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A novel strontium(II)-modified calcium phosphate bone cement stimulates human-bone-marrow-derived mesenchymal stem cell proliferation and osteogenic differentiation in vitro

M. Schumacher [a], A. Lode [a,*], A. Helth [b], M. Gelinsky [a]

[a] Centre for Translational Bone, Joint and Soft Tissue Research, Medical Faculty and University Hospital, Technische Universität Dresden, Dresden, Germany
[b] Leibniz-Institute for Solid State and Materials Research IFW Dresden, Dresden, Germany

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ABSTRACT

In the present study, the in vitro effects of novel strontium-modified calcium phosphate bone cements (SrCPCs), prepared using two different approaches on human-bone-marrow-derived mesenchymal stem cells (hMSCs), were evaluated. Strontium ions, known to stimulate bone formation and therefore already used in systemic osteoporosis therapy, were incorporated into a hydroxyapatite-forming calcium phosphate bone cement via two simple approaches: incorporation of strontium carbonate crystals and substitution of Ca^{2+} by Sr^{2+} ions during cement setting. All modified cements released 0.03–0.07 mM Sr^{2+} under in vitro conditions, concentrations that were shown not to impair the proliferation or osteogenic differentiation of hMSCs. Furthermore, strontium modification led to a reduced medium acidification and Ca^{2+} depletion in comparison to the standard calcium phosphate cement. In indirect and direct cell culture experiments with the novel SrCPCs significantly enhanced cell proliferation and differentiation were observed. In conclusion, the SrCPCs described here could be beneficial for the local treatment of defects, especially in the osteoporotic bone.

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1. Introduction

Osteoporosis is a systemic disease "characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" [1], which results from an impaired balance of bone resorption and formation by osteoclasts and osteoblasts, respectively. For the year 2000, the number of primary osteoporosis-related fractures was estimated to be 9 million worldwide, with ~61% of fractures occurring in women [2]. Considering the worldwide demographic development, the burden of osteoporosis will further increase in the near future. Various therapies have been developed for the clinical treatment of osteoporosis based on either the inhibition of bone resorption (e.g. by bisphosphonates, strontium ranelate SrR, RANKL antibody denosumab) or the anabolic stimulation of bone formation (e.g. by parathyroid hormone peptides, SrR) [3,4]. The impact of strontium(II), acting both as an inhibitor of resorption as well as a stimulus of bone formation, has already been demonstrated in vitro and in vivo [4–6]. Strontium was shown to affect cellular processes via the membrane-bound calcium sensing receptor (CaSR), not only in osteoblasts but also in cells of the osteoclasts lineage, where CaSR interaction with strontium can inhibit pre-osteoclast maturation and induce apoptosis in mature osteoclasts [7,9,10]. Mesenchymal stem cell as well as pre-osteoblast proliferation and differentiation into bone-forming osteoblasts is enhanced by the presence of strontium as well as the rate of extracellular matrix formation and mineralization (new bone deposition) [4,5,7]. This effect is based on an influence of Sr^{2+} on the Wnt/β-catenin pathway resulting in enhanced extracellular matrix formation [4] and triggering of mitogenic signalling [8]. Furthermore, Bakker et al. recently found evidence that SrR affects the signalling between osteocytes and both osteoblasts and osteoclasts [11].

In vivo, a significant increase of bone mineral density associated with a decrease of osteoporosis-related vertebral and non-vertebral fracture risk was demonstrated in two phase 3 clinical trials under the administration of 2 g day^{-1} strontium ranelate [12,13]. Still, the bioavailability of SrR is relatively low (~20% [14]) and the actual concentration of strontium at a specific remodelling site cannot be measured and therefore remains unknown. Since high-dose administration of strontium ranelate has sometimes been associated to osteomalacia in rats [15,16], a controlled, local release of divalent strontium ions might be preferable to the systemic administration in the treatment of osteoporotic bone defects. Therefore, strontium has been incorporated in apatite coatings of orthopedic or dental implants [17–19], or polymer-based bone cements [20]. Special attention has been given to

* Corresponding author.
E-mail address: anja.lode@tu-dresden.de (A. Lode).

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calcium phosphate bone cements, which are frequently used in the treatment of bone defects and complicated fractures (also in osteoporotic bone). Calcium phosphate bone cements can integrate into the physiological bone remodelling process due to their solubility and bio-degradability and are therefore ideal to locally release Sr^{2+} into a defect of the bone [21]. Thus, the effect of Sr^{2+} incorporated into different bone cement formulations has been investigated in several studies [22–27]. However, most of these approaches involve the synthesis of Sr-containing calcium phosphate species and therefore require high temperature processing or elaborate precipitation techniques.

In our work, we used two different routines to introduce up to 8.37 wt.% strontium into a well-characterized, hydroxyapatite-forming bone cement based on α-tricalcium phosphate [28]: either the addition of strontium carbonate ($SrCO_3$, A-type modification) or the substitution of $CaCO_3$ by $SrCO_3$ (S-type) in the cement powder. While set A-type cements therefore are composed of a hydroxyapatite matrix filled with $SrCO_3$ clusters, S-type cements are characterized by a homogeneous phase of Sr-substituted hydroxyapatite, as described recently in detail in a complementary study [29].

Here, we describe the positive influence of these novel strontium(II) modifications on in vitro proliferation and osteogenic differentiation of human mesenchymal stem cells (hMSCs), the progenitors of osteoblasts which play a crucial role in bone regeneration. The release of Sr^{2+} from the modified cements was measured and correlated with proliferation and differentiation of hMSC cultured under the influence of different Sr^{2+} concentrations. Secondly, ionic interactions ($[Sr^{2+}]$, $[Ca^{2+}]$ and pH) between the cell culture medium and different cement variations as well as the effect of these variations on hMSC were studied. Finally, hMSCs were cultured in direct contact with the different cement types and characterized with biochemical and molecular biological methods regarding their proliferation and osteogenic differentiation.

2. Materials and methods

2.1. Sample preparation

In this study, a hydroxyapatite-forming calcium phosphate cement (CPC) as first described by Khairoun et al. [28] was used as starting material and control. The CPC precursor consisted of 58 wt.% α-tricalcium phosphate (α-TCP, α-$Ca_3(PO_4)_2$), 24 wt.% calcium hydrogen phosphate (monetite, $CaHPO_4$), 8.5 wt.% calcium carbonate ($CaCO_3$) and 8.5 wt.% hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and was supplied by InnoTERE GmbH, Radebeul, Germany. During ageing in the presence of water, the precursor gradually sets into Ca-deficient hydroxyapatite, with a conversion ratio of ~70% after 7 days. Four different strontium modifications were prepared as described previously in detail [29]. Briefly, in samples referred to as "A-type" strontium carbonate ($SrCO_3$) was added in two concentrations (5 and 10 wt.%, labelled as A5 and A10, respectively). Upon setting, A-type cements form Ca-deficient hydroxyapatite matrix (comparable to CPC) with cluster-like $SrCO_3$ agglomerates. Samples denoted as S50 and S100 were obtained by 50 and 100 wt.% substitution of $CaCO_3$ by $SrCO_3$ in the above-mentioned precursor formulation (see Table 1). By this "S-type" approach, a homogeneous substitution of Ca^{2+} by Sr^{2+} ions in the hydroxyapatite lattice of the set cement could be obtained, due to the formation of Sr-substituted hydroxyapatite, as has been shown recently [29].

Cement powder was mixed manually with 4 wt.% aqueous disodium hydrogen phosphate (Na_2HPO_4) solution using a powder-to-liquid ratio of 400 μl g^{-1} to form a mouldable paste. Disc-shaped specimens designed to fit into standard 48-well tissue culture plates (10 mm diameter and ~1 mm height) were formed using silicone moulds and cured for 4 days in water-saturated atmosphere in a sealed container at 37 °C, instead of immersion in water or aqueous solutions, which would lead to a partial release of strontium ions already during cement setting. Subsequently, samples were air dried and sterilized by γ-radiation at 25 kGy.

2.2. Cement characterization

2.2.1. Cement surface characterization

Cement surface was characterized prior to cell seeding by scanning electron microscopy (SEM, Phillips ESEM XL30, Eindhoven, The Netherlands) on samples coated with carbon (Leica EM SCD005, Leica Microsystems, Wetzlar, Germany). Furthermore, surface roughness was assessed on samples after sterilization as well as on samples immersed in basal cell culture medium (α-MEM containing 9% FCS, 10 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1% L-glutamine, all purchased from Biochrom, Berlin, Germany) for 3 days using white light interferometry (FRT MicroProf, CHR 150 N) and evaluated using the FRT Mark III software (V3.9.10, both Fries Research & Technology, Bergisch Gladbach, Germany).

2.2.2. Ion concentration measurement

Ion release from the different cements as well as pH in the medium was investigated during immersion of set, disc-shaped cement samples in 1 ml basal cell culture medium (α-MEM containing 9% FCS, 10 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1% L-glutamine, all purchased from Biochrom, Berlin, Germany) in semi-dynamic mode (regular complete medium change). A second set of samples was immersed in medium containing osteogenic supplements (OS+, see Section 2.3) and a third set of liquid samples was collected during cell culture (see Section 2.3.3). After 3, 5, 7, 14 and 21 days of incubation at 37 °C and 5% CO_2, pH was measured (pH spear, Eutech Instruments, Nijkerk, The Netherlands), supernatants were completely removed from the sample and collected for ion quantification. Subsequently, 1 ml fresh medium was added to the cement samples. The supernatants were stored for subsequent analysis by inductively coupled plasma mass spectrometry (ICP-MS, IRIS Intrepid II XUV, Thermo Fisher Scientific, Waltham, USA). For analysis, samples were diluted in 13.5 ml water and 0.5 ml HNO_3 (Carl Roth, Karlsruhe, Germany) and filtered using 0.45 μm filter (TPP, Trasadingen, Switzerland) to remove possible cement debris.

Table 1
Composition and relative Sr^{2+} content of strontium modified bone cement precursor powders.

Label	Description	Composition (wt.%)					Sr (wt.%)
		α-TCP	DCPA	HA	$CaCO_3$	$SrCO_3$	
CPC	Pure cement	58.00	24.00	8.50	8.50	0.00	0.00
A5	5 wt.% $SrCO_3$ addition	55.80	23.10	8.20	8.20	4.80	2.83
A10	10 wt.% $SrCO_3$ addition	53.20	22.00	7.80	7.80	9.20	5.40
S50	50% substitution of $CaCO_3$ by $SrCO_3$	58.00	24.00	8.50	4.25	4.25	4.28
S100	100% substitution of $CaCO_3$ by $SrCO_3$	58.00	24.00	8.50	0.00	8.50	8.37

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