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Efficacy of the biomaterials 3 wt%-nanostrontium-hydroxyapatite-enhanced calcium phosphate cement (nanoSr-CPC) and nanoSr-CPC-incorporated simvastatin-loaded poly(lactic-*co*-glycolic-acid) microspheres in osteogenesis improvement: An explorative multi-phase experimental in vitro/vivo study

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ABSTRACT

Aims: The purpose of this multi-phase explorative in vivo animal/surgical and in vitro multi-test experimental study was to (1) create a 3 wt%-nanostrontium hydroxyapatite-enhanced calcium phosphate cement (Sr-HA/CPC) for increasing bone formation and (2) creating a simvastatin-loaded poly(lactic-*co*-glycolic acid) (SIM-loaded PLGA) microspheres plus CPC composite (SIM-loaded PLGA + nanostrontium-CPC). The third goal was the extensive assessment of multiple in vitro and in vivo characteristics of the above experimental explorative products in vitro and in vivo (animal and surgical studies).

Methods and results pertaining to Sr-HA/CPC: Physical and chemical properties of the prepared Sr-HA/CPC were evaluated. MTT assay and alkaline phosphatase activities, and radiological and histological examinations of Sr-HA/CPC, CPC and negative control were compared. X-ray diffraction (XRD) indicated that crystallinity of the prepared cement increased by increasing the powder-to-liquid ratio. Incorporation of Sr-HA into CPC increased MTT assay (biocompatibility) and ALP activity ($P < 0.05$). Histomorphometry showed greater bone formation after 4 weeks, after implantation of Sr-HA/CPC in 10 rats compared to implantations of CPC or empty defects in the same rats ($n = 30$, ANOVA $P < 0.05$).

Methods and results pertaining to SIM-loaded PLGA microspheres + nanostrontium-CPC composite: After SEM assessment, the produced composite of microspheres and enhanced CPC were implanted for 8 weeks in 10 rabbits, along with positive and negative controls, enhanced CPC, and enhanced CPC plus SIM ($n = 50$). In the control group, only a small amount of bone had been regenerated (localized at the boundary of the defect); whereas, other groups showed new bone formation within and around the materials. A significant difference was found in the osteogenesis induced by the groups sham control (16.96 ± 1.01), bone materials (32.28 ± 4.03), nanostrontium-CPC (24.84 ± 2.6), nanostrontium-CPC-simvastatin (40.12 ± 3.29), and SIM-loaded PLGA + nanostrontium-CPC (44.8 ± 6.45) (ANOVA $P < 0.001$). All the pairwise comparisons were significant (Tukey $P < 0.01$), except that of nanostrontium-CPC-simvastatin and SIM-loaded PLGA + nanostrontium-CPC. This confirmed the efficacy of the SIM-loaded PLGA + nanostrontium-CPC composite, and its superiority over all materials except SIM-containing nanostrontium-CPC.

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1. Introduction

In 1980s, calcium phosphate cements (CPCs) were discovered by LeGeros and Chow et al. [1]. Calcium phosphate cements are used as the bone substitute materials and can serve as injectable pastes to fill defects, while being biocompatible and osteoconductive [2,3]. A bioactive material is one that can bind with the surrounding bone without the formation of fibrous tissue [4]. Bioactivity, together with the perfect adaptability of the cement paste leads to a stable connection between defect and implant and boost bone healing process [5,6]. The main reaction of calcium phosphate cements is the cementing action of acidic and basic calcium phosphate compounds in an aqueous solution [1,2,7]. Calcium phosphate cements are formed by a chemical reaction between two phases: the dry powder phase which is a combination of calcium orthophosphate, and the liquid phase which is water or a calcium or phosphate-containing aqueous solution [1,2,4,7,8]. After mixing the powder phase with the liquid phase, a paste forms that sets and hardens into a solid mass. One of the advantages of calcium phosphate cements is that no heat is generated during cementation reaction and the cementation process is not exothermic (thus, no risk of hyperthermia) [2,3,7,9,10]. Furthermore calcium phosphate cements are intrinsically microporous, as a result of extra aqueous solution leaving the material after hardening. This property can be used for carrying of biological fluids into CPCs and causes degradation and replacement of CPCs by bone. Degradation rate depends on the composition and microstructure of the cement. Degradation products are well absorbed by the surrounding tissues [11–14].

Despite having many advantages, calcium phosphate cements have some drawbacks including poor mechanical properties. Like most ceramics, they are brittle. Furthermore, due to their intrinsic porous structure, their strength is lower than acrylic cements. This drawback has limited their application [11,15–17].

There are two main calcium phosphate cements: hydroxyapatite and dicalcium phosphate dehydrate (brushite). The final product formed by the liquid and solid phase depends on pH of the solution. Hydroxyapatite forms in pHs higher than 4.2 while brushite (DCPD) forms when the pH is lower than 4.2. Solubility of brushite is greater than hydroxyapatite at physiological pH. In fact brushite is metastable under physiological conditions and can be resorbed more quickly than hydroxyapatite [2,8,18].

Brushite cements have generally short setting times (about 30 to 60 s) that limit their use as orthopedic applications. To make these cements suitable for orthopedic usage, specific setting retardants (such as pyrophosphate ions or citrates) are usually added to slow down the setting process by inhibiting nucleation and growth of calcium phosphate crystals [7,16,19,20]. Moreover, some ions like Sr, Zn, and Mg can serve as enzyme cofactors in bone regeneration process [21–23]. Thus, the incorporation of these ions in biomaterials can improve bone tissue healing [24,25].

Strontium can stimulate osteoblast differentiation and inhibit osteoclast activity and therefore is a valuable ion in treatment of osteoporosis. Hence, there are interests in incorporating strontium in calcium phosphate cements. Incorporation of strontium affects the reactivity of the cement but can also modify the final composition of the material [24–26].

A recent advent in pharmacology is solid polymer biodegradable particulates such as micro and sub-micron spheres with high dissolution rates, which might be of use in locally targeted pharmaceutical delivery and regeneration of injured tissues [27–29]. Because of their biodegradation potential and physiological removal, poly(lactic-*co*-glycolic acid) (PLGA) microspheres might be a proper candidate for such drug delivery systems [28,30,31]. Simvastatin (SIM) is a hypolipidemic drug capable of inducing bone regeneration; hence it might be loaded into PLGA microspheres to possibly improve stability of microspheres after formation while also improving the bone regeneration [28,32,33].

In this study, the structural, physicochemical and biological properties of the type of calcium phosphate cements modified by strontium ion were evaluated. Also its effect on bone generation was examined. Afterwards, a new form of SIM-PLGA was created by the addition of nanostrontium-enhanced CPC. Its efficacy in improving osteogenesis was assessed.

2. Materials and methods

2.1. Overview

In the first phase of this study (in vitro), an optimum ratio of power to liquid (P/L) was selected (among three available ratios) for producing 3 wt% nanostrontium-enhanced CPC with the best in vitro properties. The recommended ratio was then used in next phases: in the second phase, the 3 wt% nanostrontium-enhanced CPC was assessed with various in vitro biological tests and then was compared against CPC as well as negative controls in rat. In the third phase (in vitro), the nanostrontium-enhanced CPC was composited with simvastatin-loaded poly(lactic-*co*-glycolic acid) (SIM-loaded PLGA) microspheres. In the last phase the properties of this "SIM-loaded PLGA + 3 wt% nanostrontium-enhanced CPC" composite biomaterial was evaluated in vitro. Then efficacy of this composite material in ontogenesis induction was evaluated in vivo (rabbit) against the materials: 3 wt% nanostrontium-enhanced CPC and 3 wt% nanostrontium-enhanced CPC plus simvastatin, a negative control (sockets left empty after bone trephining) as well as a positive control (experimental bone defects filled with the animal's trephined bone transplanted from the negative control sites). This study was conducted in accordance with the regulations and approval of the Institutional Animal Care and Ethical Committee of the Tehran University of Medical Sciences.

2.2. Preparation of nanostrontium-enhanced CPC

To prepare calcium phosphate cement powder in this research, one gram of tetracalcium phosphate ($Ca_4P_2O_9$, TTCP) with a controlled mean particle size of 10 μm was synthesized following the method from the reaction of dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$, DCPD; Merck Co. Kenilworth, New Jersey, United States) and calcium carbonate ($CaCO_3$). Afterwards, disodium hydrogen phosphate (Na_2HPO_4; Merck) was added to the mixture; three different amounts of Na_2HPO_4 were added, in order to create 3 different powder-to-liquid ratios 1.205 ml (P/L = 0.83), 0.8 ml (P/L = 1.25), and 0.645 ml (P/L = 1.55). Finally, 3 wt% of synthesized nanostrontium-substituted hydroxyapatite (Sr-HA) (10% of calcium in hydroxyapatite was replaced with strontium) was added to the prepared cement. The calcium phosphate cement powder was mechanically ground to the mean particle size distribution of 3 mm; then it was vacuum-packed and g-ray-sterilized (20 kGy). The size of nanoparticles is evident in Fig. 1.

2.3. Characterization of nanostrontium-enhanced CPC

2.3.1. X-ray diffraction analysis

XRD patterns of the prepared calcium phosphate cements (CPCs) were obtained at room temperature using a very high-resolution Cu-K_α radiation diffraction system (Equinox3000, INEL, Artenay, France) operating at a voltage of 40 kV and a current of 30 mA. CPCs were analyzed in the 2θ angle range of 0–80°, and their patterns were studied to determine the crystal phases present in the samples.

2.3.2. Fourier transform infrared spectroscopy (FTIR) analysis

Infrared spectroscopy was carried out to determine the chemical composition of the prepared microspheres using FTIR (Nicolet, USA)