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Bone formation induced by strontium modified calcium phosphate cement in critical-size metaphyseal fracture defects in ovariectomized rats☆

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ABSTRACT

The first objective was to investigate new bone formation in a critical-size metaphyseal defect in the femur of ovariectomized rats filled with a strontium modified calcium phosphate cement (SrCPC) compared to calcium phosphate cement (CPC) and empty defects. Second, detection of strontium release from the materials as well as calcium and collagen mass distribution in the fracture defect should be targeted by time of flight secondary ion mass spectrometry (TOF-SIMS). 45 female Sprague–Dawley rats were randomly assigned to three different treatment groups: (1) SrCPC ($n = 15$), (2) CPC ($n = 15$), and (3) empty defect ($n = 15$). Bilateral ovariectomy was performed and three months after multi-deficient diet, the left femur of all animals underwent a 4 mm wedge-shaped metaphyseal osteotomy that was internally fixed with a T-shaped plate. The defect was then either filled with SrCPC or CPC or was left empty. After 6 weeks, histomorphometric analysis showed a statistically significant increase in bone formation of SrCPC compared to CPC ($p = 0.005$) and the empty defect ($p = 0.002$) in the former fracture defect zone. Furthermore, there was a statistically significant higher bone formation at the tissue–implant interface in the SrCPC group compared to the CPC group ($p < 0.0001$). These data were confirmed by immunohistochemistry revealing an increase in bone-morphogenic protein 2, osteocalcin and osteoprotegerin expression and a statistically significant higher gene expression of alkaline phosphatase, collagen10a1 and osteocalcin in the SrCPC group compared to CPC. TOF-SIMS analysis showed a high release of Sr from the SrCPC into the interface region in this area compared to CPC suggesting that improved bone formation is attributable to the released Sr from the SrCPC.

1. Introduction

The treatment of osteoporotic fractures and particularly of osteoporotic fractures with bone defects remains a critical challenge. Biomaterials with the potential to stimulate bone healing have gained interest to improve healing and the treatment outcome for patients with osteoporotic fractures [1]. Injectable calcium phosphate cements have been used in bone surgery since many years based on their osteoconductive properties to stimulate new bone formation [2]. Strontium (II) (Sr^{2+}) has been shown to effectively both stimulate bone formation and inhibit osteoclastic activity and has been introduced into all day clinical practice as oral strontium ranelate medication against osteoporosis [3,4]. Local administration of strontium mainly from functionalized titanium implant surfaces [5–10] or from strontium-substituted hydroxyapatite coatings [11,12] gained interest due to the positive effects of strontium on new bone formation for better implant fixation.

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We recently showed the possibility to generate a strontium (II) modified calcium α-tricalcium phosphate based phosphate cement (SrCPC) in which the $CaCO_3$ portion in the precursor mixture is replaced completely with strontium carbonate ($SrCO_3$, 99.994%), resulting in Sr/Ca ratio of 0.123 [13]. The intention of this recently developed composite material is to use the osteoconductive calcium phosphate cement as a drug carrier for the local release of strontium into bone defects in order to leverage the osteoanabolic and anti-osteoclastic activity not in a systemic but local environment to achieve high strontium concentrations with subsequent enhancement of new bone formation.

In vivo evaluation of SrCPC effects on new bone formation in osteoporotic bone requires a clinically relevant animal model that mimics osteoporotic fractures. Recently, we published a rat model with a critical size defect in the distal metaphyseal femur in which biomaterials can be tested [14]. This model shows important reduction in bone mineral density (BMD) of both spine and femur after bilateral ovariectomy and special diet deficient in calcium, phosphorus and vitamin D3-, soy- and phytoestrogen-free compared to sham animals. The osteotomy addresses the metaphyseal region of the distal femur respecting the fact that mainly metaphyseal regions are affected by osteoporotic fractures and uses the clinically relevant surgical technique of plate fixation of such a fracture defect.

Time of flight secondary ion mass spectrometry (TOF-SIMS) originates from materials science with increasing applications in life science due to its ability to assess chemical composition of solid surfaces down to about 100 nm lateral resolution [15,16]. In brief, a high energetic cluster ion beam is scanning over the sample surface in order to release molecules, atoms and ions that subsequently fly away from the sample surface due to the ion impact. The ions can then be collected by an electrical field and are analyzed in a time of flight analyzer by their mass to charge ratio which enables to visualize strontium and calcium distribution in bone.

The first intention of this study was to evaluate the effects on new bone formation of strontium modified calcium phosphate cement (SrCPC) compared to a strontium free calcium phosphate cement of otherwise similar composition (CPC) and an empty defect control group with the use of the above mentioned clinically relevant rat model [14]. The second intention was to use TOF-SIMS technology to detect strontium release from the materials as well as calcium and collagen mass distribution in the defect area.

2. Materials and methods

2.1. Study design and general information

All interventions were performed in full compliance with the institutional and German protection laws and approved by the local animal welfare committee (Reference number: V 54 – 19 c 20-15 (1) GI 20/28 Nr. 108/2011). 45 female Sprague–Dawley rats were randomly assigned to three different treatment groups: (1) strontium modified calcium phosphate (SrCPC) ($n = 15$), (2) calcium phosphate cement (CPC) ($n = 15$), and (3) empty defect control ($n = 15$). The animals underwent induction of osteopenic bone status by bilateral ovariectomy combined with a multi-deficient diet as previously described [14,17,18]. Three months after ovariectomy and multi-deficient diet, a 4 mm defect in the distal femur metaphysis was created that was stabilized with a mini-plate. The defect was subsequently filled either with SrCPC, CPC or left empty (Fig. 1). The femurs were harvested after 6 weeks and histomorphometrical assessment including immunohistochemistry, enzyme histochemistry, molecular-biological analysis and TOF-SIMS analysis for detection of strontium, calcium and collagen was performed.

2.2. Calcium phosphate cement (CPC) and strontium (II)-modified calcium phosphate cement (SrCPC)

Calcium phosphate cement (CPC) as well as a strontium (II)-modified cement (SrCPC) were used in this study. Synthesis and material properties have been described recently in detail elsewhere [13]. In brief, CPC precursor was composed of α-tricalcium phosphate (α-TCP; α-$Ca_3(PO_4)_2$), calcium hydrogenphosphate ($CaHPO_4$), calcium carbonate ($CaCO_3$) and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) as first described by Driessens and co-workers [19]. In case of strontium-containing SrCPC, $CaCO_3$ was replaced completely with strontium carbonate ($SrCO_3$, 99.994%, Alfa Aesar, Karlsruhe, Germany), resulting in the formation of a Sr^{2+}-substituted apatite cement matrix with a Sr/Ca ratio of 0.123. Cement precursor powders were supplied by InnoTERE GmbH (Radebeul, Germany) and were sterilized by γ-radiation at 25 kGy. Prior to implantation, cement powder was manually mixed with 4 wt.% aqueous Na_2HPO_4 solution using a liquid-to-powder-ratio of 0.40 and 0.35 ml/g for CPC and SrCPC, respectively.

2.3. Animals and surgical procedure

10 weeks old healthy female Sprague–Dawley rats were purchased from Charles River (Sulzfeld, Germany), with an initial weight of 250–290 g. All animals were randomly assigned to the three treatment groups. Animals underwent an acclimatization period of four weeks before induction of osteopenic bone status as described previously [17,18]. Briefly, rats were ovariectomized bilaterally with a dorsal approach. Thereafter, the animals received a low calcium-, phosphorus- and vitamin D3-, and soy- and phytoestrogen-free multi-deficient diet (Altromin-C1034, Altromin Spezialfutter GmbH, Lage, Germany), for 12 weeks.

A wedge-shaped osteotomy was performed on the distal end of the left femur as previously described [14]. In brief, the distal femur was fixed laterally with 7-hole T-shaped mini-plate (Leibinger® XS-mini-plate, Stryker, Schökirchen, Germany). Then the osteotomy at the distal metaphyseal area with creation of a wedge-shaped defect with a lateral length of 4 mm and a medial gap of 0.35 mm using an ultrasound bone saw (Piezosurgery® 3, Saw blade OT7S-3, Mectron, Köln, Germany) was performed. The defect was then treated according to the study protocol and the animals either received SrCPC, CPC or no biomaterial (Fig. 1). Postoperatively, animals continued receiving the deficient diet until euthanized at 6 weeks post femur surgery. Animals were euthanized under inhalation of CO_2 after general anesthesia.

2.4. End-point and specimen preparation

Animals were euthanized 6 weeks post femur surgery. The left femora were then harvested, and all surrounding soft-tissue was removed. Only samples with intact fixation plates were used for further analysis.

2.5. TOF-SIMS

Measurements were done with a TOF-SIMS 5–100 machine (IONTOF, Münster, Germany) on Technovit section (see chapter "Histological analysis"). The machine is equipped with a 25 keV Bi-cluster ion gun for surface analysis. The maximum dimension for the primary ion raster mode is 500 × 500 μm². For survey images, so called stage scans, were used. Single ion images of 300 × 300 μm² were merged to a big mass image of 7.0 × 14.5 mm² for the empty defect, 6.7 × 14.2 mm² for CPC and 8.1 × 13.6 mm² for the SrCPC group. With a pixel density of 100/mm, the primary ion gun runs in the high current bunched (hc-bu) mode with highest mass resolution and a lateral resolution of about 10 μm for this large survey images. For more detailed images, the low current bunched (lc-bu) mode with a lateral resolution of 2 μm was used. The pixel density was 1000/mm and single frames of 300 × 300 μm² were assembled to survey images of 1.8 × 0.9 mm². As primary ion species Bi^{3+} was used. Data evaluation was done with the Surface Lab 6.3 software (IONTOF, Münster, Germany) delivering detailed mass maps of the analyzed surface. In one mass image usually only the distribution of one selected mass is shown. Within this image, bright and dark pixels represent a high and low count rate of this mass, respectively. For more detailed information about this method the reader is referred to the literature [16].

Mass distribution analysis of Sr^+, Ca^+, and of $C_4H_8N^+$ which was shown to correlate with collagen I [20] was performed. Furthermore, an overlay image of the collagen and Ca signal was depicted.

2.6. Histological analysis and histomorphometry

For histological analysis, the harvested femurs were fixed in phosphate- buffered 4% paraformaldehyde and stored at 4 °C until processing. Samples were then embedded in Technovit® 9100 NEU according to the manufacturers protocol (Heraeus Kulzer, Hanau, Germany). After embedding, Technovit blocks were sectioned into 5 μm thick slices with the aid of Kawamoto's film (Section-Lab Co. Ltd., Japan) to keep the biomaterials intact. For histological and histomorphometrical analysis these sections were stained with movat-pentachrome as described earlier [21].

Two regions of interest (ROIs) were used for histomorphometric evaluations (Fig. 2). The first ROI was made by directly tracing over the material followed by a 100 pixels increase to include the biomaterial–tissue interface. The second ROI comprised the entire initial wedge-shaped osteotomized defect area to assess the new bone formation within the former fracture defect area. With the help of Adobe Photoshop CS6, the measurements for area of bone, ROIs, implant, and the void were made respectively to determine bone versus tissue ratio (BV/TV). A count for tartrate-resistant acid phosphatase (TRAP) positive cells was also performed. The consecutive sections were then used for all described methods. The measurements were done blind folded with regards to the test groups.