SYSTEMATIC REVIEW PROTOCOL FOR ANIMAL INTERVENTION STUDIES

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Item #	Section/Subsection/Item	Description	Check for approval
1.	Title of the review	**The influence of bioinorganic elements included in calcium phosphate based bone substitutes on bone regeneration: a systematic review and meta-analysis**	
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5.	Funding sources/sponsors	None	
6.	Conflicts of interest	None	
7.	Date and location of protocol registration		
8.	Registration number (if applicable)		
9.	Stage of review at time of registration	Selection of articles	
	Background		
10.	What is already known about this disease/model/intervention? Why is it important to do this review?	The proper bone mass is achieved by presence of living cells (osteoblasts, osteoclasts, and osteocytes), organic matrix (mainly I-type collagen) and inorganic minerals (apatites). The inorganic composition of bone (bone mineral) is primarily formed from salts of calcium and phosphate, the major salt being hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The exact composition of the matrix may change over time and with nutrition, with the ratio of calcium to phosphate varying between 1.3-2 (per weight), and trace minerals such as **Magnesium (Mg), Zinc (Zn), Strontium (Sr) and Fluorine (F)** also being found [1]**.** Trace minerals generally reduce the crystallinity of apatite, except for fluoride, which mainly increases the crystallinity of apatite. As a	

		consequence of the reduction of crystallinity, bone mineral becomes more soluble [2,3]. **Magnesium** is an essential element and the tenth most abundant element in the human body, with approximately 65% of the total magnesium contained in the bones and teeth [4]. In vivo studies have noted that calcium phosphate cement (CPC) doped with magnesium phosphate in the maxillary sinus floor elevation showed greater biodegradability and excellent osteoconductivity when compared to control CPC [5]. **Zinc** is required for the growth, development and maintenance of healthy bones, it stimulates osteoblasts, inhibits osteoclasts function and enhances bone protein synthesis, what increases bone mass and growth [6,7]. In studies with rats, the increase in bone zinc content resulted from lactation of maternal rats fed diet containing zinc [8,9]. Osteoporosis patients have been shown to have lower levels of skeletal zinc than the normal individuals [10]. Zinc supplementation could have beneficial effects on the bone density [8]. Zinc has been known to play an important role in various physiological processes. Zinc is utilized in structural, catalytic or regulatory actions of metalloenzymes, one of such enzymes is alkaline phosphatase (ALP). ALP is vital for the maturation of new bone formation. Furthermore, zinc deficiency is associated with a number of skeletal anomalies in fetal and postnatal development [11]. A number of studies have been performed in vitro to study the effect of the incorporation of zinc into calcium phosphate ceramics on the biological processed related to bone formation and turnover. One study investigated the osteogenic ability of rat and human BMSCs cultured in HA/TCP ceramics containing zinc in amounts varying between 0 and 1.3 wt%. Both rat and human BMSCs cultured in an osteogenic medium showed an increase in ALP expression with increasing zinc content in the HA/TCP ceramic [12]. In another study, a positive effect on the proliferation of the MC3T3 osteoblastic cell line was observed in HA/TCP ceramics containing up to 1.3 wt% zinc, whereas higher concentrations caused cytotoxicity [13]. **Strontium** can enhance bone regeneration when incorporated into synthetic bone grafts. Because it is similar to calcium in size, it is thought to displace calcium ions in osteoblast mediated processes. It is found that strontium most likely stimulates bone formation by a dual mode of action. First by activating the calcium sensing receptor (CaSR) in osteoblasts, which simultaneously increases osteoprotegerin (OPG) production and decreases the receptor activator of nuclear factor kappa beta ligand (RANKL) expression [14]. **Fluorine** enhances the stability of apatite lattice in bones and teeth [15]. Other elements present in bone tissue, such as **Copper**	

		(Cu) and Silicon (Si) also play an important role in bone formation. The presence of these elements in hydroxylapatite is important, because they cocreate bone cells: osteoblasts and osteoclasts, which take part in bone turnover and remodeling. **Copper** is important in collagen maturation [15, 16–18]. Copper is known for its stimulatory effect on angiogenesis in endothelial cells. Copper deficiency is potentially life threatening. Enhanced activity and proliferation of osteoblastic cells was observed when copper ions were loaded on CPC scaffolds. Mesoporous bioactive glass (MBG) scaffolds showed multifunctional characteristics, such as angiogenesis potential, osteostimulation, and antibacterial properties [19]. Other trace elements and minerals that are present in the human body and might also play a role in bone formation are **Sulfur (S) and Chlorine (Cl)**. Among beneficially elements, there are also Cadmium (Cd), Lead (Pb), and Aluminium (Al), which may be toxic and cause bone demineralization and damage [20–22]. 1. Hall, A.C., Guyton, J.E. (2005) Textbook of medical physiology (11th ed.) W.B. Saunders, Philadelphia. 2. Farbod, K. et al. (2014) Interactions Between Inorganic and Organic Phases in Bone Tissue as a Source of Inspiration for Design of Novel Nanocomposites. Tissue Engineering: Part B 20(2) 3. Morgan, E.F., Barnes, G.L. and Einhorn, T.A. The bone organ system: form and function. In: Marcus, R. Feldman, D., Nelson, D., and Rosen, C.J., eds. Fundamentals of Osteoporosis. San Diego, CA: Elsevier Academic Press, 2008, pp. 1-25. 4. Rude R.K. et al. (2005) Dietary magnesium reduction to 25% of nutrient requirement disrupts bone and mineral metabolism in the rat. Bone 37(2):211-219. 5. Zeng D. et al. (2012) Maxillary sinus Floor elevation using a tissue-engineerd bone with calcium-magnesium phosphate cement anda bone marrow stromal cells in rabbits. Tissue Eng Part A 18(7-8):870-881. 6. Yamaguchi, M., Gao, Y.H., and Ma, Z.J. (2000) Synergistic effect of genistein and zinc on bone components in the femoral-metaphyseal tissues of female rats. J. Bone Miner. Metab. 18, 77–83. 7. Bougle, D.L., Sabatier, J.P., Guaydier-Souquieres, G., Guillon-Metz, F., Laroche, D., et al. (2004) Zinc status and bone mineralisation in adolescent girls. J. Trace Elements Med. Biol. 18, 17–21. 8. Ma, Z.J. and Yamaguchi, M. (2001) Role of endogenous zinc in the enhancement of bone protein synthesis associated with bone growth of newborn rats. J. Bone Miner. Metab. 19, 38–44.	

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	Research question		
11.	Specify the disease/health problem	Bone defects	

	of interest		
12.	Specify the population/species studied	All animal models	
13.	Specify the intervention/exposure	Bioinorganic elements included in calcium phosphate based bone substitutes	
14.	Specify the control population	Bone substitutes without bioinorganic elements	
15.	Specify the outcome measures	Bone formation	
16.	State your research question (based on items 11-15)	Hypothesis: the presence of various bioinorganic elements in calcium phosphate based bone substitutes is associated with the enhancement of new bone formation due to various, element-associated properties. Research question: does the addition of bioinorganic elements to calcium phosphate based bone substitutes increase the quantity of bone formation in animals with bone defects?	
	Search and study identification		
17.	Identify literature databases to search (*e.g.* Pubmed, Embase, Web of science)	☒ **MEDLINE via PubMed** ☐ Web of Science ☐ SCOPUS ☒ **EMBASE** ☐ Other, namely: ☐ Specific journal(s), namely:	
18.	Define electronic search strategies (*e.g.* use the step by step search guide[15] and animal search filters[20, 21])	When available, please add a supplementary file containing your search strategy: **Search 17052017 V2.0**	
19.	Identify other sources for study identification	☒ **Reference lists of included studies** ☐ Books ☒ **Reference lists of relevant reviews** ☐ Conference proceedings, namely: ☐ Contacting authors/ organisations, namely: ☐ Other, namely:	
20.	Define search strategy for these other sources	Screen the reference lists of included studies and relevant reviews for articles that should be included.	
	Study selection		
21.	Define screening phases (*e.g.* pre-screening based on title/abstract, full text screening, both)	**1. Pre-screening based on title and abstract** **2. Full text screening**	
22.	Specify (a) the number of reviewers per screening phase and (b) how discrepancies will be resolved	Every screening phase will be carried out by two reviewers. Discrepancies will be resolved by discussion between the reviewers.	
	Define all inclusion and exclusion criteria based on:		
23.	Type of study (design)	Inclusion criteria: primary research paper presenting pre-clinical animal data (*in vivo*). Exclusion criteria: research paper presenting *in vitro* data or clinical data; reviews, conference abstracts, posters	
24.	Type of animals/population (*e.g.* age, gender, disease model)	Inclusion criteria:	

		• Data should be acquired in healthy animals or animals with a systemic disease that is proven to have a negative effect on bone healing; • Animals used should have reached the age of skeletal maturity Exclusion criteria: • Data acquired in animals with a disease of which the influence on bone healing properties is not clear; • Skeletally immature animals	
25.	Type of intervention (*e.g.* dosage, timing, frequency)	• Inclusion criteria: Data should be presented for animals that received a calcium phosphate based bone substitute with inclusion of one (not multiple) bioinorganic element (i.e. Mg, Sr, Zn, F, Cu, Si, S or Cl) (experimental group); Data should be presented for animals that received a calcium phosphate based bone substitute without inclusion of a bioinorganic element (control group); Exclusion criteria: • No calcium phosphate based bone substitute • Multiple bioinorganics added to a calcium phosphate based bone substitute • No relevant control group	
26.	Outcome measures	Inclusion criteria: histological or (histo)morphometrical data about bone formation should be presented (i.e. percentage of material remnants in the region of interest and percentage of new bone in the region of interest) Exclusion criteria: only other outcome measures	
27.	Language restrictions	Inclusion criteria: all languages Exclusion criteria: none	
28.	Publication date restrictions	Inclusion criteria: articles dated up to May 2017 Exclusion criteria: none	
29.	Other	Inclusion criteria: none Exclusion criteria: none	
30.	Sort and prioritize your exclusion criteria per selection phase	**Selection phase:** **1.** *in vitro* or clinical data; reviews, conference abstracts, posters; no calcium phosphate based bone substitute; no bioinorganic added or multiple bioinorganics added per calcium phosphate based bone substitute; no bone	

		defect **2.** Data acquired in animals with a c disease of which the influence on bone healing properties is not clear; skeletally immature animals; no relevant control group; only outcome measures other than histological and histomorphometrical data;	
	Study characteristics to be extracted (for assessment of external validity, reporting quality)		
31.	Study ID (*e.g.* authors, year)	First author & year of publication	
32.	Study design characteristics (*e.g.* experimental groups, number of animals)	• Number of animals per group.	

33.	Animal model characteristics (*e.g.* species, gender, disease induction)	• Species/strain;
		• Male or female;
		• Age
		• Healthy animals or animals with a disease impairing bone-healing.
		  ○ In case of the latter: name of the disease.
		  ○ Method of disease induction
		• Location (orthotopic (implantation in bone)));
		• Size of defect	

34.	Intervention characteristics (*e.g.* intervention, timing, duration)	• Characteristics of experimental group: calcium phosphate based bone substitute with inclusion of one bioinorganic element:
		  ○ Type of calcium phosphate based bone substitute;
		  ○ Type of bioinorganic element;
		• Control group: calcium phosphate based bone substitute without inclusion of a bioinorganic element	

35.	Outcome measures	• Time period (follow-up) • Histological and histomorphometrical data about bone formation and material remnants • Other outcome measures	
36.	Other (*e.g.* drop-outs)	• Reason for drop-out	
	Assessment risk of bias (internal validity) or study quality		

37.	Specify (a) the number of reviewers assessing the risk of bias/study quality in each study and (b) how discrepancies will be resolved	(a) 2 reviewers assessing the risk of bias/study quality in each study (b) Discrepancies will be resolved in a discussion between the reviewers; if necessary by consulting a 3rd party	
38.	Define criteria to assess (a) the internal validity of included studies (*e.g.* selection, performance, detection and attrition bias) and/or (b) other study quality measures (*e.g.* reporting quality, power)	~~☐~~ **By use of SYRCLE's Risk of Bias tool[4]** ☐By use of SYRCLE's Risk of Bias tool, adapted as follows: ☐By use of CAMARADES' study quality checklist, e.g [22] ☐By use of CAMARADES' study quality checklist, adapted as follows: ☐Other criteria, namely:	
	Collection of outcome data		
39.	For each outcome measure, define the type of data to be extracted (*e.g.* continuous/dichotomous, unit of measurement)	histological data: continuous data; descriptive histomorphometrical data about bone formation: continuous data; percentage of new formed bone in a Region Of Interest (ROI).	
40.	Methods for data extraction/retrieval (*e.g.* first extraction from graphs using a digital screen ruler, then contacting authors)	• Data extraction from tables, graphs and text: search for values of material remnants and new bone formation / using a digital screen rule • If necessary: contact authors for missing data	
41.	Specify (a) the number of reviewers extracting data and (b) how discrepancies will be resolved	(a) One reviewer extracting the data, second reviewer checking the data extraction of a random selection of articles (b) By discussion and if necessary consulting a 3rd party	
	Data analysis/synthesis		
42.	Specify (per outcome measure) how you are planning to combine/compare the data (*e.g.* descriptive summary, meta-analysis)	histological data: continuous data → descriptive summary histomorphometrical data about bone formation and material remnants: continuous data; percentage of new formed bone / material remnants in a Region Of Interest (ROI) → meta-analysis	
43.	Specify (per outcome measure) how it will be decided whether a meta-analysis will be performed	A minimum of 3	
	If a meta-analysis seems feasible/sensible, specify (for each outcome measure):		
44.	The effect measure to be used (*e.g.* mean difference, standardized mean difference, risk ratio, odds ratio)	(standardized) Mean difference	
45.	The statistical model of analysis (*e.g.* random or fixed effects model)	Random	
46.	The statistical methods to assess heterogeneity (*e.g.* I^2, Q)	I^2	
47.	Which study characteristics will be examined as potential source of heterogeneity (subgroup analysis)	Healthy vs. not healthy, species, bone type and bioinorganics	

48.	Any sensitivity analyses you propose to perform	Type of defect	
49.	Other details meta-analysis (*e.g.* correction for multiple testing, correction for multiple use of control group)	Correction for multiple use of control group	
50.	The method for assessment of publication bias	Funnel plots, visual inspection	