Web of Science, and Cochrane Library) with no date restriction, but limited to publications in the languages of the authors, English, Portuguese, Italian and Spanish. The search was carried out up to June 5, 2022, and was performed with MeSH terms/entry terms as follows: “(large segmental bone defect OR large bone defect OR critical-sized bone defect) AND (bioengineering OR bone tissue engineering OR biomaterials OR stem cells OR scaffolds OR

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grafts OR growth factor) AND (clinical OR imaging OR microct OR ctscan OR xray OR histological OR histomorphometric OR biomechanical)". In addition, an independent manual search was conducted by using terms adapted for each database, including the grey literature and relevant journals in the field. The manual search was also conducted on the reference lists of relevant review studies. Alerts were established for each database to maintain the search strategy up to date.

Eligibility criteria

The PICO framework [29] was used to target our focused question as follows:

(P) population: large animals (all species, all sexes) with critical-sized segmental bone defects;

(I) intervention: bone tissue engineering (scaffolds, cells, growth factors, bioreactors), biomaterials (ceramics, polymers, composites), 3D printing and/or 3D bioprinting for treatment of critical size segmental bone defects;

(C) comparison: untreated bone defects; and

(O) outcome: histological analysis, histomorphometry, biomechanical and imaging analysis (micro-CT or X-ray).

Included in this systematic review were: (a) papers utilizing *in vivo* large animal models (swine, sheep, goat) to study segmental bone defects (determined as greater than 2.5 cm), (b) treatment with tissue engineering, biomaterials, and/or 3D printing/3D bioprinting, (c) including control groups, and (d) with at least a histological analysis of the changes occurring after the intervention.

Studies conducted in small animal models (rodents), rabbits, or with non-CSBD (<2.5 cm), non-segmental bone defects, or designated as purely *in vitro*, or observational and clinical (case reports, case series, controlled trials, human studies, and randomized controlled clinical trials), review papers, abstract-only papers, and unavailable full texts were excluded.

Study selection

For this purpose, all the references retrieved from databases were imported to the Rayyan - Intelligent Systematic Review platform (https://www.rayyan.ai/). Initially, cross-checking eliminated all duplicates, and two reviewers (N.R. and M.B.S.) independently assessed all titles and abstracts for inclusion using the inclusion criteria described above. In case of a disagreement, a third reviewer (F.P.S.G.) was consulted and the final decision was settled by consensus. The kappa coefficient value was calculated to determine inter-reader agreement. Finally, a full-screen process was performed of the remaining articles that met the inclusion and exclusion criteria.

Data extraction

The following information was recorded: author(s), year of publication, animal species, strain/breed, age (days, weeks, months, or years), gender, weight (grams or kilograms), type and size of the defect (description), experimental groups, types of cells used, periods of analysis (days or weeks), imaging analysis (micrometers or millimeters), histology, immunohistochemistry, molecular biology analysis, and main finding. In the case of missing data, one attempt to contact the corresponding author was performed.

Quality and risk of bias assessments

For the assessment of quality, each of the studies included was evaluated through compliance with the ARRIVE guidelines [30]. A grading system was used to check the 20 items included in the ARRIVE checklist based on the criteria adopted by Schwarz et al. (2012) [31] and Monteiro et al. (2020) [32]. Also, the risk of bias in the included studies was analyzed according to the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) which provides a risk-of-bias tool for animal studies [33].

Results

The detailed screening process is shown in Figure 1. In total, 7668 records were identified through the electronic search, including 5,043 in PubMed, 2,445 in Web of Science, 176 in Embase, and 4 in Cochrane library. After 2,694 duplicates were removed, 4,974 records were screened by title and abstract leading to inclusion of 70 articles. The

full texts of these articles were assessed, and 60 of these articles were excluded for at least one of the following reasons: bone defect < 2.5 cm (n=20), no segmental defect (n= 7), no control group (n= 17), not assessing BMSCs nor biomaterials as a therapeutic option (n=12), published in Chinese (n=1), published in German (n=1), retracted article (n=1), not a large animal model (n=1). In total, 10 studies were included in the final qualitative synthesis.

The different animal model-related findings are reported in Table 1. All ten studies used animals from the Caprine: sheep (n = 5) [34-38]; goats (n = 4) [39-42], and non-specified (n = 1) [43]. The age of the animals is reported in only six studies with variability between them: Berner et al [34,35] used older sheep in both studies (7-8 and 6-7 years old, respectively), whereas Dai et al. [39], Gardel et al. [40], Viateau et al. [38] and Xu et al. [42] used animals between 1 and 2 years old. Conversely, Liu et al. did not report the age of the animals but specified that skeletally mature animals were used [41]. Regarding Only five studies reported gender with a slight prevalence of male animals (male = 3 [34,35,37] over female = 2 [38,40]).

In most studies, the critical-sized bone defect was created in the tibia, except for Szivek et al. [37] and Viateau et al. [38], who chose the femur and metatarsal bone, respectively. The length of the bone defect was between 2.5 cm and 3.5 cm in 8 studies [34,35], while two studies created a more extensive defect (4.2 cm) [37,40]. Five studies used plate fixation [34,35,38,40,43], two used circular external fixators [39,41], two used intramedullary nail fixation [37,42], and 1 study used an axial external fixator [36]. The follow-up period was varied between studies and ranged from a minimum of 4 weeks [43] to a maximum of 52 weeks, post-surgery [34].

Overall, the outcomes were evaluated using imaging, biomechanical testing, histology, immunohistochemistry, and molecular biology (Table 3). For imaging analysis, all studies performed radiographic imaging (X-ray) but only 6 studies [34-37,40,41] utilized microCT as an analysis tool, and scanning electron microscopy (SEM) analysis with or without energy-dispersive X-ray spectroscopy (EDS) was performed only by Berner et al 2015 [34] and Gardel et al. (2014) [40]. Biomechanical testing was not performed in all studies; however, torsional test and torsional stiffness [34,35,42], compressive strength and elastic modulus [39],

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Young's modulus and bending strength [41], bone stiffness [36,37], and shear modulus, and maximal angular deformation [36] were used in different studies. Qualitative histological analysis was done using different types of staining, such as hematoxylin and eosin (H&E) [34,35,39-43], Von Kossa, Goldner's trichrome, and Mc Neal's tetrachrome [34,35], Masson's trichrome [40,42], Alcian blue and Sirius red [36], Villanueva's mineralized bone stain [37], Stevenel blue and Van Gieson picro-fuchsin [38], and Indian ink perfusion [42]. Furthermore, only Dai et al. [39] and Viateau et al. [38] performed quantitative analysis, the former to determine the average of trabecular bone and absorptivity of the biomaterial, and the latter to determine the volume of newly formed bone. Immunohistochemistry was also performed by Berner et al 2015 [34] for collagen type I (Col1A1), osteocalcin (OCN), and endothelium-related von Willebrand factor (vWf), and by Berner et al 2013 [35] only for collagen type I (Col1A1) and osteocalcin (OCN). Molecular biology was used by Szivek et al. (2019) [37], to quantify C-terminal telopeptide crosslink (CTX-1) with an ELISA kit.

The type of cells, biomaterials used and cell seeding are reported in Table 4. The data showed that all mesenchymal stem cells (MSCs) were harvested from caprine (sheep (n=5) [34-38], goats (n=4) [39-42], and non-specified sub-family in one study [43]), specifically from the bone marrow from the iliac crest, except by Szivek et al. [37], who harvested MSCs from inguinal and tail fat of sheep. Most interestingly, eight studies [34-36,38,40-43] used MSCs that underwent osteogenic induction before surgical implantation by supplementing the media with β-glycerol phosphate, ascorbic acid, and dexamethasone, whereas Dai et al (2005) [39] and Szivek et al. [37] who did not perform osteogenic differention before usage. In most studies the cells were seeded into scaffolds by pipette or by immersion as performed by Xu et al (2009) [42], and Smith et al (2015) [36]. Three studies [37,38,40] utilized a bioreactor system to cultivate the cells instead of the static culture. Berner et al. (2015) [34] was the only study that implanted the cells 4 weeks after scaffold implantation, wheas the other studies [35-43] included seeded the cells before the implantation. A carrier, platelet-rich plasma (PRP), was used only by Berner et al. (2013) [35] to deliver cells to the scaffolds. Only one study [39] used a genetic modification technique for transducing recombinant human bone morphogenetic protein-2 (rhBMP-2) into cells.

In reviewed publications, it is noted that scaffolds were typically ceramic, except for Xu et al. (2009) [42], who used an allogenic demineralized bone matrix. Scaffolds based on polycaprolactone associated with hydroxyapatite [34], with beta-tricalcium phosphate [35,43] with starch [40], and with poly L-lactic acid [36] were used. Also, scaffolds based only on biphasic calcified bone [39], or only beta-tricalcium phosphate [37,41], and natural coral (Porites) [38] were found.

Main findings

The main findings are reported in Table 3. In general, X-ray analysis demonstrated that BMSCs-seeded scaffolds leads to better callus formation and bone bridging compared to scaffold-alone or empty defect groups. [34-38,40-43] It is important to highlight that three studies [34,35,39] showed callus formation in the early stage of healing. Even though Gardel et al. [40] did not show any early or late complete bone healing or callus formation, they underline that the experimental groups had less bone resorption at the bone-screws-plate and bone interface compared to the negative control group. Dai et al. (2005) [39] reported improved bone healing rates when the rhBMP-2 transduced BMSCs-seeded scaffold group is compared to the transduced-alone BMSCs, untransduced BMSCs-seeded scaffold, and scaffold-alone groups.

Viateau et al. (2007), Liu et al. (2008) and Huang et al.(2011) [38,41,43] found similar bone quality between the autologous bone group and the BMSCs-seeded scaffold group from micro-CT analysis. Szivek et al. (2019) [37] and Smith et al. (2017) [36] demonstrated incomplete bone union at 6 months and 4 months respectively, for the BMSCs-seeded scaffold group or scaffold-alone group. Nevertheless, both the studies showed a trend towards increasing bone formation compared to the empty defect group. Liu et al. (2008) [41] observed new bone formation, bone union and no marrow cavity in the BMSCs seeded group compared to both the control groups.

Histological analysis showed that BMSCs enhance the mineralization process and bone formation in 9/10 studies [34-37,39-43] except for Viateau et al.(2007) [38] where histomorphometric data was not reported. It is worth emphasizing that an inflammatory response and pus formation was seen in only one study [43] for the scaffold-alone group.

Overall, there is a tendency for improved bone regeneration and remodeling for BMSCs-seeded scaffold group. The results also demonstrated more positive staining for Collagen I and Osteocalcin for the BMSCs-seeded scaffold group [34,35].

In studies reporting biomechanical analysis, Liu et al (2008) [41] and Smith et al (2017) [36] could not make measurements from the untreated group and scaffold-alone group due to non-union. In general, biomechanical performance was higher for intact bone or autologous bone graft, except for Dai et al. (2005) [39] which did not have any intact bone or autologous bone graft for comparison. When the BMSCs-seeded scaffold group is compared to the scaffold-only group, higher torsional strength [42], mechanical strength [36], and maximal compressive strength [39] were observed.

Compliance with the ARRIVE guidelines

Compliance with the ARRIVE guidelines for all studies included in the qualitative synthesis is shown in Table 5. Only one study clearly reported the species, strain, sex, age, weight and source of animals [38]. None of the studies provided clear details of housing, husbandry and welfare-related assessments and interventions that were carried out prior to, during, or after the experiment. Eight studies gave satisfactory information about the study design, the number of experimental and control groups and the steps taken to minimize bias [36-43]. Only one study provided clear details of sample size, calculation used and information about characteristics and health status of animals prior to treatment [36]. None of the studies clearly defined their primary and secondary outcomes in the methods section.

SYRCLE risk-of-bias tool

The results of the attribution of bias based on each domain of the SYRCLe tool are shown in Figure 2. All studies failed to clearly explain whether the animal allocation into group was concealed, if the animals were randomly housed during the experiments, if proper blinding of the caregivers/investigators with respect to which intervention each animal received during the experiment was performed, or if there was a random selection of the animals for outcome assessment. Only two studies adequately generated and

applied the allocation sequence and only one stated that the outcome assessor was blinded when performing outcome analysis. Importantly, all studies were free of selective outcome and presented no other clear problems that could result in high risk of bias.

Discussion

Bone is considered to be a tissue with excellent healing properties and many bone defects can heal spontaneously under appropriate conditions. Extreme bone loss can hinder the remodeling and regenerative processes [44]. A bone defect is considered of critical size when surgical augmentation is needed. An agreement on the size which renders a defect ''critical'' has not been reached. Based on the existing literature, a critical defect has been defined as CSBD of a length that exceeds 2.5 times the diameter of the injured bone [45]. Due to the poor functional, mechanical, and clinical outcomes of the current treatment options for CSBD, it is crucial to provide an environment that could mimic the bone's natural healing process and increase its regenerative potential. Over the last decades, the orthopedic field has moved towards the era of cell-based and tissue engineering therapies to improve biological and functional outcomes. The combination of MSCs and tissue engineering strategies, for example, have already shown therapeutic potential for cartilage and tendon regeneration [46,47].

The combination of BMSCs, if delivered into the bone defect, and a scaffold, has led to successful outcomes in small animals [48,49]. However, it is essential to translate these theories into large weight-bearing animal models in order to better recapitulate the clinical scenario. The extensive preparation methods and limited supply represent a challenge for the use of autologous BMSCs in the clinical routine. BMSCs are considered immune-privileged cells, and allogenic BMSCs have been used extensively in oncology [50,51]. For this reason, translating the use of allogenic cell transplantation from oncology to orthopedics may represent an opportunity for regenerative musculoskeletal medicine. Berner et al. (2013) [35] analyzed the differences between autologous and allogenic BMSCs in a critical-sized bone defect. They found no significant radiological, biomechanical and histological difference in bone formation. Besides the osteogenic potential, this was the first study that

focused on the immunological response of allogenic BMSCs in a large animal model for bone defect regeneration demonstrating the safety of this technique.

To overcome some shortcomings of using BMSCs for the regeneration of large segmental bone defects, alternative sources of MSCs have been proposed mainly due to: (1) the significant amount of cells required to be delivered in bone defects to achieve proper healing; (2) the presence of other cell types other than MSCs; and (3) the invasive nature of harvesting BMSCs [52,53]. Adipose-derived stem cells (ADSCs) have been of interest and can be easily used for osteogenic differentiation due to their straightforward isolation process and abundance in the human body [54]. They have been demonstrated to have a high proliferative capacity and the ability to resist senescence, retaining their differentiation potential for a more extended period [55-57]. Nevertheless, pre-clinical and clinical studies investigating the possibility of ADSCs in enhancing bone regeneration have shown different results [52,53]. The high heterogeneity of manipulation methods of ADSCs and the confounding outcomes underline the need for more investigations to make these cells a reliable therapy for bone regeneration.

Another potential source of cells used in regenerative medicine is dental pulp stem cells (DPSCs) [58]. The isolation of DPSCs was first reported by Gronthos et al. [59], using impacted third molars. Recent findings demonstrate that these cells can contribute to the regeneration of different tissue types and specifically they present a strong osteogenic differentiation capacity [60,61]. Thus, there is increasing interest in the use of DPSCs for *in vivo* bone regeneration [62]. Results have shown that DPSCs can induce the generation of adult bone tissue with an integral blood supply and that there is a possibility to associate these cells with scaffolds, growth factors, platelet concentrates, hydrogels, and recombinant human bone morphogenetic protein 2 (rhBMP-2) to enhance tissue regeneration [63-65]. Interestingly, a recent systematic review pointed effectiveness of DPSCs in the craniofacial area for 1) alveolar socket preservation and 2) cleft lip and palate [62].

In the craniofacial area the most challenging scenarios for surgeons and scientists have been the treatment of large critical-sized bone defects in the mandible and the calvaria of large preclinical animal models (i.e., swine, sheep). Several studies have shown

promising results in the regeneration of swine and sheep mandibular critical-sized defects [66-70] using bone scaffolds made of different biomaterials (i.e., ceramics, polymers), seeded with BMSCs or ADSCs, the use of bioreactors (i.e., ex vivo, in vivo) to generate a functional and vital bone graft, and 3D printing technology to produce customized bone scaffolds. A recent study has also shown the use of dental pulp neural crest MSCs or bone marrow aspirate combined with 3D-printed ceramic scaffolds supported the regeneration of high-quality bone in critical-sized swine calvarial defect [71].

In the early stages of bone healing, the microenvironment is associated with a low cell survival rate and a high presence of platelets and macrophages. On the other hand, the fibrocartilaginous callus formation phase is associated with a progressive increase in endothelial cells, MSCs, and chondrocytes. Hence, understanding the most favorable timing for delivery of BMSCs into a scaffold can be considered the first step toward an appropriate bone defect repair. It is evident from this review that BMSCs can improve the mineralization process and enhance bone formation (Table 5). Berner et al. (2015) [34] showed that the injection of BMSCs four weeks after defect formation led to significantly improved bone regeneration compared to pre-seeded scaffolds. Biomechanical testing and CT scans showed comparable results to the clinical gold standard (autogenous bone grafts).

Moreover, the bone healing process depends mainly on osteogenesis, osteoinduction, and osteoconduction [44]. As demonstrated by Szivek et al. (2019) [37], treating large bone defects with the scaffold-alone results in a more disorganized tissue without lamellar organization compared to the MSCs-seeded tricalcium phosphate scaffold. This may indicate that the remodeling phase of bone healing can be targeted when regenerative medicine techniques are used.

Mechanical stability is an essential element when a large segmental defect is associated with load-bearing bones. Among the different fixation methods, it is known that the intramedullary nail fixation can impair the regeneration potential of the endosteal area due to a blockage of the medullary cavity leading to detrimental effect on the mechanical proprieties of the new bone. Of the ten studies included in this systematic review, two

used intramedullary nail fixation methods [37,42]. Szivek et al. (2019) [37], demonstrated that the experimental groups showed less stiffness compared to intact bone. Xu et al (2009) [42], showed that the BMSCs-seeded scaffold group treated had superior biomechanical proprieties (torsional stiffness) with respect to scaffold-alone and empty defect. However, no comparison to the intact bone was performed.

Biocompatibility, degradability, and a porous structure are the main characteristics for a promising bone repair scaffold to enhance the process of "bone remodeling" and reduce adverse events [22]. The report by Huang et al. (2011) [43] was the only study to report an inflammatory response in the scaffold-alone group. They underline the importance of normal blood flow in the osteogenic scaffold to remove acidic monomers from the lesion site. In their control group of laminated scaffold-alone the scaffold collapsed before the 8th week. The severe inflammatory response may have occurred due to the accumulation of acidic prepolymer or monomer of PLLA.

It is well known that the extent of the defect also impacts new bone formation and the time course of healing and intergration [72]. Among the ten studies included in this review, Gardel et al. (2014) [40] and Szivek et al. (2019) [37] created a defect of 4.2 cm. Both these studies did not achieve bone union after 12 and 24 weeks, respectively. The same problem was reported by Smith et al. (2017) [36] with a defect of 3.5 cm at 12 weeks of follow-up.

Among the studies included in this systematic review, there is no homogeneity between treatment groups. It is important to emphasize that Gardel et al [40] was the only study where a scaffold-alone group was not added. Moreover, only one study has a positive and negative control and a scaffold-alone group [38]. Berner et al. 2015 [34] and 2013 [35] included only a positive control group. The lack of homogeneity between treatment groups is a significant limitation since it does not allow direct comparisons to be drawn.

The continuous development of different biomaterial scaffolds and the fabrication of new technologies highlight the importance of preclinical large animal models to investigate their capacity to provide a suitable environment for healing CSBD in a weight-bearing structure.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Conclusion

This review has highlighted the efficacy of tissue engineering strategies for the repair of extensive bone defects in pre-clinical large-animal models. In particular, the use of BMSCs, combined with bio-scaffolds appears to be a successful method in comparison with the use of cell-free scaffolds. While the evidence of efficacy from a scientific point of view is solid, concerns remain regarding translation to broad clinical application due to the high cost of procedures that use *in vitro* cultured cells. It will also be interesting to verify the impact of less expensive procedures that use concentrated bone marrow cells without *in vitro* manipulation and their effectiveness in comparison to those that use *in vitro* cultivation that directs the cells towards an osteogenic phenotype. Furthermore, after critically reviewing the articles, we observed the lack of standardization across all studies. Important methodological aspects such as animal species, strain, age, sex, weight, must be provided by authors. Establishment of better control groups and follow up time points must be considered as well. This will increase the validity and will improve the reproducibility of animal experiments by different groups interested in the same topic.

Authors' Contributions

N.R. contributed to conception, design, data acquisition and interpretation, drafted, and critically revised the article; H.H. contributed to conception, design, data acquisition and interpretation, drafted, and critically revised the article; M.B.S. contributed to conception, design, data acquisition and interpretation, drafted, and critically revised the article; G.M.P. contributed to data interpretation, drafted, and critically revised the article; M.A.R. contributed to data interpretation, drafted, and critically revised the article; R.W.R. contributed to data interpretation, drafted, and critically revised the article; F.P.S.G. contributed to conception, design, data acquisition and interpretation, drafted, and critically revised the article.

All authors gave their final approval and agreed to be accountable for all aspects of the work.

Disclosure Statement

All authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Tables

Table 1. Animal models' characteristics. Abbreviations: y, years; SM, skeletally mature; NR, not reported; kg, kilograms; wks, weeks.

Animal model and defect characteristics

Characteristics / Studies	**Animal**	**Strain/ Breed**	**Age (years)**	**Gender**	**Weight**	**Location**	**Length of Defect**	**Type of Fixation**	**Period of Analysis**
Berner et al. 2015	Sheep	Merino	7-8 y	Male	40-50 kg	Tibial	3 cm	Plate fixation	52 wks
Berner et al. 2013	Sheep	Merino	6-7 y	Male	45 ± 2 kg	Tibial	3 cm	Plate fixation	12 wks
Dai et al. 2003	Goats	NR	1 y	NR	18.6-31.5 kg	Tibial	2.6 cm	Circle external fixation	26 wks
Gardel et al. 2014	Goats	NR	2 y	Female	23-27.5 kg	Tibial	4.2 cm	Plate fixation	6 and 12 wks
Huang et al. 2011	Caprine	Chinese	NR	NR	NR	Tibial	3 cm	Plate fixation	4, 8, and 12 wks
Liu et al. 2008	Goats	NR	SM	NR	22.3 ± 4.1 kg	Tibial	2.6 cm	Circle external fixation	16 and 32 wks

Smith et al. 2015	Sheep	Northern mule	NR	NR	60-85 kg	Tibial	3.5 cm	Axial external fixation	12 wks
Szivek et al. 2018	Sheep	NR	NR	Male	NR	Femoral	4.2 cm	Intramedullary nail fixation	12 and 24 wks
Viateau et al. 2007	Sheep	Pré-Alpes	2 y	Female	60 kg	Metatarsal	2.5 cm	Plate fixation	6 and 24 wks
Xu et al. 2009	Goats	NR	0.9-1.1 y	NR	19.6-25.2 kg	Femur	3 cm	Intramedullary nail fixation	12 and 24 wks

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Table 2. Summary of excluded articles.

Reason for exclusion	Number
Bone defect < 2.5 cm	20
No segmental defect	7
No control group	17
Not assessing Bone Marrow Mesenchymal Stem Cells (BMSCs) nor biomaterials as a therapeutic option	12
Published in Chinese	1
Published in German	1
Retracted article	1
Not large animal model	1

Table 3. Groups, outcome parameters, and main findings.

Studies	Groups	Outcome parameters				Main findings
		Imaging	Histology	Biomechanical testing	Immunohistochemistry	
Berner et al. 2015	1) Positive control (ABG) 2) PCL-HA scaffold + delayed injection of 100 million allogeneic BMSCs 4 weeks after scaffold implantation 3) PCL-HA scaffold	1) X-ray 2) Micro-CT	1) H&E. 2) Goldner's trichrome 3) Von Kossa/Mc Neal's tetrachrome	1) Maximum torsional moment 2) torsional stiffness	1) Collagen type I 2) Osteocalcin 3) Endothelium-Related Von Willebrand Factor	Delayed injection of BMSc in tissue engineering approaches is a capable technique for large bone defect regeneration with biomechanical, radiological, and micro-CT results comparable to

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						the current gold standard (ABG).
Berner et al. 2013	1) Positive Control (ABG) 2) Scaffold alone 3) Autologous MPCs + Scaffold 4) Allogenic MPCs + Scaffold	1) X-ray 2) Micro-CT 3) CT-scan	1) H&E 2) Goldner's trichrome 3) Von Kossa/McNeal's Tetrachrome	1) Maximum torsional moment 2) Torsional stiffness	1) Collagen type I 2) Osteocalcin	Allogenic bone marrow-derived MPCs can be safely delivered for scaffold (mPCL-TCP) with no detectable foreign body reaction or immune response. No significant differences in bone

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						formation and mechanical proprieties between the autologous and allogenic groups.
Dai et al. 2003	1) Negative control (empty defect) 2) AdvhBMP-2/BMSCs/BCB group 3) Adv-ßgal/BMSCs/BCB group 4) Not transduced BMSCs/BCB group 5) Single BCB group	1) X-ray	1) H&E	1) Compressive strength 2) Elastic modulus	N/A	Genetically engineered implants had better area and biomechanical strength of the callous in the bone defect compared to non-genetically

						engineered implants.
Gardel et al. 2014	1) Negative control (empty defect) 2) Scaffold-seeded BMSCs cultured in a bioreactor 3) Scaffold seeded with BMSCs cultured statically	1) X-ray 2) Micro CT	1) H&E 2) Masson's Trichrome	N/A	N/A	Bone development showed better micro-CT outcomes with the perfusion culture of the constructs, demonstrating the importance of the culturing conditions in the *in vivo* functionality of the constructs

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						composed of GBMSC and SPCL scaffolds.
Huang et al. 2011	1) Negative control (empty defect) 2) PLLA/b-TCP + BMSCs 3) PLLA/b-TCP without BMSCs	1) X-ray	1) H&E	N/A	N/A	Implantation of allogeneic BMSCs loaded in a PLLA/b-TCP laminated scaffold facilitated bone repair of large defects.
Liu et al. 2008	1) Negative Control (empty defect) 2) Porous b-TCP + BMSCs 3) Porous b- TCP	1) X-ray 2) micro-CT	1) H&E	1) Young's module 2) Bending strength	N/A	Critical-sized segmental defects of the goat tibia could be repaired by biodegradable

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						b-TCP scaffolds combined with osteogenically induced autologous BMSCs. Meanwhile, b-TCP scaffolds alone were not sufficient to repair the defect.
Smith et al. 2015	1) Negative control (empty defect) 2) Autologous SSC-seeded polymer scaffold 3) Polymer scaffold	1) X-ray 2) micro-CT	1) Alcian blue 2) Sirius red	1) Shear modulus 2) Bone stiffness 3) Maximum torque 4) Maximum shear stress	N/A	Both the scaffold and scaffold+SSC groups showed

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				5) Maximal angular deformation at failure		enhanced quantitative bone regeneration with micro-CT analysis; however, this was found to be significant in the scaffold+SSCs group only.
Szivek et al. 2018	1) Negative control (empty defect) 2) Standard scaffold + TCP + and MSCs 3) Scaffold without TCP without MSC	1) X-ray 2) micro-CT	1) Villanueva's mineralized bone stain	1) Bone-scaffold stiffness	N/A	Based on x-ray and micro-CT results, The MSC-seeded, TCP-coated, inverse trabecular

						scaffold successfully facilitated the bridging of the critical-sized defect. Moreover, the results of the experimental sheep demonstrated a strong, relatively positive impact on the quantity and quality of cortical bone formation.

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Viateau et al. 2007	1) Negative control (empty defect) 2) Positive control (autogenic cortico-cancellous graft) 3) Coral scaffolds loaded with MSCs 4) Defect filled with plain coral scaffolds	1) X-ray	1) Stevenel blue 2) Van Gieson picro-fuchsin	N/A	N/A	Coral scaffold loaded with MSCs showed good degradation time along with formation of new bone.
Xu et al. 2009	1) Negative control (empty defect) 2) DBM-BMSC 3) DBM	1) X-ray	1) H&E 2) Masson staining	1) Torsional test to failure	N/A	The quantity and quality of newly formed bone was superior in the DBM/MSC group compared to the other groups.

Table 4. Type of cells, biomaterials, and cell seeding.

	Type of cells	Biomaterial/scaffold	Seeding
Berner et al. 2015	BMSC from Merino Sheep (Iliac crest) were harvested and cultivated until 10^{-3} cells per cm^2.	Biodegradable Scaffold produced by fused deposition method manufactured in polycaprolactone (80%) and hydroxyapatite (20%) and coated with CaP layer (16mm diameter, 30mm height, 8mm inner diameter).	Scaffold present 74% porosity and a 0/90 lay/down pattern. Cells received osteogenic induction and were injected four weeks after scaffold implantation.
Berner et al. 2013	BMSC from Merino Sheep (Iliac crest) were harvested and cultivated until 10^{-3} cells per cm^2.	Biodegradable Scaffold produced by fused deposition method and manufactured in polycaprolactone (80%) and b-tricalcium phosphate (20 %), and with 20 mm of outer diameter, 30mm height, and 8 mm inner diameter.	Cells received osteogenic induction and were injected four weeks after scaffold implantation. For the 3D culture, $35x10^{-6}$ cells were mixed with PRP (1.2 ml) and then seeded in an mPCL+TCP scaffold.

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Dai et al. 2003	BMSCs were harvested from a goat (iliac crest) and cultivated until passage number 2.	Scaffolds were manufactured using biphasic calcined bone (BCB) by a 16mm trephine. The scaffold had a 7mm central canal. Scaffolds were coated with Collagen (3mg/mL).	Cells received osteogenic induction and were transduced with hBMP-2 using adenovirus. Then 10^{-8} BMSC in 2 mL of media were loaded in BCB scaffolds.
Gardel et al. 2014	BMSC from goats (Iliac crest) were harvested and cultivated until 10^{-3} cells per cm^2 until passage 4.	Scaffold manufactured with starch + polycaprolactone (SPCL). They were produced from melt-spun fibers by a fiber-bonding method into mesh structures with a fiber diameter ranging from 120 to 500 mm and with a porosity of 75%, with 97.5% pore interconnectivity consisting of a mean pore size of 275 mm. The final presentation of the scaffold: disks of 16 mm in diameter and 3 mm in thickness with a 6-mm interior hole.	Cells received osteogenic induction and were seeded in the scaffolds using two types of culture: static and dynamic.
Huang et al. 2011	BMSC from caprine (Iliac bone) were harvested and cultivated until 10^{-3} cells	Sandwich-structured, PLLA/b-TCP/PLLA composite scaffold.	Cells received osteogenic induction, and then $7x10^{-6}$ cells were pipetted

	per cm^2 until passage number 3.	b-TCP powders were mixed with the milled polystyrene resin particles in a 6:1 weight ratio, along with 5% polyvinyl alcohol aqueous solution as an adhesive agent; and PLLA ($Mw=4x10^{-4}$) with NaCl. The porosity of PPLA was controlled by the weight ratio of NaCl and PPLA (from 4:1 to 10:1). The final presentation: The cylinder-shaped scaffold has a length of 3 cm, an outer diameter of 1.2 cm, and an inner diameter of 0.4 cm.	onto the outer surface of the cylinder, and 5 million cells onto the inner surface, then cultured in vitro for three days.
Liu et al. 2008	BMSC from goats (Iliac crest) were harvested and cultivated until 10^{-5} cells per cm^2 until passage 4.	Scaffold manufactured with b-TCP cylinders using polymeric sponge method (26 mm high,15 mm diameter).	Cells received osteogenic induction, and then $2x10^{-7}$ cells/mL were injected into a B-TCP cylinder and incubated for four hours, followed by seven days of culture in vitro before *in vivo* implantation.

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Smith et al. 2015	BMSCs from 'cull' ewe sheep (Northern Mule) (iliac crest) were cultivated at a density of $1x10^{-7}$ cells/T175 flasks.	Scaffold manufactured with poly (L-lactic acid)/ poly(ε-caprolactone) (PLLA/PCL), 20/80 by solution blending process (diameter 23 mm, length 35 mm, and with an 8 mm longitudinal medullary canal).	Cells received osteogenic induction, and then the scaffold was seeded by immersion in 20 ml of its respective autologous skeletal stem cells (SSC) solution for 2 hours with a total of $1x10^{-7}$ cells/scaffold.
Szivek et al. 2018	MSC from sheep (inguinal and tail fat) was cultivated for 2 weeks until passage 2.	Cylindrical scaffold was printed using a Stratasys 1650 Fused Deposition Modeler from 4um tricalcium phosphate (TCP) particles. Trabecular cores from the ovine femoral head were scanned and used to create a pattern for the Scaffold. Scaffolds were 42 +/- 1 mm in length, 22+/- 2 mm in diameter, and had an 11+/- 1 mm channel through the center.	Cells received osteogenic induction, and then $2x10^{-6}$ cells were seeded in the scaffolds for 3 hours and then cultivated in a bioreactor for two days before the surgery.
Viateau et al. 2007	BMSCs from ewes (iliac crest) were cultivated until passage 4.	Scaffolds manufactured with (3 x 3 x 3 mm) natural coral (Porites). This material presented 99% of calcium	Cells received osteogenic induction and were seeded onto the scaffolds at a ratio of $2.5x10^{4}$ cells for 4 hours.

		carbonate in the form of aragonite with 1% organic material (amino acids), with pores of 250 um diameter and porosity of 49%+/- 2%.	Afterward, the scaffolds were cultivated for ten days in a bioreactor until surgery.
Xu et al. 2009	BMSCs from goats (iliac crest) were cultivated at a density of $1x10^{-5}$ cells/cm^2 until passage 2.	Scaffolds based on an Allogenic demineralized bone matrix (aDBM) were prepared (10 mm x 10 mm x2 mm).	Cells received osteogenic induction, and then Scaffolds were seeded by immersion on the media with 10^{-6} cells per mL for 4 hours and then cultivated in a flask for seven days before implantation.

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Table 5. ARRIVE Guidelines.

	Berner et al. 2015	Berner et al. 2013	Dai et al. 2003	Gardel et al. 2014	Huang et al. 2011	Liu et al. 2008	Smith et al. 2015	Szivek et al. 2018	Viateau et al. 2007	Xu et al. 2009
TITLE (0, inaccurate/not concise; 1, accurate/concise)	1	1	1	1	1	1	1	1	1	1
ABSTRACT Summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings, and conclusions of the study (0, clearly inaccurate; 1, possibly accurate; 2, clearly accurate)	1	2	2	2	1	2	2	2	1	2

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INTRODUCTION Background e objectives, experimental approach and rationale, relevance to human biology (0, clearly insufficient; 1, possibly sufficient; 2, clearly sufficient)	2	2	1	1	1	2	2	2	1	2
INTRODUCTION Objectives e primary and secondary (0, not clear; 1, clear)	1	1	1	0	1	1	1	0	0	0
METHODS Ethical statement e nature of the review permission, relevant licenses, national and institutional guidelines for the care and use of	2	2	2	2	1	1	2	2	2	2

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animals (0, clearly insufficient; 1, possibly sufficient; 2, clearly sufficient)										
METHODS Study design e number of experimental and control groups, any steps taken to minimize bias (i.e., allocation concealment, randomization, blinding) (0, clearly insufficient; 1, possibly sufficient; 2, clearly sufficient)	0	0	1	1	1	1	1	1	1	1
METHODS Experimental procedure e precise details (i.e., how, when, where, why) (0, clearly insufficient; 1,	2	2	0	2	0	1	2	1	2	2

possibly sufficient; 2, clearly sufficient)										
METHODS Experimental animals e species, strain, sex, developmental stage, weight, source of animals (0, clearly insufficient; 1, possibly sufficient; 2, clearly sufficient)	1	1	1	1	1	1	1	0	2	1
METHODS Housing and husbandry e conditions and welfare-related assessments and interventions (0, clearly insufficient; 1, possibly sufficient; 2, clearly sufficient)	0	0	0	1	0	1	1	1	0	0

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METHODS Sample size e total number of animals used in each experimental group, details of calculation (0, clearly inadequate; 1, possibly inadequate; 2, clearly adequate)	0	0	1	1	0	1	2	0	0	1
METHODS Allocation animals to experimental groups e randomization or matching, order in which animals were treated and assessed (0, no; 1, yes)	0	0	0	0	0	1	0	0	0	0
METHODS Experimental outcomes and definition of primary	1	1	1	1	1	1	1	1	1	1

and secondary outcomes (0, no; 1, unclear/not complete; 2, yes)										
METHODS Statistical methods and details and unit of analysis (0, no; 1, unclear/not complete; 2, yes)	1	1	0	2	1	2	2	0	2	1
RESULTS Baseline data characteristics and health status of animals (0, no; 1, yes)	0	0	0	0	0	0	1	0	0	0
RESULTS Numbers analyzed absolute numbers in each group included in each analysis, explanation for exclusion (0, clearly	1	1	0	0	0	2	2	2	2	1

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inadequate; 1, possibly inadequate; 2, clearly adequate)										
RESULTS Outcomes and estimation results for each analysis with a measure of precision (0, no; 1, unclear/not complete; 2, yes)	1	1	0	1	1	2	1	2	2	2
RESULTS Adverse events details and modifications for reduction (0, no; 1, unclear/not complete; 2, yes)	0	0	0	0	0	0	2	0	2	2
DISCUSSION Interpretation/scientific implications study	1	2	1	1	1	1	2	1	2	1

limitations including animal model, implications for the 3Rs (0, clearly inadequate; 1, possibly inadequate; 2, clearly adequate)										
DISCUSSION Generalizability/translation relevance to human biology (0, clearly inadequate; 1, possibly inadequate; 2, clearly adequate)	2	2	1	2	1	1	2	1	2	2
DISCUSSION Funding sources, role of the funders (0, clearly inadequate; 1, possibly inadequate; 2, clearly adequate)	2	2	2	1	2	2	2	2	2	2

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Figure legends

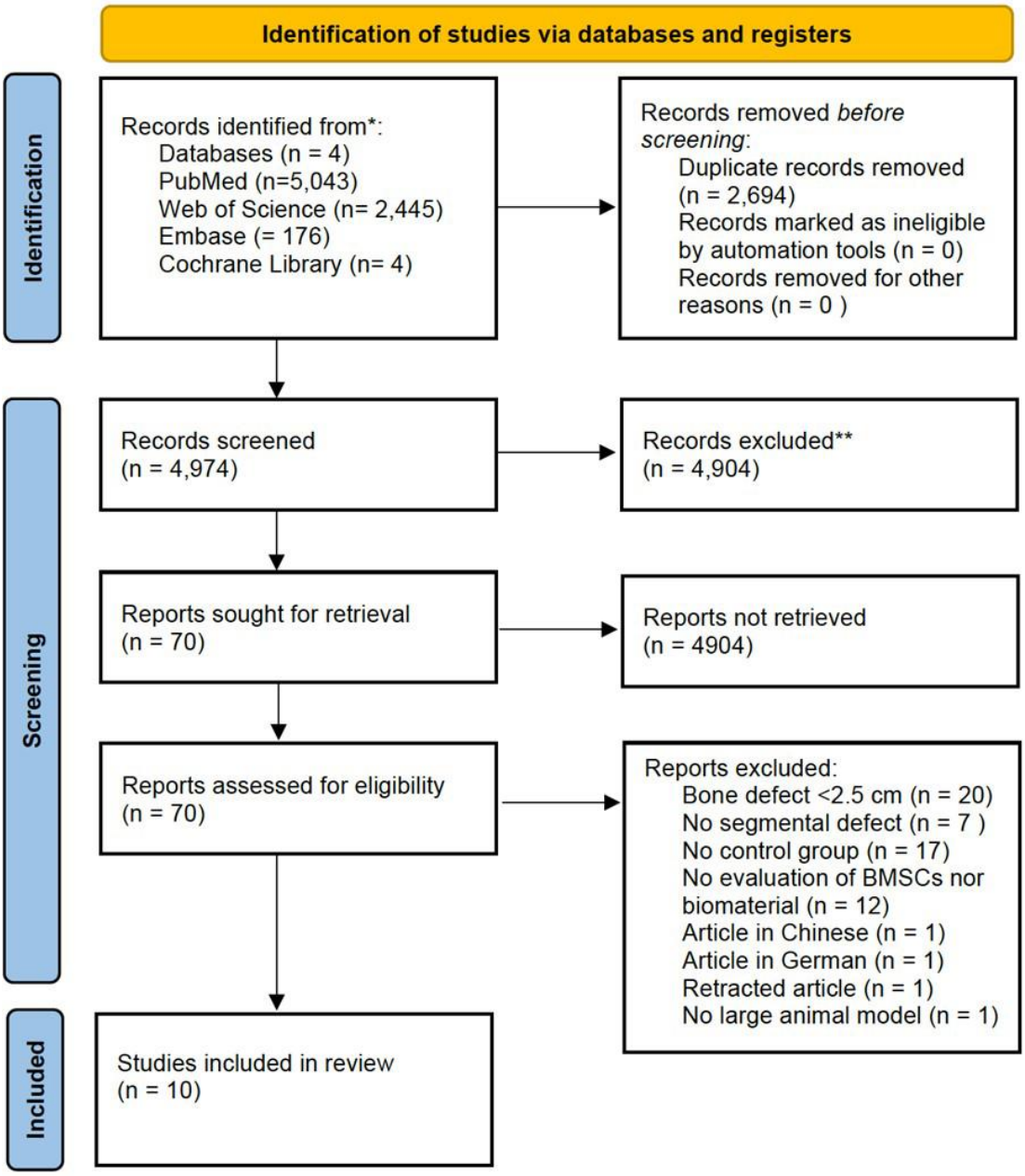
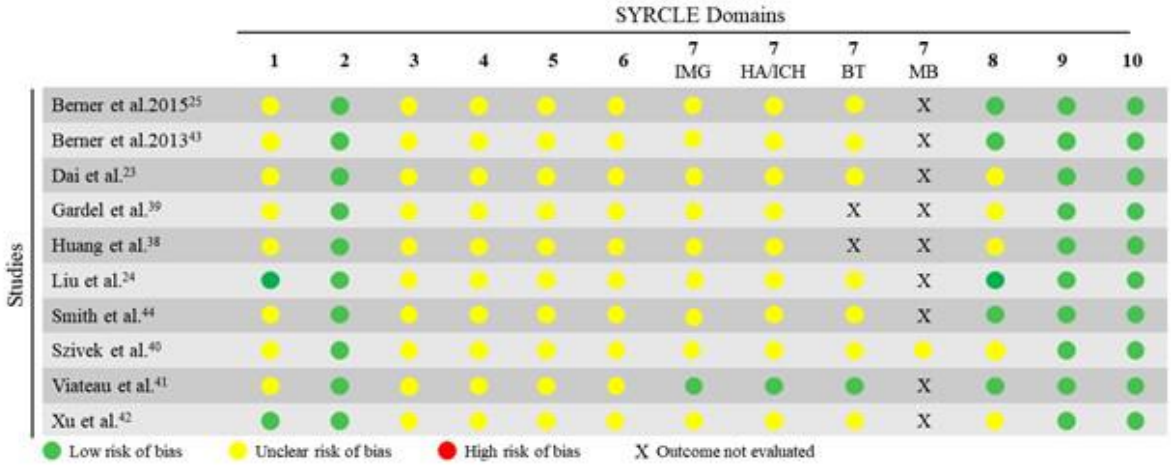


Figure 1: Prisma flow diagram. Search strategy and selection process of the included studies.

*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).

**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.



The risk of bias of the individual animal studies are included. SYRCLE's RoB tool was used to address the following domains: 1, Was the allocation sequence adequately generated and applied?; 2, Were the groups similar at baseline or were they adjusted for confounders in the analysis?; 3, Was the allocation to the different groups adequately concealed?; 4, Were the animals randomly housed during the experiment?; 5, Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?; 6, Were animals selected at random for outcome assessment?; 7, Was the outcome assessor blinded?; 8, Were incomplete outcome data adequately addressed?; 9, Are reports of the study free of selective outcome reporting?; 10, Was the study apparently free of other problems that could result in high risk of bias?. Abbreviations and symbols: green circle, low risk of bias; yellow circle, nuclear risk of bias; red circle, high risk of bias; X, outcome not evaluated; IMG, imaging analysis; HA, histologic analysis; IHC, immunohistochemistry; BT, biomechanical testing; MB, molecular biology

Figure 2: The risk of bias in the individual animal studies is included. SYRCLE's RoB tool was used to address the following domains: 1, Was the allocation sequence adequately generated and applied?; 2, Were the groups similar at baseline, or were they adjusted for confounders in the analysis?; 3, Was the allocation to the different groups adequately concealed?; 4, Were the animals randomly housed during the experiment?; 5, Were the caregivers and/or investigators blinded from knowledge of which intervention each animal received during the experiment?; 6, Were animals selected at random for outcome assessment?; 7, Was the outcome assessor-blinded?; 8, Were incomplete outcome data adequately addressed?; 9, Are reports of the study free of selective outcome reporting?; 10, Was the study apparently free of other problems that could result in high risk of bias?. Abbreviations and symbols: green circle, low risk of bias; yellow circle, nuclear risk of bias; red circle, high risk of bias; X, outcome not evaluated; IMG, imaging analysis; HA/IHC, histologic and immunohistochemistry analysis; BT, biomechanical testing; MB, molecular biology analysis.

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