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ABOUT COVER

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Oxysterols as promising small molecules for bone tissue engineering: Systematic review

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Abstract

BACKGROUND

Bone tissue engineering is an area of continued interest within orthopaedic surgery, as it promises to create implantable bone substitute materials that obviate the need for autologous bone graft. Recently, oxysterols – oxygenated derivatives of cholesterol – have been proposed as a novel class of osteoinductive small molecules for bone tissue engineering. Here, we present the first systematic review of the *in vivo* evidence describing the potential therapeutic utility of oxysterols for bone tissue engineering.

AIM

To systematically review the available literature examining the effect of oxysterols on *in vivo* bone formation.

METHODS

We conducted a systematic review of the literature following PRISMA guidelines. Using the PubMed/MEDLINE, Embase, and Web of Science databases, we queried all publications in the English-language literature investigating the effect of oxysterols on *in vivo* bone formation. Articles were screened for eligibility using PICOS criteria and assessed for potential bias using an expanded version of the SYRCLE Risk of Bias assessment tool. All full-text articles examining the effect of oxysterols on *in vivo* bone formation were included. Extracted data included: Animal species, surgical/defect model, description of therapeutic and control treatments, and method for assessing bone growth. Primary outcome was fusion rate for spinal fusion models and percent bone regeneration for critical-sized defect models. Data were tabulated and described by both surgical/defect model and oxysterol employed. Additionally, data from all included studies were aggregated to posit the mechanism by which oxysterols may mediate *in vivo* bone

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PRISMA 2009 Checklist statement: PRISMA guidelines were followed for this systematic review.

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formation.

RESULTS

Our search identified 267 unique articles, of which 27 underwent full-text review. Thirteen studies (all preclinical) met our inclusion/exclusion criteria. Of the 13 included studies, 5 employed spinal fusion models, 2 employed critical-sized alveolar defect models, and 6 employed critical-sized calvarial defect models. Based upon SYRCLE criteria, the included studies were found to possess an overall “unclear risk of bias”; 54% of studies reported treatment randomization and 38% reported blinding at any level. Overall, seven unique oxysterols were evaluated: 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, Oxy4/Oxy34, Oxy18, Oxy21/Oxy133, and Oxy49. All had statistically significant *in vivo* osteoinductive properties, with Oxy4/Oxy34, Oxy21/Oxy133, and Oxy49 showing a dose-dependent effect in some cases. In the eight studies that directly compared oxysterols to rhBMP-2-treated animals, similar rates of bone growth occurred in the two groups. Biochemical investigation of these effects suggests that they may be primarily mediated by direct activation of Smoothened in the Hedgehog signaling pathway.

CONCLUSION

Present preclinical evidence suggests oxysterols significantly augment *in vivo* bone formation. However, clinical trials are necessary to determine which have the greatest therapeutic potential for orthopaedic surgery patients.

Key words: Oxysterol; Bone tissue engineering; Critical-sized defect; Biomaterial; Orthopaedic surgery; Systematic review

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Core tip: Here we present the first systematic review evaluating the utility of oxysterols for bone tissue engineering. Thirteen preclinical studies examining seven unique oxysterols were evaluated; all examined compounds were found to have statistically significant *in vivo* osteoinductive properties, with some showing dose-dependent effects. Importantly, eight studies found oxysterols to have similar osteoinductive properties to rhBMP-2 in treated animals. These effects are thought to occur through direct activation of Smoothened in the Hedgehog signaling pathway. Future clinical work is necessary to determine the exact therapeutic utility of these molecules in orthopaedic surgery patients.

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INTRODUCTION

Each year, millions of patients undergo bone grafting as part of orthopaedic, neurosurgical, or maxillofacial procedures^[1,2]. The present “gold standard” bone graft material is autologous bone, which possesses the three classic properties of an ideal graft: Osteoconduction, osteoinduction, and osteogenesis^[2-4]. However, local autologous bone supply is often insufficient for adequate grafting, and orthotopic grafting requires an additional surgery for graft harvest, which may result in donor-site morbidity^[4,5]. Furthermore, donor material may fail to engraft, leading to nonunion in a nontrivial proportion of treated patients^[6-8].

To address these issues, extensive work has been performed over the past several decades, creating the field of bone tissue engineering, which comprises all efforts aimed at creating implantable bone substitutes^[9]. In their most basic form, bone tissue engineering products constitute a biological scaffold to facilitate new bone growth often combined with bioactive molecules and/or cells that induce new bone formation^[9-11]. One prime example is the Infuse® Bone Graft (Medtronic Spinal &

Biologics; Memphis, TN) – a collagen sponge with adsorbed recombinant human bone morphogenetic protein-2 (rhBMP-2)^[12] that has been used in more than a million neurosurgical and orthopaedic patients since receiving Food and Drug Administration (FDA) approval in 2002^[12,13]. Despite this widespread use, the Infuse® product can add significantly to surgical costs, has a concerning side effect profile, and does not ensure bony union, creating the opportunity for other, superior bone graft materials to be developed^[14–16].

Recently, a class of steroid derivatives called oxysterols (or hydroxycholesterols) have been studied for their potential osteoinductive effects^[17,18]. Common nomenclature of these molecules has yet to be standardized, with multiple “Oxy” names existing for some identical species^[19,20]. At their core, oxysterols are simply cholesterol oxidation products, which occur naturally in the human body^[18,21]. These compounds, such as 27-hydroxycholesterol (the most abundant circulating oxysterol in humans), have been described in a number of clinical contexts, though are perhaps best known for their role in cholesterol homeostasis, inflammation, and apoptosis^[22,23]. Yet it was not until 2004 that the first description of the osteogenic activity of oxysterols was made, when Kha *et al.*^[24] demonstrated that specific oxysterols could induce osteogenic differentiation and matrix mineralization in *in vitro* preparations of murine mesenchymal stem cells (MSCs).

In vivo support for the osteogenic potential of oxysterols was subsequently reported in a rat model of critical-sized calvarial defects^[17]. Investigation into these molecules has subsequently increased, including several studies exploring oxysterols as an adjuvant to or replacement for rhBMP-2, the current “gold standard” osteoinductive factor employed for bone tissue engineering. Given the newness of these investigations, there has yet to be a review examining the effect of oxysterols on *in vivo* bone formation. To address this, here we provide the first systematic review of the effect of oxysterols on *in vivo* bone formation as a means of evaluating the potential therapeutic utility of oxysterols for bone tissue engineering.

MATERIALS AND METHODS

Electronic literature search

Based upon the PRISMA guidelines^[25] we queried the PubMed/MEDLINE, Embase, and Web of Science databases for all English-language literature examining the effect of oxysterols on *in vivo* bone formation. An example search query employed for the PubMed/MEDLINE database was: ("oxysterol" OR "hydroxycholesterol") AND "bone". We also queried the bibliographies of the included studies for additional sources. Studies identified from the databases were screened for eligibility using the PICOS criteria (Population, Intervention, Comparators, Outcomes, and Study Type)^[26], as follows: Population – laboratory animals; intervention – use of oxysterol-containing biomaterials to promote *in vivo* bone formation; comparators – biomaterials with no bioactive agents or non-oxysterol osteoinductive agents; outcomes – overall fusion rate (spinal fusion studies) and percent bone regeneration (critical-sized defect studies); study type – studies presenting primary *in vivo* results. Studies were excluded if they presented only *in vitro* data, did not present primary data, or did not have full-text availability. Eligible studies were screened against these criteria by two reviewers (Cottrill E and Lazzari J) with a third reviewer (Pennington Z) resolving any discrepancies between the first two reviewers.

Quality assessment

Study methodological quality was assayed using an adapted version of the SYRCLE Risk of Bias tool^[27,28]. Additionally, we included two items from the consensus statement of “Good laboratory practice: Preventing introduction of bias at the bench”^[29] to evaluate overall methodological quality: (1) Was a sample-size calculation provided? and (2) Was a statement on potential conflicts of interest provided? All included studies were screened against these fourteen items by two reviewers (Cottrill E and Lazzari J) with a third reviewer (Ehresman J) resolving any discrepancies.

Data extraction

From full texts we extracted details regarding animal species, surgical/defect model, oxysterol/graft materials used, control/comparative treatment (if available), primary method of assessing bony growth, and primary outcome. For studies employing a spinal fusion model, the primary outcome was fusion rate at last follow-up and for alveolar and calvarial defect studies the primary outcome was percent bone

regeneration. These outcomes were chosen a priori as objective, study-appropriate metrics of *in vivo* bone formation to standardize data collection. In cases where these primary outcomes were not available, other objective metrics of *in vivo* bone formation (e.g., bone volumes) were recorded.

Data were tabulated and described by both surgical/defect model and oxysterol employed. Additionally, data from all included studies were aggregated to posit the mechanism by which oxysterols may mediate *in vivo* bone formation.

RESULTS

Our search identified 267 unique articles, of which 27 underwent full-text review and 13 studies met criteria for inclusion^[17,19-21,30-38] (Figure 1). All fourteen excluded studies were eliminated for lack of full-text availability. Of the 13 included studies – all preclinical – five employed spinal fusion models^[19-21,31,33] (Table 1), two employed critical-sized alveolar defect models^[35,37] (Table 2), and six employed critical-sized calvarial defect models^[17,30,32,34,36,38] (Table 3). Across all studies there were 7 unique oxysterols evaluated (Table 4).

Quality assessment

Methodological quality assessment of the included studies is summarized in Figure 2. Though all included studies were found to have a low risk of both attrition and reporting biases, they all presented an “unclear risk of bias” with regard to group allocation and intervention blinding. Additionally, 62% of studies had an “unclear risk” of assessment blinding bias, creating an overall unclear risk of bias among the included studies. Considering quality indicators, 54% of studies reported randomization at any level and 38% reported blinding at any level. Although no study provided a sample size calculation, 92% provided conflict of interest statements that at minimum identified sources of funding.

Oxysterols in spinal fusion models

Descriptive summaries of the identified spinal fusion studies are provided in Table 1. Of these 5 studies ($n = 213$ animals), four employed rat models ($n = 191$) and one utilized a rabbit model ($n = 22$). All studies used posterolateral inter-transverse process lumbar fusion models with four employing a one-level model (L4-L5) and the fifth utilizing a two-level model (L3-L5). For grafting, all studies used a collagen sponge scaffold, with the exception of Scott *et al.*^[31], who employed iliac crest autograft. In total, four unique oxysterols were evaluated: Oxy4/Oxy34, Oxy18, Oxy21/Oxy133, and Oxy49 (Oxy4 and Oxy34 are identical, and Oxy21 and Oxy133 are identical).

Oxysterols significantly augmented arthrodesis rates in all four studies with a negative control group, with Oxy4/Oxy34 and Oxy21/Oxy133 showing a dose-dependent effect. Furthermore, in all four studies with a rhBMP-2 positive control group, similar osteoinductive effects were observed between rhBMP-2- and oxysterol-treated animals (Table 1). These findings are most strongly highlighted by Johnson *et al.*^[21], who utilized a collagen scaffold supplemented with 0.2, 2, or 20 mg of Oxy34 in a rat model^[21]. These different doses led to reported fusion rates of 0%, 50%, and 100% (10 animals per group), respectively. The oxysterol group receiving the highest dose had an identical fusion rate to controls treated with 5 μ g of rhBMP-2, suggesting non-inferiority. Both agents significantly improved fusion relative to that seen in negative controls treated with inert dimethyl sulfoxide (DMSO). Similar findings were reported by Montgomery *et al.*^[19], who reported fusion rates of 0%, 50%, and 86% using a collagen scaffold supplemented with 0.2, 2, and 20 mg of Oxy133, respectively, compared to rates of 100% using rhBMP-2 and 0% using a control solution. Across the five identified studies, fusion rates of 100% were achieved in some cases with the use of Oxy34, Oxy49, and Oxy133. Stappenbeck *et al.*^[20] reported significantly larger bone volumes using Oxy21 (Oxy133) relative to Oxy4 (Oxy34) in a rat spinal fusion model.

Oxysterols in critical-sized alveolar defect models

Table 2 contains descriptive summaries of the 2 studies examining oxysterols in critical-sized alveolar defect models, both of which employed rat models. Lee *et al.*^[35] employed a two-site defect model and injected solutions of DMSO, rhBMP-2, or a combination of 20(S)-hydroxycholesterol and 22(S)-hydroxycholesterol into the extracted sockets. These treatments produced mean bone regeneration of 45%, 53%, and 65%, respectively^[35]. While suggesting oxysterols to have osteoinductive properties, the regeneration rates were not significantly different across groups.

Table 1 Descriptive summaries of the identified studies investigating the effect of oxysterols on bone formation in spinal fusion models (*n* = 5)

Ref. (level of evidence)	Species (<i>n</i>)	Surgical model and scaffold(s) used	Experimental groups (<i>n</i>)	Assessment method and time point	Fusion rate (no. fused/total)
Johnson <i>et al</i> ^[21] , 2011 (V)	Rat (<i>n</i> = 59)	L4–L5 PLF using a collagen sponge supplemented with Oxy34 ¹ , Oxy49, or rhBMP-2	A. DMSO only (control) (<i>n</i> = 9) B. 5-μg rhBMP-2 (<i>n</i> = 10) C. 0.2-mg Oxy34 (<i>n</i> = 10) D. 2-mg Oxy34 (<i>n</i> = 10) E. 20-mg Oxy34 (<i>n</i> = 10) F. 20-mg Oxy49 (<i>n</i> = 10)	No motion bilaterally <i>via</i> manual palpation at 9 wk post-operatively	A. 0% (0/9) B. 100% (10/10) C. 0% (0/10) D. 50% (5/10) E. 100% (10/10) F. 100% (10/10)
Stappenbeck <i>et al</i> ^[20] , 2012 (V)	Rat (<i>n</i> = 30)	L4–L5 PLF using a collagen sponge supplemented with Oxy4 ¹ , Oxy18, or Oxy21 ²	A. 2-mg Oxy4 (<i>n</i> = 10) B. 2-mg Oxy18 (<i>n</i> = 10) C. 2-mg Oxy21 (<i>n</i> = 10)	Fusion rate was not reported; however, <i>via</i> μ-computed tomographic analysis, treatment with Oxy21 led to significantly larger de novo bone volumes compared to treatment with Oxy4, which was comparable to treatment with Oxy18	
Montgomery <i>et al</i> ^[19] , 2014 (V)	Rat (<i>n</i> = 38)	L4–L5 PLF using a collagen sponge supplemented with Oxy133 ² or rhBMP-2	A. DMSO only (control) (<i>n</i> = 7) B. 5-μg rhBMP-2 (<i>n</i> = 8) C. 0.2-mg Oxy133 (<i>n</i> = 8) D. 2-mg Oxy133 (<i>n</i> = 8) E. 20-mg Oxy133 (<i>n</i> = 7)	No motion bilaterally <i>via</i> manual palpation at 8 wk post-operatively	A. 0% (0/7) B. 100% (8/8) C. 0% (0/8) D. 50% (4/8) E. 86% (6/7)
Scott <i>et al</i> ^[31] , 2015 (V)	Rabbit (<i>n</i> = 22)	L4–L5 PLF using iliac crest autograft supplemented with Oxy133 or rhBMP-2	A. Saline only (control) (<i>n</i> = 5) B. 30-μg rhBMP-2 (<i>n</i> = 6) C. 20-mg Oxy133 (<i>n</i> = 6) D. 60-mg Oxy133 (<i>n</i> = 5)	No motion bilaterally <i>via</i> manual palpation at 8 wk post-operatively	A. 0% (0/5) B. 83.3% (5/6) C. 83.3% (5/6) D. 80% (4/5)
Buser <i>et al</i> ^[33] , 2017 (V)	Rat (<i>n</i> = 64)	L3–L5 posterolateral fusion (PLF) using a collagen sponge supplemented with Oxy133 and/or rhBMP-2	A. DMSO only (control) (<i>n</i> = 32, 8 rats/4 segments each) B. 0.5-μg rhBMP-2 (<i>n</i> = 32, 8 rats/4 segments each) C. 5-μg rhBMP-2 (<i>n</i> = 32, 8 rats/4 segments each) D. 5-mg Oxy133 (<i>n</i> = 32, 8 rats/4 segments each) E. 20-mg Oxy133 (<i>n</i> = 32, 8 rats/4 segments each)	No motion at the operated segment <i>via</i> manual palpation at 8 wk post-operatively	A. 0% (0/32) B. 69% (22/32) C. 100% (32/32) D. 100% (32/32) E. 100% (32/32)

	segments each)	
	F. 0.5- μ g rhBMP2 + 5-mg Oxy133 (n = 32, 8 rats/4 segments each)	F. 100% (32/32)
	G. 0.5- μ g rhBMP2 + 20-mg Oxy133 (n = 32, 8 rats/4 segments each)	G. 100% (32/32)
	H. 5- μ g rhBMP2 + 20-mg Oxy133 (n = 32, 8 rats/4 segments each)	H. 100% (32/32)

¹Oxy4 and Oxy34 are identical.

²Oxy21 and Oxy133 are identical. PLF: Posterolateral inter-transverse process spinal fusion; rhBMP-2: Recombinant human bone morphogenetic protein-2; DMSO: Dimethyl sulfoxide.

Table 2 Descriptive summaries of the identified studies investigating the effect of oxysterols on bone formation in critical-sized alveolar defect models ($n = 2$)

Ref. (level of evidence)	Species (<i>n</i>)	Surgical model and scaffold(s) used	Experimental groups (<i>n</i>)		Assessment method and time point	Bone regeneration (%):	
Lee <i>et al</i> ^[35] , 2017 (V)	Rat (<i>n</i> = 9)	Regeneration of critical-size alveolar defects (left and right maxillary first molars) using equimolar amounts of 20(S)-OHC and 22(S)-OHC (dissolved in 1% DMSO in PBS) or rhBMP-2 in solution	A. DMSO only (control) (<i>n</i> = 3)		Bone regeneration as assessed <i>via</i> μ-CT at 15 days of healing.	A. Approximately 45%	
			B. 15-μg rhBMP-2 (<i>n</i> = 3)			B. Approximately 53%	
			C. 0.40-μg of 20(S)-OHC and 22(S)-OHC (<i>n</i> = 3)			C. Approximately 65%	
Bakshi <i>et al</i> ^[37] , 2019 (V)	Rat (<i>n</i> = not reported)	Regeneration of a critical-size alveolar defect (7 mm × 4 mm × 3 mm) using a collagen sponge supplemented with rhBMP-2 or a mixture of 20(S)-OHC and 22(R)-OHC oxysterols	A. No treatment		Bone regeneration as assessed <i>via</i> μ-CT at 8 weeks of healing.	A. Approximately 65%	
			Collagen sponge	B. No additive		B. Approximately 72%	
				C. 12.5-μg of rhBMP-2		C. Approximately 92% ^a	
				D. 20-mg of 20(S)-OHC and 22(R)-OHC		D. Approximately 90% ^a	

¹Percent bone regeneration of critical-sized defect.

^a $P < 0.05$, compared to collagen sponge only and to no treatment. rhBMP-2: Recombinant human bone morphogenetic protein-2; OHC: Hydroxycholesterol; DMSO: Dimethyl sulfoxide; PBS: Phosphate buffer solution; μ -CT: Micro-computed tomography.

In contrast, using a one-site defect model, Bakshi *et al*^[37] found significant differences in bony regeneration rates among animals treated with rhBMP-2 (92%) or oxysterol-supplemented collagen scaffolds (90%) relative to negative controls that were

Table 3 Descriptive summaries of the identified studies investigating the effect of oxysterols on bone formation in critical-sized calvarial defect models (*n* = 6)

Ref. (level of evidence)	Species (<i>n</i>)	Surgical model and scaffold(s) used	Experimental groups (<i>n</i>)	Assessment method and time point	Bone regeneration (%): [†]
Aghaloo <i>et al</i> ^[17] , 2007 (V)	Rat (<i>n</i> = 15)	Regeneration of critical-sized, bilateral parietal defects (5-mm) using PLGA (porosity: 92%) scaffolds supplemented with 20(S)-OHC and 22(S)-OHC.	A. PBS only (control) (<i>n</i> = 12 defects) B. 70-ng 20(S)-OHC + 70-ng 22(S)-OHC (<i>n</i> = 9 defects) C. 700-ng 20(S)-OHC + 700-ng 22(S)-OHC (<i>n</i> = 9 defects)	Bone regeneration as assessed <i>via</i> μ -CT at 6 weeks of healing.	A. approximately 12% B. approximately 21% ^a C. approximately 22% ^a
Hokugo <i>et al</i> ^[30] , 2014 (V)	Rat (<i>n</i> = not reported)	Regeneration of critical-sized, right parietal defects (5-mm) using a gelatin hydrogel soaked with 20(S)-OHC or incorporating 20(S)-OHC-containing micelles.	A. No implantation (empty defect) (<i>n</i> = 4 defects) B. Gelatin hydrogel alone (<i>n</i> = 4 defects) (mass not reported) C. 20(S)-OHC-gelatin hydrogel (without micelle) (<i>n</i> = 4 defects) (mass not reported) D. 20(S)-OHC-micelle-gelatin hydrogel (<i>n</i> = 4 defects) (mass not reported)	Percent bone regeneration was not reported; however, <i>via</i> μ -CT observation at 6 wk, “no” bone regeneration was observed in untreated defects, “some” bone formation was noted in the periphery of defects implanted with gelatin hydrogel and gelatin hydrogel + 20(S)-OHC, and “robust” bone formation was observed in defects treated with gelatin hydrogel + 20(S)-OHC-micelle.	
Hokugo <i>et al</i> ^[32] , 2016 (V)	Rabbit (<i>n</i> = 25)	Regeneration of critical-sized cranial defect (6-mm; 4 defects/cranium) using a collagen sponge supplemented with rhBMP-2 and/or Oxy49.	A. No treatment (empty defect) (<i>n</i> = 5) B. Collagen sponge only (<i>n</i> = 5) C. 75- μ g rhBMP-2 (<i>n</i> = 5) D. 1-mg Oxy49 (<i>n</i> = 5) E. 10-mg Oxy49 (<i>n</i> = 5)	Bone regeneration as assessed <i>via</i> μ -CT at 6 weeks of healing.	A. approximately 10% ^a B. approximately 35% C. approximately 65% ^a D. approximately 55% ^a E. approximately 65% ^a
Li <i>et al</i> ^[36] , 2017 (V)	Rabbit (<i>n</i> = not reported)	Regeneration of critical-sized cranial defect (8-mm; 4 defects/cranium) using a collagen sponge supplemented with Oxy133 ² and/or rhBMP-2.	A. No treatment (<i>n</i> \geq 6 defects) B. Collagen sponge only (<i>n</i> \geq 6 defects) C. 7- μ g rhBMP-2 (<i>n</i> \geq 6 defects) D. 1-mg Oxy133 (<i>n</i> \geq 6 defects) E. 10-mg Oxy133 (<i>n</i> \geq 6 defects)	Percent bone regeneration was not reported; however, <i>via</i> micro-CT analysis at 6 wk, significantly greater bone formation was observed following treatment with either 10-mg Oxy133 or 7- μ g rhBMP-2 relative to treatment with collagen alone.	
Cui <i>et al</i> ^[34] , 2017 (V)	Mouse (<i>n</i> = 12)	Regeneration of critical-sized, right side cranial defects (3-mm) using MeGC hydrogels supplemented with BMSCs and either SA/Cholesterol or SA/20(S)-OHC sterosomes.	A. No treatment (empty defect) (<i>n</i> = 4) B. SA/Cholesterol sterosome (<i>n</i> = 4)	Bone regeneration as assessed <i>via</i> μ -CT at 6 wk of healing.	A. 7% B. 31%

Huang <i>et al</i> ^[38] , 2019 (V)	Rabbit (<i>n</i> = 6)	Regeneration of critical-sized cranial defect (6-mm; 4 defects/cranium) using an inorganic bovine bone graft (Bio-Oss) supplemented with SVA and/or 20(S)-OHC.	= 4) (mass not reported)	
			C. SA/20(S)-OHC sterosome (<i>n</i> = 4) (mass not reported)	C. 61% ^a
			A. No supplementation (Bio-Oss only; control) (<i>n</i> = 6 defects)	Percent bone regeneration was not reported; however, <i>via</i> histological analysis at 4 wk, bone regeneration was increased following treatment with SVA, 20(S)-OHC, and, especially, SVA + 20(S)-OHC, relative to no supplementation (Bio-Oss only control). A synergistic effect was observed with combinatorial treatment of SVA and 20(S)-OHC.
			B. 0.5-mg of SVA (<i>n</i> = 6 defects)	
			C. 1.0-μg of 20(S)-OHC (<i>n</i> = 6 defects)	
			D. 0.5-mg of SVA + 1.0-μg of 20(S)-OHC (<i>n</i> = 6 defects)	

¹Percent bone regeneration of critical-sized defect.

²Oxy133 and Oxy21 are identical.

^a*P* < 0.05 compared to PBS only (Aghaloo *et al*^[17]), collagen sponge only (Hokugo *et al*^[32]), or no treatment (empty defect) (Cui *et al*^[34]). rhBMP-2: Recombinant human bone morphogenetic protein-2; PLGA: Poly(lactic-co-glycolic acid);

SVA: Simvastatin; OHC: Hydroxycholesterol; μ-CT: Micro-computed tomography; MeGC: Methacrylated chitosan; SA: Stearylamine; BMSCs: Bone marrow-derived mesenchymal stem/stromal cells.

untreated (65%) or treated with unadulterated collagen scaffolds (72%). Similar to the findings of Johnson *et al*^[21] and Montgomery *et al*^[19] in the spinal fusion models, bony regeneration rates were equivocal in animals treated with rhBMP-2 and those receiving a combination of 20(S)-hydroxycholesterol and 22(R)-hydroxycholesterol *et al*^[37].

Oxysterols in critical-sized calvarial defect models

Descriptive summaries of the critical-sized calvarial defect studies are provided in Table 3. Of the six studies, one employed a mouse model, two employed rat models, and three employed rabbit models. Two studies employed single defect models (one defect per skull), 1 study employed a two-defect model, and 3 studies employed four-defect models. Scaffold materials varied widely across studies and included poly(lactic-co-glycolic acid) polymer, gelatin hydrogel, collagen, methacrylated chitosan (MeGC) hydrogel, and inorganic bovine bone graft. A single study co-implanted bone marrow-derived mesenchymal stromal/stem cells (BMSCs) with oxysterols^[34]. In total, four unique oxysterols were evaluated: 20(S)-hydroxycholesterol, 22(S)-hydroxycholesterol, Oxy49, and Oxy133.

As seen in Table 3, the studies cumulatively suggested oxysterols significantly increase bone regeneration relative to control treatments. The largest study by Hokugo *et al*^[32] compared bony regeneration rates in 25 rabbits receiving no treatment or treatment with a collagen sponge. Animals treated with collagen sponges supplemented by either oxysterols or rhBMP-2 had improved bony regeneration rates relative to controls, with the 1 mg Oxy49, 10 mg of Oxy49, and 75 μg rhBMP-2 groups

Table 4 Summary of unique oxysterols investigated for *in vivo* bone tissue engineering in animal models

Oxysterol name	Change from cholesterol ¹	Effect on <i>in vivo</i> bone formation (defect model)	Ref.
20(S)-OHC	OH group at C20(S)	Increase ² (alveolar and calvarial)	Aghaloo <i>et al</i> ^[17] , 2007; Hokugo <i>et al</i> ^[30] , 2014; Lee <i>et al</i> ^[35] , 2017; Cui <i>et al</i> ^[34] , 2017; Bakshi <i>et al</i> ^[37] , 2019; Huang <i>et al</i> ^[38] , 2019
22(R)-OHC	OH group at C22(R)	Increase ^{2,3} (alveolar)	Bakshi <i>et al</i> ^[37] , 2019
22(S)-OHC	OH group at C22(S)	Increase ^{2,3} (calvarial)	Aghaloo <i>et al</i> ^[17] , 2007
Oxy4/Oxy34	OH group at C20(S), single bond between C5 and C6, OH group at C6(S)	Increase ² (spinal fusion)	Johnson <i>et al</i> ^[21] , 2011; Stappenbeck <i>et al</i> ^[20] , 2012
Oxy18	OH group at C20(S), single bond between C5 and C6, OH group at C6(S), deuterated carbons at C22 and C23	Increase ⁴ (spinal fusion)	Stappenbeck <i>et al</i> ^[20] , 2012
Oxy21/Oxy133	OH group at C20(S), single bond between C5 and C6, OH group at C6(S), n-hexane at C20(S)	Increase ² (spinal fusion, calvarial)	Stappenbeck <i>et al</i> ^[20] , 2012; Montgomery <i>et al</i> ^[19] , 2014; Scott <i>et al</i> ^[31] , 2015; Li <i>et al</i> ^[36] , 2015; Buser <i>et al</i> ^[33] , 2017
Oxy49	OH group at C20(S), single bond between C5 and C6, OH group at C6(S), double bond between C25 and C27	Increase ² (spinal fusion, calvarial)	Johnson <i>et al</i> ^[21] , 2011; Hokugo <i>et al</i> ^[32] , 2016

¹(S) and (R) denote stereoisomerism. Refer to [Figure 3](#) for molecular structures.

²Statistically significant relative to control.

³Used in combination with 20(S)-OHC.

⁴Significantly greater bone formation relative to Oxy4/Oxy34 (internal positive control). OHC: Hydroxycholesterol.

having mean percent bone regeneration of approximately 55%, 65%, and 65%, respectively. Animals treated with collagen alone experienced only approximately 35% bone regeneration, which was significantly less than all treated groups^[32].

Similarly, in a murine single-defect model, Cui *et al*^[34] directly compared bony regeneration in untreated animals to those treated with MeGC hydrogel scaffolds supplemented with BMSCs and sterosomes containing either cholesterol or 20(S)-hydroxycholesterol. Bone regeneration was greater in animals treated with 20(S)-hydroxycholesterol (61%) versus cholesterol (31%), and both provided better bony regeneration than was seen in untreated controls (7%)^[34]. The above results combined with the four remaining studies showed significant osteoinductive effects in animals treated with 20(S)-hydroxycholesterol, 22(S)-hydroxycholesterol, Oxy49, and Oxy133.

Osteoinductive effects by specific oxysterol

The osteoinductive effects seen across all studies are summarized for each specific oxysterol in [Table 4](#). To date, seven oxysterols have been investigated, all of which have been shown to enhance bone formation in at least one *in vivo* model. Of these seven oxysterols, 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 22(S)-hydroxycholesterol are naturally occurring species, where the remaining four are synthetic analogues designed for enhanced osteoinductive properties ([Figure 3](#)).

DISCUSSION

Globally, millions of patients undergo bone grafting each year^[1,2]. Though autologous bone remains the “gold standard” for grafting procedures, it is by its very nature in limited supply and does not ensure optimal bony outcomes. Additionally, the harvest of orthotopic autograft (*e.g.*, iliac crest graft) is associated with increased surgical risk and donor-site complications, making it a less-than-ideal option in more medically complex patients^[2-5]. Consequently, there exists an obvious need for implantable bone substitute materials, the creation of which is the focus of bone tissue engineering^[9,10]. Despite prodigious efforts by the field, relatively few commercially viable substitutes have been generated that are capable of providing outcomes equivalent or superior to autograft. One such product is the rhBMP-2 treated collagen sponge (Infuse®, Medtronic Spinal & Biologics; Memphis, TN), which has been used in hundreds of

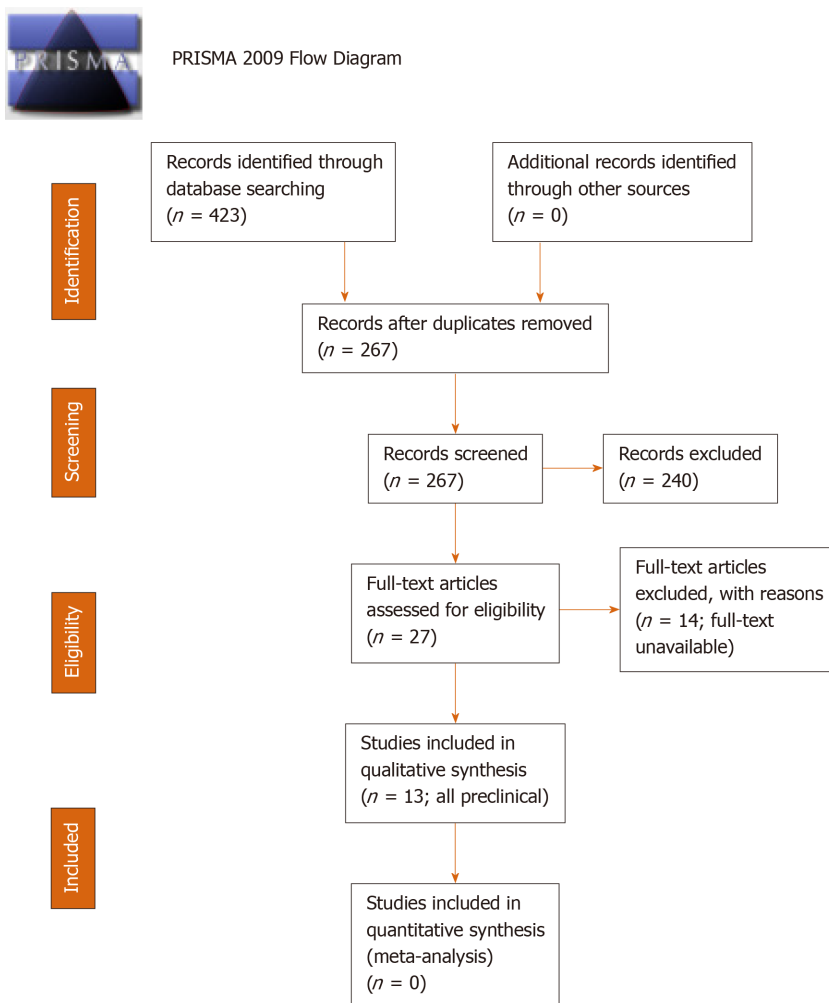


Figure 1 PRISMA 2009 flow diagram for article selection.

thousands of patients and generated billions of dollars in revenue since its market introduction in 2002^[12]. Along these same lines, though, it can significantly increase care costs, has limited FDA-approved indications, possesses a worrisome side effect profile, and does not ensure optimal bony outcomes^[14,15]. Consequently, there remains an outstanding need for additional osteoinductive and osteoconductive agents, of which oxysterols are one promising candidate currently undergoing preclinical investigations.

Here, we present the first systematic review of the available evidence for oxysterols as osteoinductive agents. Our analysis included 13 preclinical *in vivo* studies investigating 7 unique oxysterols in three common animal models of bone healing – spinal fusion, calvarial critical-sized defect, and alveolar critical-sized defect. In aggregate these studies found that oxysterols produced significantly greater arthrodesis rates and bony regeneration relative to the rates seen in untreated animals. Additionally, in the subset of studies directly comparing oxysterol-treated animals to those treated with rhBMP-2 containing scaffolds, non-inferior outcomes were seen in the oxysterol group, suggesting that they may have clinically comparable osteoinductive properties to rhBMP-2. Importantly, though, these properties appear to be stereoisomer-specific^[39].

A visual representation of the proposed mechanism by which oxysterols exert their osteoinductive effects is illustrated in **Figure 4**. At present, it is believed that oxysterol-mediated osteoinduction occurs *via* activation of the Hedgehog (Hh) signaling pathway, a signaling pathway known to be essential for normal bone development^[19,20,31,34-37,40,41]. Though multiple signaling cascades have been demonstrated for Hh signaling, canonical signaling involves the interaction of the transmembrane proteins Patched (Ptc) and Smoothened (Smo)^[40]. Under normal circumstances, Ptc binds to and represses Smoothened, thereby inhibiting Smo signaling. But upon binding Hh ligand, Ptc is inactivated, allowing for Smo activation^[40]. Activated Smo increases levels of the active forms of Gli transcription

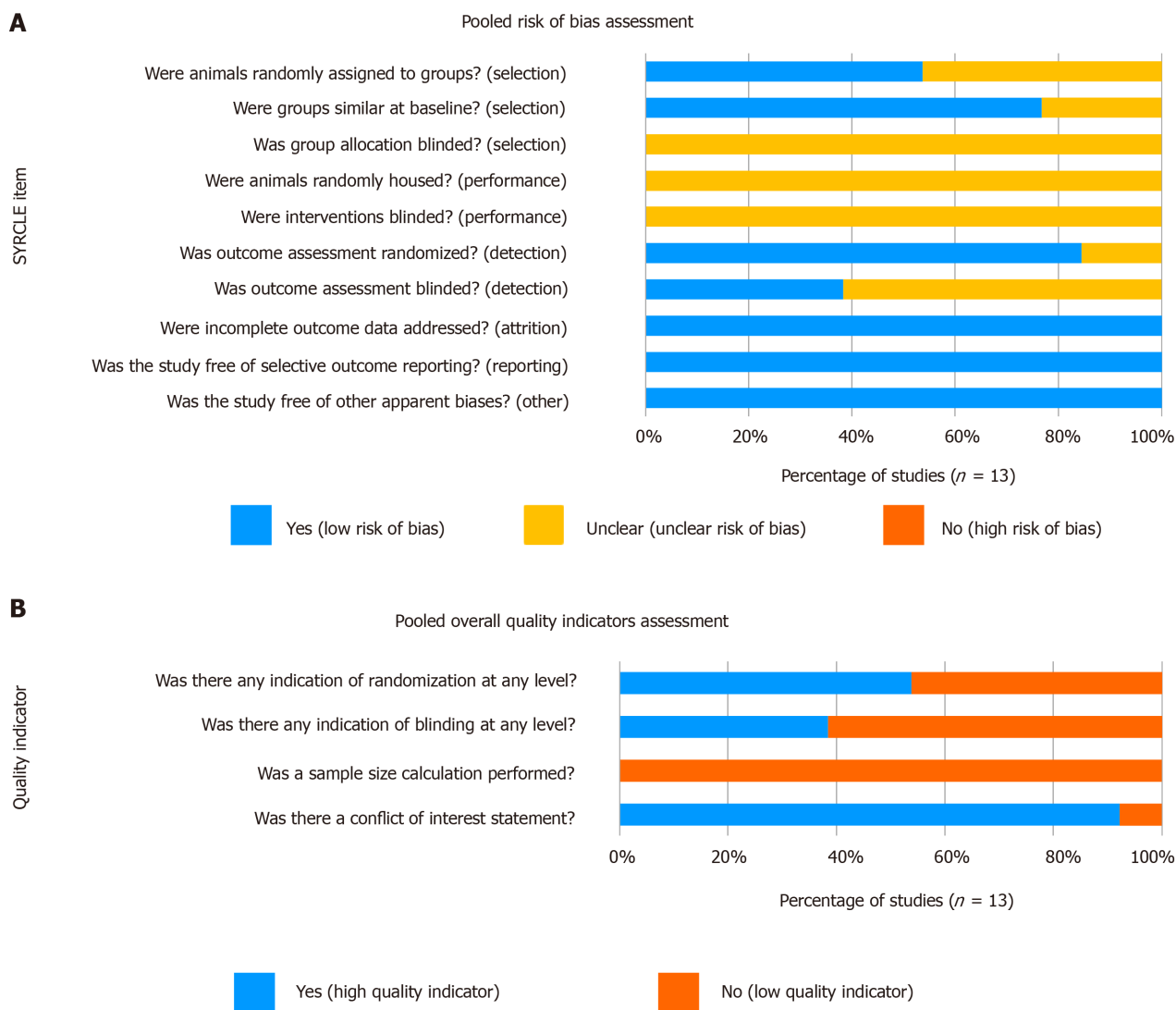


Figure 2 Quality assessment summary of the included studies (*n* = 13). A: SYRCL risk of bias items; B: Overall quality indicators.

factors, which translocate to the nucleus and upregulate multiple genes, including those key for bone formation^[40]. It is well-known that Smo activity can be modulated by small molecules^[42,43], and more recent evidence has suggested oxysterols may directly activate Smo, thereby promoting Hh signaling^[39,44]. Consistent with this, it has been shown that treatment of bone marrow-derived MSCs with both sonic Hh (a member of the Hh ligand family) and Oxy133 results in synergistic activation of Hh signaling and upregulation of osteogenic differentiation markers *in vitro*^[19]. As a possible negative feedback mechanism, it is thought that activated Ptc transports oxysterols away from Smo to maintain Smo inactivation^[44].

Conventional growth factors, such as rhBMP-2, have several limitations that render them suboptimal for many clinical applications. In general, they are limited by high dose requirements, poor shelf-stability, low *in vivo* half-life, and high costs^[45-47]. They are also potentially immunogenic and have low diffusion potential owing to their large size^[45-47]. By contrast, as small molecules, oxysterols are attractive alternatives. Small molecules are low molecular weight (< 1000 Dalton) organic compounds that are generally highly diffusible, allowing for better tissue penetration than growth factors. These small molecules may additionally be designed to activate the same intracellular signaling cascades as growth factors^[45-47]. Small molecules also frequently possess the advantages of high shelf-stability and lower manufacturing cost, making them financially advantageous relative to peptides. Unlike peptides, small molecules are not prone to denaturation and accordingly can be transported more easily with lower logistical costs, making them potentially more desirable from an efficacy and cost perspective^[45]. Furthermore, small molecules are less immunogenic^[46] and are highly amenable to chemical modification, allowing for better control of their bioactivity and tissue targeting^[19,21,48]. Lastly, the pharmaceutical industry has greater experience with

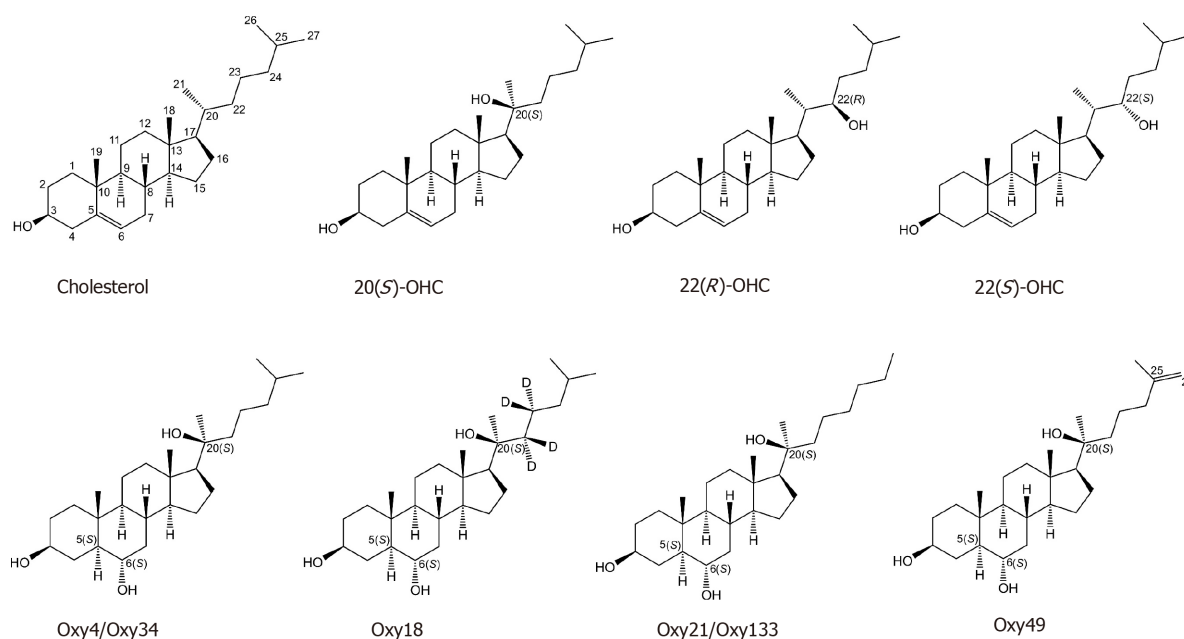


Figure 3 Molecular structures of the identified oxysterols that have been investigated for *in vivo* bone tissue engineering in animal models. Cholesterol is shown for reference. OHC: Hydroxycholesterol.

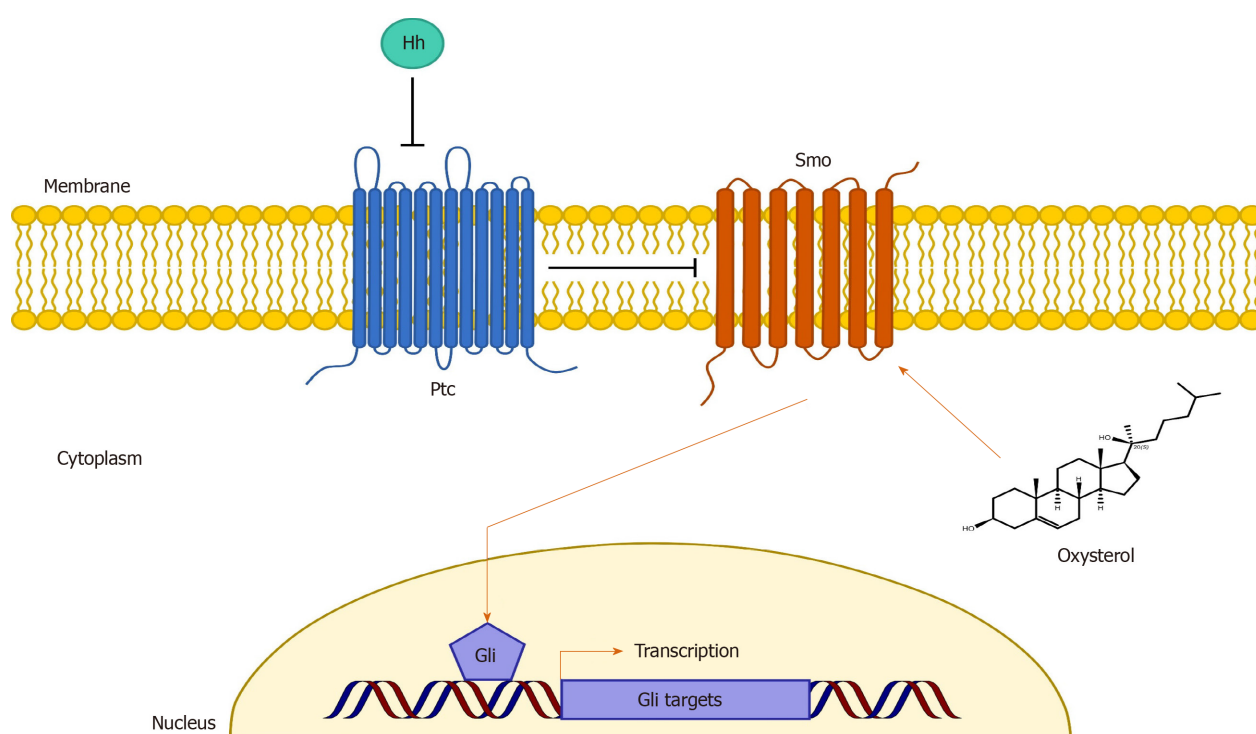


Figure 4 Conceptual illustration of the proposed primary mechanism by which oxysterols exert their osteoinductive effects. It is believed that oxysterol-mediated osteoinduction occurs *via* activation of the Hedgehog (Hh) signaling pathway, which is known to be essential for normal bone development. In canonical Hh signaling, the transmembrane protein Patched (Ptc) inhibits Smoothened (Smo) activity. However, upon binding Hh ligand, Ptc is inactivated, allowing for Smo activation. Activated Smo increases levels of the active forms of Gli transcription factors, which translocate to the nucleus and upregulate multiple genes, including those keys for bone formation. In a stereoisomer-specific manner, it is believed that oxysterols may directly activate Smo, thereby promoting Hh signaling and osteoinduction. Ptc: Patched; Smo: Smoothened; Hh: Hedgehog.

small molecules and there is therefore a concordantly larger knowledge base from which targeted delivery mechanisms can be developed. These mechanisms can reduce the likelihood of off-target effects and optimize pharmacokinetic properties for optimal therapeutic effect^[45-47].

For all of the above reasons, interest in small molecules for bone tissue engineering

has been growing rapidly, with a near-exponential rise in articles published on this topic since the early 2000s^[46]. It is likely that this literature will continue to expand, as high-throughput screening allows for the concomitant testing of thousands of compounds, accelerating the identification of osteogenic compounds^[45,49,50]. To exemplify this, Genthe *et al*^[51] recently used high-throughput screening of a library of approximately 600000 small molecules to identify a new class of osteogenic small molecules, called “ventromorphins”. These previously undescribed agents were found to directly activate BMP signaling^[51]. Other groups around the world have used similar techniques to identify promising small molecules for bone tissue engineering across a wide-range of drug classes^[50,52-55]. Future studies are necessary to evaluate the osteoinductive effects and safety profiles of these and other small molecules. Yet it is likely that some of these osteogenic small molecules – potentially oxysterols – will see clinical translation for the purposes of augmenting *in vivo* bone formation. Whether this is as an adjunct or alternative to growth-factor based therapies remains unclear.

There are several limitations to our study. First, our analysis was limited to a primary outcome of macroscopic evidence of bony fusion or regeneration. We did not consider changes in osteoblast, osteocyte, or osteoclast gene expression or proliferation, nor did we consider histological findings. To definitively conclude that it is the oxysterols mediating improved bony healing through osteoinduction we would likely need to include these endpoints. However, said outcomes are not readily available in the *in vivo* literature and from a clinical perspective, evidence of bone formation *via* imaging or manual palpation is considered the “gold standard” for the models considered. Furthermore, we were unable to perform a meta-analysis secondary to the limited and heterogeneous nature of the available data. Along these lines, the data we were able to include in the review was found to have an overall “unclear risk of bias” as assessed by the SYRCLE Risk of Bias tool. Consequently, the generalizability of our findings may be limited. However, given the consistent findings across the included studies, it appears there is sufficient *in vivo* evidence to support the osteogenic nature of oxysterols. Despite this, the absence of available clinical data means that we are unable to determine the clinical effectiveness of these agents in mediating *in vivo* bone formation. Given these limitations, it is apparent that additional, high-quality research is needed to more fully evaluate the efficacy, safety, and cost-effectiveness of oxysterols on bone tissue engineering.

In conclusion, oxysterols appear to be promising osteoinductive small molecules with potential for bone tissue engineering in the fields of orthopaedic surgery, neurosurgery, and oral maxillofacial surgery. The present preclinical literature has an unclear risk of bias, but the presence of positive outcomes across multiple animal species and surgical models suggests there is sufficient evidence to explore their application in larger animal models and eventually early phase clinical trials. Based upon prior *in vitro* work and the observation of dose-dependency, it appears that the observed effects are secondary to oxysterol-mediated upregulation of osteogenic gene products. Future work is necessary however to identify the therapeutically optimal oxysterol and to optimize its pharmacokinetic properties for use in orthopaedic surgery.

ARTICLE HIGHLIGHTS

Research background

Bone grafting is performed on millions of patients each year, and autologous bone remains the “gold standard” bone graft material. However, autologous bone is limited in supply, is associated with increased surgical risk and donor-site complications and does not ensure optimal bony outcomes. Consequently, there exists an obvious need for implantable bone substitute materials, the creation of which is the focus of bone tissue engineering.

Research motivation

Recently, oxysterols – oxygenated derivatives of cholesterol – have been proposed as a novel class of osteoinductive small molecules for bone tissue engineering. However, there has yet to be a review examining the effect of oxysterols on *in vivo* bone formation. To address this, here we provide the first systematic review of the effect of oxysterols on *in vivo* bone formation as a means of evaluating the potential therapeutic utility of oxysterols for bone tissue engineering.

Research objectives

Following PRISMA guidelines, we aimed to systematically review the available literature examining the effect of oxysterols on *in vivo* bone formation.

Research methods

Using the PubMed/MEDLINE, Embase, and Web of Science databases, we queried all publications in the English-language literature investigating the effect of oxysterols on *in vivo* bone formation. Articles were screened for eligibility using PICOS criteria and assessed for potential bias using an expanded version of the SYRCLE Risk of Bias assessment tool. All full-text articles examining the effect of oxysterols on *in vivo* bone formation were included. Extracted data included: Animal species, surgical/defect model, description of therapeutic and control treatments, and method for assessing bone growth. Primary outcome was fusion rate for spinal fusion models and percent bone regeneration for critical-sized defect models. Data were tabulated and described by both surgical/defect model and oxysterol employed. Additionally, data from all included studies were aggregated to posit the mechanism by which oxysterols may mediate *in vivo* bone formation.

Research results

Thirteen studies (all preclinical) met our inclusion/exclusion criteria. Of the 13 included studies, 5 employed spinal fusion models, 2 employed critical-sized alveolar defect models, and 6 employed critical-sized calvarial defect models. Based upon SYRCLE criteria, the included studies were found to possess an overall “unclear risk of bias”; 54% of studies reported treatment randomization and 38% reported blinding at any level. Overall, seven unique oxysterols were evaluated: 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, Oxy4/Oxy34, Oxy18, Oxy21/Oxy133, and Oxy49. All had statistically significant *in vivo* osteoinductive properties, with Oxy4/Oxy34, Oxy21/Oxy133, and Oxy49 showing a dose-dependent effect in some cases. In the eight studies that directly compared oxysterols to rhBMP-2-treated animals, similar rates of bone growth occurred in the two groups. Biochemical investigation of these effects suggests that they may be primarily mediated by direct activation of Smoothened in the Hedgehog signaling pathway.

Research conclusions

Present preclinical evidence suggests oxysterols significantly augment *in vivo* bone formation. Based upon prior *in vitro* work and the observation of dose-dependency, it appears that the observed effects are secondary to oxysterol-mediated upregulation of osteogenic gene products. The present preclinical literature has an unclear risk of bias, but the presence of positive outcomes across multiple animal species and surgical models suggests there is sufficient evidence to explore their application in larger animal models and eventually early phase clinical trials.

Research perspectives

Oxysterols appear to be promising osteoinductive small molecules with potential for bone tissue engineering in the fields of orthopaedic surgery, neurosurgery, and oral maxillofacial surgery. Future work is necessary to identify the therapeutically optimal oxysterol and to optimize its pharmacokinetic properties for use in orthopaedic surgery.

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