ISSN 1998 – 409X

REVIEW ARTICLE

Hydroxyapatite-Based Three-Dimensional Bioprinted Osseous Scaffolds for Bone Regeneration: A Systematic Review of Animal Studies

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Abstract

Introduction: The application of three-dimensional (3D) bioprinting technology using hydroxyapatite (HA)-containing bioinks has become a promising approach for designing patient-specific osseous scaffolds by combining osteoconductive mineral chemistry with the precision of additive manufacturing technology for improved human health and function. Whether such an approach results in improved bone regeneration *in vivo* rather than *in vitro* has not yet been systematically assessed. **Methods:** This systematic review was registered with PROSPERO, the International Prospective Register of Systematic Reviews, with Registration No: CRD420261469466. *In vivo* animal studies investigating the use of 3D bioprinted HA-containing scaffolds in the repair of bone defects have been retrieved from six databases (PubMed, Scopus, Web of Science, Embase, Cochrane Central, and IEEE Xplore). Articles have been screened according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses methodology, and the risk of bias has been assessed using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) approach in 10 different fields. **Results:** Among 842 screened papers, three studies (with 47 total animal specimens from rat, sheep, and rabbit models) fulfilled the inclusion criteria. Cell viability/biocompatibility was significantly increased for all three studies in comparison to acellular/scaffold control groups. Mechanical strength, osteogenesis, and *in vivo* bone formation were noted for two out of three studies (femoral rats and rabbits with calvarial bone); the third study (maxillary sinus of sheep) did not find any difference for those parameters but noted enhanced osteoconductive properties on imaging. Vascularization benefits were observed in only one study. All three studies demonstrated high overall risk of bias on the SYRCLE tool due to inadequate description of randomization, allocation concealment, and blinding procedures. **Conclusions:** The inclusion of biological components into HA-based 3D-bioprinted scaffolds enhances their cell viability *in vivo*, and the combination with cellular elements and biomimetics (periosteum, for example) improves bone regeneration results in comparison to acellular scaffolds. However, the results are obtained on a small evidence base with high heterogeneity of methodology and risk of bias and need further investigation.

Key words: animal model, bioink, bone regeneration, health, hydroxyapatite, medicine, scaffold, systematic review, systematic review centre for laboratory animal experimentation, three-dimensional bioprinting

INTRODUCTION

Oral bone defects are a common sequela following pathologies, trauma, and congenital anomalies, greatly affecting the form and function of the affected tissues. Traditionally, bone grafting techniques and guided bone regeneration techniques have been employed to treat such conditions to improve the overall masticatory efficiency, thereby enhancing health and quality of life.[1] Although autografts have been considered the gold standard graft material, they have been associated with morbidity and limited availability, whereas allografts and xenografts have been associated with the risk

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Received: 05-08-2026
Revised: 10-09-2026
Accepted: 20-09-2026

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of immune rejection and virus-mediated disease transfer.[2] To overcome these limitations, synthetic bone grafts have been employed; however, they lack the biomimetic aspect associated with the other graft options. This has led to the application of tissue engineering principles to achieve a more predictable and complete regenerative outcome.[3-5]

Three-dimensional (3D) bioprinting has emerged as a transformative additive manufacturing technology that is rooted in tissue engineering principles.[6] It enables the precise layer-by-layer fabrication of biomimetic tissue constructs using bioinks. Unlike conventional 3D printing, which employs mineral and polymer matrices and provides passive structural scaffolds, 3D bioprinting enables the fabrication of biologically functional constructs through the precise deposition of biomaterials, with the potential to incorporate cells, growth factors, and other bioactive molecules either during the printing process or through subsequent cellularization and maturation.[7] 3D bioprinting therefore encompasses both cell-laden approaches, in which living cells are incorporated directly into the bioink during fabrication, and cell-free scaffold-based approaches, in which an acellular scaffold is first 3D bioprinted and subsequently seeded with cells and maintained under appropriate culture conditions to promote cellular attachment, proliferation, differentiation, and tissue maturation.[8,9] Thus, 3D-bioprinted constructs can be designed to provide both a biomimetic 3D architecture and a biologically active microenvironment conducive to tissue regeneration.

Various natural, synthetic, and composite cellular and acellular bioinks have been developed for the bioprinting of osseous scaffolds.[10-12] Each constituent of the bioink serves a distinct role, collectively enhancing scaffold properties, including mineralization potential, biocompatibility, hydrophilicity, degradability, structural integrity, and antimicrobial properties.[13] Among these, mineralization potential is a key functional property required of bioprinted osseous scaffolds to promote effective bone regeneration. Among the multitude of bioink constituents, bioactive ceramics, particularly calcium phosphate–based materials such as hydroxyapatite (HA), are widely recognized for enhancing the mineralization potential of bioprinted osseous scaffolds owing to their compositional biomimicry of the native bone mineral phase, inherent osteoconductivity, and bioactivity.[14] Despite its excellent bioactivity, HA alone exhibits inherent brittleness and lacks the mechanical versatility required to fabricate patient-specific constructs with the precise, interconnected porosity necessary for effective cell infiltration and vascularization.[15] To overcome these limitations, HA is frequently combined with natural or synthetic polymers, cell-laden hydrogels, growth factors, or decellularized extracellular matrix (dECM), thereby improving its mechanical performance while retaining its osteoconductive and bioactive properties. In this context, additive manufacturing has emerged as a highly suitable fabrication approach, enabling the production of composite scaffolds with precisely controlled architectures and interconnected pore networks tailored for bone regeneration.

A wide range of HA-based bioinks has been reported in the literature, combining HA with polymers such as poly(lactic-co-glycolic acid) (PLGA), polycaprolactone, poly-L-lactic acid (PLLA), and polyethylene glycol (PEG)[16] and with biological components such as mesenchymal stromal cells, amniotic-derived cells, dECM,[17] and osteogenic growth factors.[18] Early examples of this approach include a hybrid-bioprinted PLGA/HA/PEG construct tested in a rat femoral defect,[19] a biphasic calcium-phosphate scaffold seeded with amniotic-derived cells in a sheep sinus-augmentation model,[20] and a co-bioprinted PLLA/HA bone-periosteum construct tested in a rabbit calvarial defect[21] – illustrating that such constructs are already being tested across a spectrum of animal models, from small rodents to large animals,[22] and across defect types ranging from long-bone segmental defects to craniofacial and sinus defects.

Despite the growing body of literature, there remains limited synthesis of the performance of HA-based 3D bioprinted scaffolds across different animal models, defect sites, bioink formulations, and comparator groups, together with a lack of comprehensive appraisal of the methodological quality of the available *in vivo* evidence. Existing reviews have largely combined conventionally 3D-printed scaffolds without cellular incorporation or cell seeding with true bioprinted constructs, thereby obscuring the unique biological advantages conferred by the direct incorporation of cells and bioactive molecules during fabrication.[23,24] Consequently, the specific regenerative potential of HA-based 3D bioprinted scaffolds, independent of conventional scaffold fabrication approaches, remains insufficiently characterized. Furthermore, the methodological rigor of the available pre-clinical studies, including aspects such as randomization, blinding, allocation concealment, and outcome assessment, has not been systematically evaluated, limiting confidence in the strength and reproducibility of the reported findings.

Pre-clinical animal studies constitute a critical stage in the translational pathway of regenerative biomaterials, providing essential evidence on biological safety, osteoconductivity, biodegradation, vascularization, and bone regenerative efficacy within physiologically relevant environments that cannot be adequately replicated *in vitro*.[25] The quality and consistency of these studies directly influence the reliability of efficacy estimates and the likelihood of successful clinical translation. A systematic synthesis of pre-clinical evidence not only identifies the most promising scaffold compositions, bioink formulations, and fabrication strategies but also highlights methodological limitations that may compromise reproducibility and translational validity. Such evidence is fundamental for guiding the optimization of scaffold design, informing regulatory and clinical decision-making, and establishing a robust scientific foundation for future human clinical trials aimed at developing safe and effective therapies

for bone regeneration. This systematic review therefore narrows its focus specifically to true HA-based 3D bioprinted scaffolds and aims (i) to synthesize the *in vivo* evidence on their efficacy for bone regeneration in animal models and (ii) to formally appraise the risk of bias of the identified studies.

METHODS

Review protocol and reporting

The systematic review process in the present study was done according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses criteria for study identification, selection, and reporting,[26] whereas the risk of bias for animal experiments included in this analysis was assessed according to Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) criteria,[27] in compliance with the recommended format for pre-clinical systematic review.[28] The review protocol was registered with PROSPERO, the International Prospective Register of Systematic Reviews (Registration No: CRD420261469466).

PICO framework

The research question adopted a PICO format as summarized in Table 1 below. The population under consideration was animals only since pre-clinical animals act as surrogates for human bone healing physiology and are therefore essential for any pre-clinical research prior to translating new biomaterials to clinical use.[29] This restriction was intended to capture whole-organism bone regeneration rather than outcomes derived from *in vitro* experimental surrogates.

Search strategy

A structured e-search was conducted in six databases – PubMed, Scopus, Web of Science, Embase, Cochrane Central Register of Controlled Trials, and IEEE Xplore. The search strategy comprised controlled terms and free text terms related to four concept areas: hydroxyapatite, 3D bioprinting/additive manufacturing, bone regeneration, and animal models. Clinical trials and human studies were excluded during the initial search stage if the syntax of the database permitted. The database-specific search strategies are presented in Table 2.

Eligibility criteria

Studies were screened against the inclusion and exclusion criteria summarized in Table 3, applied first at the title/abstract level and then confirmed at full-text review.

Criteria were applied in the stated order; a record failing any single inclusion criterion was excluded regardless of its performance on the others.

Table 1: PICO framework used to define the review question.

Element	Definition applied in this review
Population (P)	Animal studies (any species), *in vivo* bone defect models
Intervention (I)	3D-bioprinted scaffolds fabricated using HA-based composite bioinks
Comparison (C)	Scaffolds printed with non-HA-based bioinks, conventional (non-printed) bone grafts, or untreated/empty-defect controls
Outcome (O)	Bone regeneration efficacy, assessed by micro-CT, histopathological examination, and special staining, was reported

HA: Hydroxyapatite, micro-CT: Micro-computed tomography, 3D: Three-dimensional

Study selection and data extraction

All titles and abstracts obtained from the six electronic databases were merged, and duplicates were removed before being independently screened against the inclusion criteria. The full text of records that passed the initial screening process was retrieved and reviewed for eligibility. From each included study, the following information was extracted: first author/year, animal model/strain and sample size, defect location/model, bioink composition (HA and co-printing material), fabrication method/bioprinting, comparison group(s), follow-up period, methods used for outcomes assessment, and the direction of effect on bone regeneration.

Risk-of-bias assessment

The SYRCLE criteria, which expand the Cochrane risk-of-bias tool to cover pre-clinical intervention studies, were used to assess the risk of bias independently for each paper included in the analysis. This tool includes the following ten domains: (1) Sequence generation, (2) baseline characteristics, (3) allocation concealment, (4) random housing, (5) blinding of caregivers/investigators, (6) random outcome assessment, (7) blinding of the outcome assessor, (8) incomplete outcome data, (9) selective outcome reporting, and (10) Other sources of bias. All domains received one of three ratings – low risk of bias (the criterion was well described and appropriately controlled), high risk of bias (the criterion was explicitly described as not properly managed and a source of bias), or an unclear risk of bias (either not described at all or poorly described in such a way that no judgment about its adequacy can be made).

Outcome domains for narrative synthesis

However, as it was impossible to perform meta-analysis due to the large number of heterogeneous studies involved,

Table 2: Database-specific search strategies (summarized)

Database	Search strategy (summary)
PubMed	(“hydroxyapatite”[MeSH] OR “hydroxyapatite” [tiab] OR “HAP” [tiab] OR “calcium phosphate” [tiab] OR “nano-hydroxyapatite” [tiab] OR “nHAP” [tiab]) AND (“bioprinting” [tiab] OR “3D bioprinting” [tiab] OR “three-dimensional bioprinting” [tiab] OR “extrusion-based bioprinting” [tiab] OR “extrusion bioprinting” [tiab] OR “additive manufacturing” [tiab] OR “direct ink writing” [tiab]) AND (“bone regeneration” [MeSH] OR “bone regeneration” [tiab] OR “bone tissue engineering” [MeSH] OR “bone scaffold” [tiab] OR “bone defect” [tiab] OR “bone repair” [tiab] OR “bone healing” [tiab] OR “alveolar bone regeneration” [tiab] OR “craniofacial bone” [tiab] OR “bioink” [tiab] OR “scaffold” [tiab]) AND (“animals” [MeSH] OR “animal model” [tiab] OR “rat” [tiab] OR “rats” [tiab] OR “mouse” [tiab] OR “mice” [tiab] OR “rabbit” [tiab] OR “rabbit model” [tiab] OR “dog” [tiab] OR “canine” [tiab] OR “beagle” [tiab] OR “swine” [tiab] OR “pig” [tiab] OR “sheep” [tiab] OR “ovine” [tiab] OR “*in vivo*” [tiab]) NOT (“humans” [MeSH] OR “clinical trial” [pt] OR “randomized controlled trial” [pt])
Scopus	TITLE-ABS-KEY((“hydroxyapatite” OR “HAP” OR “nano-hydroxyapatite” OR “nHAP” OR “calcium phosphate”) AND (“bioprinting” OR “3D bioprinting” OR “extrusion-based bioprinting” OR “extrusion bioprinting” OR “additive manufacturing” OR “direct ink writing”) AND (“bone regeneration” OR “bone tissue engineering” OR “bone scaffold” OR “bone defect” OR “bone repair” OR “bone healing” OR “alveolar bone regeneration” OR “craniofacial bone” OR “bioink” OR “scaffold”) AND (“animal model” OR “rat” OR “mice” OR “mouse” OR “rabbit” OR “dog” OR “canine” OR “beagle” OR “swine” OR “pig” OR “sheep” OR “ovine” OR “*in vivo*”)) AND NOT TITLE-ABS-KEY(“clinical trial” OR “randomized controlled trial” OR “human subjects”)
Web of Science	TS=(“hydroxyapatite” OR “HAP” OR “nano-hydroxyapatite” OR “nHAP” OR “calcium phosphate”) AND TS=(“bioprinting” OR “3D bioprinting” OR “extrusion-based bioprinting” OR “extrusion bioprinting” OR “additive manufacturing” OR “direct ink writing”) AND TS=(“bone regeneration” OR “bone tissue engineering” OR “bone scaffold” OR “bone defect” OR “bone repair” OR “bone healing” OR “alveolar bone regeneration” OR “craniofacial bone” OR “bioink” OR “scaffold”) AND TS=(“animal model” OR “rat” OR “mice” OR “mouse” OR “rabbit” OR “dog” OR “canine” OR “beagle” OR “swine” OR “pig” OR “sheep” OR “ovine” OR “*in vivo*”) NOT TS=(“clinical trial” OR “randomized controlled trial”)
Embase	(“hydroxyapatite”/exp OR “hydroxyapatite”:ab, ti OR “HAP”:ab, ti OR “nano-hydroxyapatite”:ab, ti OR “calcium phosphate”:ab, ti) AND (“bioprinting”:ab, ti OR “3D bioprinting”:ab, ti OR “extrusion bioprinting”:ab, ti OR “additive manufacturing”/exp OR “direct ink writing”:ab, ti) AND (“bone regeneration”/exp OR “bone regeneration”:ab, ti OR “bone tissue engineering”:ab, ti OR “bone scaffold”:ab, ti OR “bone defect”:ab, ti OR “bone repair”:ab, ti OR “bone healing”:ab, ti OR “alveolar bone regeneration”:ab, ti OR “craniofacial bone”:ab, ti OR “bioink”:ab, ti) AND (“animal model”/exp OR “animal experiment”/exp OR “rat”:ab, ti OR “mice”:ab, ti OR “mouse”:ab, ti OR “rabbit”:ab, ti OR “dog”:ab, ti OR “canine”:ab, ti OR “beagle”:ab, ti OR “swine”:ab, ti OR “pig”:ab, ti OR “sheep”:ab, ti OR “ovine”:ab, ti OR “*in vivo*”:ab, ti) NOT (“human”/exp OR “clinical trial”/exp OR “randomized controlled trial”/exp)
Cochrane Central	#1 hydroxyapatite OR “HAP” OR “nano-hydroxyapatite” OR “calcium phosphate” OR “nHAP”; #2 bioprinting OR “3D bioprinting” OR “extrusion-based bioprinting” OR “additive manufacturing” OR “direct ink writing”; #3 “bone regeneration” OR “bone tissue engineering” OR “bone scaffold” OR “bone defect” OR “bone repair” OR “bone healing” OR “alveolar bone regeneration” OR “craniofacial bone” OR bioink OR scaffold; #4 “animal model” OR rat OR mice OR mouse OR rabbit OR dog OR canine OR beagle OR swine OR pig OR sheep OR ovine OR “*in vivo*”; #5 #1 AND #2 AND #3 AND #4
IEEE Xplore	(“hydroxyapatite” OR “HAP” OR “nano-hydroxyapatite” OR “calcium phosphate”) AND (“bioprinting” OR “3D bioprinting” OR “extrusion bioprinting” OR “additive manufacturing“ OR “direct ink writing”) AND (“bone regeneration” OR “bone tissue engineering” OR “bone scaffold” OR “bone defect” OR “bone repair” OR “bone healing” OR “bioink” OR “scaffold”) AND (“animal model” OR “rat” OR “rabbit” OR “*in vivo*” OR “canine” OR “swine” OR “sheeps”)

Table 3: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
In vivo animal experiments only	*In vitro*-only studies, reviews, clinical trials, and case reports
Scaffold fabricated by 3D bioprinting/additive manufacturing using an HA-based bioink	Scaffolds not fabricated by 3D bioprinting (e.g., conventional casting, salt-leaching alone) or not HA-based
Outcome reported as bone regeneration by micro-CT, histology, or histomorphometry.	No description of the defect model, fabrication method, or *in vivo* outcome
Full text available in English	Conference abstracts, editorials, and non-peer-reviewed preprints

HA: Hydroxyapatite, micro-CT: Micro-computed tomography, 3D: Three-dimensional

outcomes were analyzed descriptively within five predefined areas – mechanical strength, cell viability/biocompatibility, osteogenesis, vascularization, and *in vivo* bone formation – with results being categorized as improved, indicating that the outcome was significantly better than the comparator, no difference found, or not measured.

RESULTS

Study selection

A total of 815 articles were found through the database searches and were further supplemented by 27 more articles sourced elsewhere (n = 842). Out of these, 227 duplicates were dropped, leaving 615 unique articles that were then evaluated based on their titles and abstracts, and 552 articles were excluded as they were clearly not relevant. Out of the remaining 63 articles, 60 were further excluded because they involved the use of non-HA bioinks, non-bioprinted (conventional fabrication method) scaffolds, or *in vitro*-only models. Three articles met all inclusion criteria [Figure 1].

Characteristics of included studies

The three selected studies involved three different species and defect models based on distinct anatomical locations (Table 4): A rat femoral segmental defect model (Machour *et al*., S1);[19] a sheep maxillary sinus augmentation model (Iezzi *et al*., S2);[20] and a rabbit calvarial critical-sized defect model (Zhao *et al*., S3).[21] All three experiments used a HA-based bioink (PLGA/HA, biphasic calcium phosphate [HA-tricalcium phosphate], and PLLA/HA, respectively), prepared through 3D bioprinting or 3D printing of a rapid prototype, and all three compared constructs enriched with cells/biology to an acellular or scaffold-only construct control. Sample sizes were generally small, ranging from 10 sheep in the maxillary sinus model to 15 rabbits across five treatment arms (each contributing bilateral defects) in the calvarial defect study, reflecting the exploratory, hypothesis-generating nature of much of this pre-clinical literature.

Risk of bias

All three articles were found to be at an overall risk of bias that is uncertain on the SYRCLE scale [Figure 2]. Inadequate information was provided for assessing whether there is any evidence of randomization of allocation, allocation concealment, random housing, caregiver/blinding of investigators, random/blinded outcome assessment, and selective outcome reporting in each of the studies, which is fairly typical for pre-clinical research on bioprinting rather than a sign of bias. One criterion – incomplete outcome data – was assessed as having a low risk of bias in the study by Iezzi *et al*., where the number of animals in the groups of CTR, oAEC, and oAFSMC was clearly stated. One criterion – other sources of bias – had a high risk of bias in the study by Zhao *et al*., where there were paired bilateral defects in the same animals in five different treatments.

Synthesis of outcomes across domains

Results have been synthesized narratively into five pre-defined domains, including mechanical strength, cellular

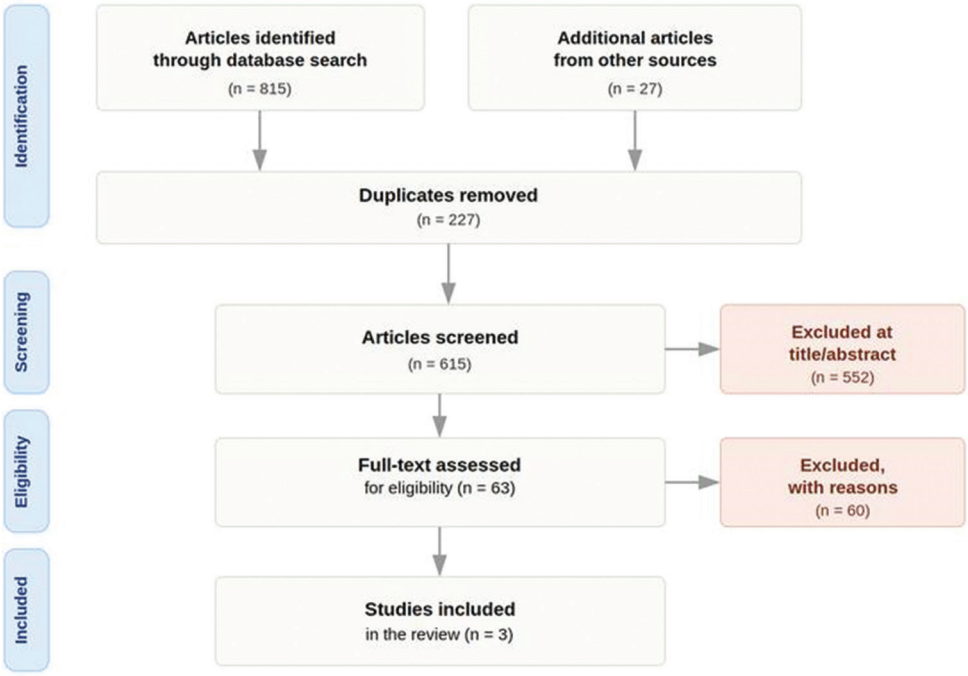


Figure 1: Preferred reporting items for systematic reviews and meta-analyses flow diagram of study identification, screening, eligibility assessment, and inclusion

SYRCLE domain	Machour	Iezzi	Zhao
1. Sequence generation (random allocation)	●	●	●
2. Baseline characteristics similar	●	●	●
3. Allocation concealment	●	●	●
4. Random housing	●	●	●
5. Blinding of caregivers/ investigators	●	●	●
6. Random outcome assessment	●	●	●
7. Blinding of outcome assessor	●	●	●
8. Incomplete outcome data addressed	●	●	●
9. Selective outcome reporting	●	●	●
10. Other sources of bias	●	●	●
Overall judgement	●	●	●

Figure 2: SYRCLE risk-of-bias assessment of the three included animal studies across the ten domains. Machour *et al.*[19] (S1), Iezzi *et al.*[20] (S2), and Zhao *et al.*[21] (S3). Green indicates low risk, yellow indicates unclear risk, and red indicates high risk of bias. Unclear risk indicates that the relevant safeguard was not reported in the study and does not necessarily mean that it was absent.

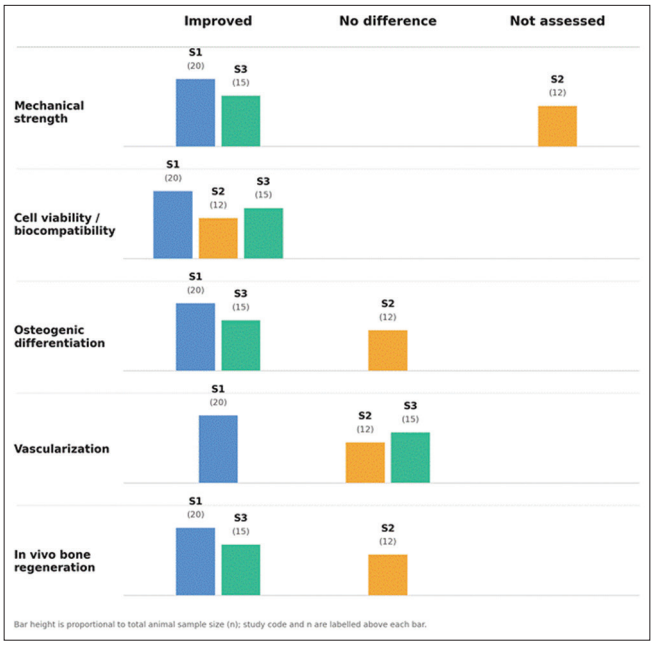


Figure 3: Harvest plot of reported outcomes across five domains in the three included studies. Bar height is proportional to the total animal sample size (n), with the study code and sample size labelled above each bar. S1 = Machour *et al.*[19] (rat, n = 20); S2 = Iezzi *et al.*[20] (sheep, *n* = 12); S3 = Zhao *et al.*[21] (rabbit, *n* = 15).

viability/biocompatibility, osteogenic differentiation, vascularization, and bone regeneration *in vivo*, and are presented as a harvest plot in Figure 3, where the height of the bars is indicative of the total number of animals used for scoring within that domain.

Mechanical strength. Only two of the three studies (S1, *n* = 20; S3, *n* = 15) have found that there was improved mechanical strength in the cell/biology-enriched graft compared to the control; S2 (*n* = 12) did not test for this outcome. Cell viability/biocompatibility. Improved cell viability/biocompatibility of the cell-laden or cell-engineered grafts was reported by the three studies (S1, S2, S3; *n* = 47), being the only domain that had consistent results. Osteogenic differentiation. S1 and S3 found improvements in osteogenic differentiation, whereas S2 found no differences in this particular parameter, even though it was more osteoconductive on imaging. Vascularization. There is only one S1 report that has found an improvement in vascular network formation; both S2 and S3 have shown that there was no difference in vascularization results compared to control groups, and this is the area of poorest evidence for effectiveness of cell engineering. Bone regeneration. S1 and S3 found bone regeneration enhancement, whereas S2 did not show any statistically significant difference in this combined result; however, there were visual results of better osteoconduction/osteoinduction.

In all five categories, the following was evident: there was a graded improvement for HA-scaffold and live cell combination studies, and in S3 also an added biomimetic periosteum layer, compared to S2, which utilized a relatively simple cell seeding technique using a rigid ceramic scaffold on a large animal load-bearing sinus study, in which benefit was seen only through imaging parameters in relation to osteoconductivity/osteoinduction but not on the distinct mechanical, osteogenic, and vascularization measurements from the harvest plot.

DISCUSSION

This review was able to find only three animal models *in vivo* for bone regeneration using 3D-bioprinting technology using HA as the bioink, even though the initial database search returned 842 papers for consideration. This high level of drop-off from 815 to 842 papers to only three studies shows how much of the literature available on the use of bioprinting using HA bioinks is, in fact, only *in vitro* characterization, not using bioprinting, or not using HA bioinks at all.

Viability and biocompatibility represented the only outcome domains in which all three articles demonstrated positive results, which can be explained by the proven osteoconductive and cytocompatibility properties of HA.[30] The inclusion of cells in a scaffold containing HA did not

Table 4: Characteristics of included studies

Study (ID)	Species/*n*	Defect model	Bioink/scaffold	Comparator (s)	Reported bone regeneration outcome
S1 – Machour *et al.*[19]	Rat; femoral defect model	Femoral (long-bone) segmental defect	Thermosensitive PLGA/HA microparticle bioink, hybrid-printed with a cell-laden ECM-based hydrogel	Acellular (scaffold-only) construct	Higher cell viability, vascular network formation, and osteogenic differentiation; superior bone regeneration than acellular controls
S2 – Iezzi *et al.*[20]	Sheep; *n*=10 (4/3/3 per group)	Maxillary sinus augmentation model	3D-printed BCP scaffold engineered with oAEC or oAFSMC	Scaffold alone (CTR, no cells)	Cell-engineered constructs showed enhanced osteoconduction/osteoinduction on synchrotron-radiation micro-CT and histology at 90 days versus scaffold-only control
S3 – Zhao *et al.*[21]	Rabbit; *n*=15 (5 groups, *n*=6, bilateral defects)	Calvarial (skull) critical-sized bone defect, two 8-mm bilateral defects/animal	Co-bioprinted PLLA/HA bone-phase scaffold with a GelMA hydrogel periosteum phase, with or without BMSCs and periosteum-derived stem cells	Blank (untreated) defect; PLLA/HA alone; PLLA/HA+GelMA	Progressive improvement in new bone formation and biomechanical repair with increasing biological complexity of the construct (cells+periosteum layer >scaffold alone >blank)

BCP: Biphasic calcium phosphate, TCP: Tricalcium phosphate, BMSCs: Bone marrow-derived mesenchymal stromal cells, CTR: Control (scaffold alone, no cells), ECM: Extracellular matrix, GelMA: Gelatin methacryloyl, HA: Hydroxyapatite, oAEC: Ovine amniotic epithelial cells, oAFSMC: Ovine amniotic fluid stem cells, PLGA: Poly (lactic-co-glycolic acid), PLLA: Poly-L-lactic acid

seem to negatively impact cell viability in comparison to acellular scaffolds in all three studies. On the other hand, the evidence regarding vascularization was quite limited, and only the experiment on the rat femoral bone using a hybrid printing technique that ensured co-localization of cell-laden hydrogel with a sinterable phase of HA/PLGA demonstrated an increase in vascularization. This result may be explained by one of the problems in bone tissue engineering – the difficulty of achieving appropriate vascular ingrowth into the scaffold core,[31,32] and therefore, simple seeding of the cells on a rigid ceramic scaffold (as in S2) cannot address this problem.

There may be a link between the level of biological complexity and the observed outcome pattern as presented in the three studies above. In Study 3,[21] the effect of the increasing level of biological complexity was directly investigated through a comparison of blank defect, acellular PLLA/HA scaffold, PLLA/HA+gelatin methacryloyl, and completely cellular and periosteum-filled constructs among the 15 rabbits included in that experiment, and progressively greater bone and biomechanical healing with increasing biological complexity was demonstrated, as is expected due to the role of periosteal progenitor cells in fracture healing.[33] Similarly, in S1, the effect of a stiff, sinterable HA-based phase and a printed cell-laden extracellular matrix hydrogel was investigated.

In contrast to S1, S2 involved the cell seeding of a prefabricated rigid biphasic calcium phosphate scaffold in a large-animal model of load-bearing sinus augmentation, which is a challenging anatomical location. Although it showed improved osteoconductivity and osteoinduction using advanced synchrotron radiation micro-CT and histology, it did not demonstrate any significant difference in terms of the mechanical, osteogenic, or angiogenic properties that were measured by harvest plot analysis. This indicates that the specific method of cell incorporation into the HA component could be crucial for the *in vivo* performance of this type of scaffolds; however, considering only three studies, this should only be treated as a hypothesis requiring confirmation in further studies.

All three studies were deemed to have an unclear risk of bias overall according to the SYRCLE tool, which is due to the absence of information regarding methodological controls including randomization, allocation concealment, and blinding of caregivers and outcome assessors, and does not indicate any documented biases. This pattern has been widely noted in pre-clinical bone tissue engineering literature despite existing reporting guidelines, with such information often missing from publications. The only instance of a high risk of bias identified (sources of bias other than the above-mentioned, Zhao *et al*.) refers to the application of paired bilateral calvarial defects in one and the same animal in multiple treatment groups, which can create confounding factors that can relate the results in two defects and affect their comparison as between-group differences.

The results suggest guarded optimism regarding the biocompatibility of HA-based 3D-bioprinted scaffolds, while the addition of cellular and biomimetic structural elements may further enhance the results of bone regeneration compared to non-cellular HA-based scaffolds. However, the perfusion results suggest that the technique of HA-based

bioprinting by itself is insufficient to overcome the perfusion issue, which poses limitations on regeneration in large defects, whereas differences in species, location of the defect, and control groups between the three papers reduce the degree of generality of the conclusions that may be drawn from each study.

These findings are broadly consistent with the wider literature on HA-based additive-manufactured bone grafts. A prior systematic review of 3D-printed HA scaffolds in animal models reported a similarly consistent direction of effect favoring HA-containing constructs over non-HA or untreated controls, but that review pooled conventionally 3D-printed and true bioprinted constructs.[23] A related scoping review focused specifically on 3D-printed HA and tricalcium phosphate scaffolds for alveolar bone regeneration reported a comparable pattern of directionally consistent but methodologically underreported pre-clinical evidence, reinforcing that incomplete risk-of-bias reporting is a feature of this literature more broadly rather than one specific to the three studies reviewed here.[24] By restricting inclusion to studies in which cells or bioactive molecules were incorporated during bioprinting, the present review focuses on a narrower and more homogeneous body of evidence, allowing the effects of biological augmentation to be evaluated within this specific context.[23,24] is reproduced consistently across three independent species and defect models.[19-21] The consistency of this direction of effect, set against the consistency of unclear risk-of-bias ratings across the same three studies, is the central comparative finding of this review. The biological rationale for HA-based bioprinting appears robust across heterogeneous pre-clinical contexts, but the underlying evidence base has not yet reached the level of methodological rigor expected for translation toward large-animal or clinical evaluation.

LIMITATIONS

- Low number of studies: only three *in vivo* studies satisfied eligibility criteria, thus making it impossible to conduct a meta-analysis and weakening statistical power for any outcome domain
- High clinical and methodological heterogeneity: the three studies differed in terms of species used, defect models (long bone, sinus augmentation, calvarial), comparator groups, and outcomes assessed
- Vague risk of bias: insufficient data regarding randomization, allocation concealment, and blinding in all included studies hampers confidence in the size, although not in the direction, of observed effects.

CONCLUSION

Among the limited, yet methodologically diverse literature base examined, all of the 3D-bioprinted, HA-based osseous scaffolds demonstrated viability of the cells *in vivo*, while constructs containing living cells and biomimetic architecture exhibited an increasing degree of improvements in terms of mechanical properties, osteogenesis and bone formation compared to acellular HA-based scaffolds. Vascularization was not convincingly shown to be better in any way, being proven only in one out of three studies and thus remaining a major problem in perfusion of the scaffold interior. The small number and heterogeneity of the included studies, as well as the consistent presence of unclear risk of bias, suggest that these results should be regarded as preliminary. More rigorous randomized and blinded pre-clinical trials, ideally conducted pre-registered according to the SYRCLE guidelines, are needed before the HA-based 3D-bioprinted scaffolds can be regarded as a scientifically validated system for bone defect treatment.

PROTOCOL REGISTRATION

The review protocol was registered with PROSPERO, the International Prospective Register of Systematic Reviews, with the Registration No.: CRD420261469466.

ACKNOWLEDGMENTS

None.

AUTHOR'S CONTRIBUTIONS

Samruddhi Gandhi: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing; Parkavi Arumugam: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review and editing; Kaarthikeyan Gurumoorthy: Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – review and editing

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Source of Support: Nil. **Conflicts of Interest:** None declared.