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# Novel therapeutic intervention for osteoporosis prepared with strontium hydroxyapatite and zoledronic acid: *In vitro* and pharmacodynamic evaluation

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ABSTRACT

Osteoporosis therapeutics has been monopolized mainly by bisphosphonates, which are potent anti-osteoporotic drugs, while they do not promote bone formation or replenish the already resorbed bone. Although strontium substituted hydroxyapatite (SrHA) has been proclaimed to improve bone properties in an osteoporotic animal model, there is no published data on direct delivery of SrHA nanoparticles by bisphosphonate-like zoledronic acid (ZOL) to the bone. Therefore, this study was designed to investigate the potential of using SrHA/ZOL nanoparticle-based drug formulation in an ovariectomized rat model of postmenopausal osteoporosis. SrHA and SrHA/ZOL nanoparticles were prepared and characterized by field-emission scanning electron microscopy (FESEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). Twelve weeks after ovariectomy, rats were treated with either single intravenous dose of SrHA/ZOL (100, 50 or 25 μg/kg); ZOL (100 μg/kg); or SrHA (100 μg/kg). Saline-treated OVX and SHAM-OVX groups served as controls. The energy-dispersive X-ray (EDX) microanalysis of bone specimen obtained from SrHA/ZOL groups yielded range between 64.3 ± 6.7 to 66.9 ± 6.8 of calcium weight (wt) % and 1.64 ± 0.6 to 1.74 ± 0.8 of calcium/phosphorus (Ca/P) ratio which was significantly higher when compared with 39.7 ± 9.3 calcium and 1.30 ± 0.2 Ca/P ratio for OVX group. Moreover, the strontium wt% in SrHA/ZOL group (between 3.1 ± 0.5 and 6.8 ± 0.4) was significantly higher than SrHA group (1.8 ± 0.9). These results confirmed targeted delivery of SrHA nanoparticles by ZOL to the bone. Therapy with SrHA/ZOL showed significant improvements in trabecular bone microarchitecture and mechanical strength as compared to ZOL or SrHA ($p < 0.05$). Moreover, treatment with SrHA/ZOL significantly precluded an increase in serum bone-specific alkaline phosphatase and tartrate-resistant acid phosphatase than either ZOL or SrHA ($p < 0.05$). These results strongly implicate that SrHA/ZOL nanoparticle-based drug formulation showed better efficacy at a much lower dose of ZOL. SrHA/ZOL drug formulation has a therapeutic advantage over ZOL or SrHA monotherapy for experimental osteoporosis.



## 1. Introduction

Osteoporosis is amongst the most common systemic skeletal diseases occurring in adults who are older than fifty years of age [1]. Bisphosphonates are the primary anti-osteoporotic agents who are shown to inhibit bone resorption activity but are unable to replenish the already lost bone, resulting in poor bone remodelling [2,3]. Research studies demonstrated that long term therapy with bisphosphonates resulted in excellent anti-fracture action [4]. However, possible increased risk of atypical fractures due to prolonged bisphosphonate therapy has been reported [5]. Furthermore, the systemic administration of bisphosphonates like zoledronic acid (ZOL) has been associated with jaw osteonecrosis which is frequent in oncologic patients who receive higher doses of this medication [6–8]. Thus, a first-rate therapeutic intervention adequate to obtain efficient osteogenesis has inspired current research in the field of osteoporosis.

These are the following rationales for this work. First, synthetic hydroxyapatite (HA) is commonly assimilated to osseous and hard tissues in humans and vertebrates [9]. However, biological HA is different from the stoichiometric HA in various components including non-stoichiometry, nano-dimensional crystals and structural order with low degree [10]. HA nanoparticles do not have negative osteoclast activity but they are classified as bioactive substances having positive osteogenic activity and osteointegration when used in orthopedic and dental

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applications [9,11]. Second, studies demonstrated that administration of strontium in the form of strontium ranelate did reduce fracture risk in patients with osteoporosis [12,13]. Strontium ranelate has been indicated as an alternate remedy following long term therapy with bisphosphonates [14]. Studies further illustrated that strontium strengthens bone development and bone mineralization by induction of osteogenesis by osteoblast cells aside from restraining osteoclastic resorption [12,13,15]. Previous *in-vitro* data showed that strontium ranelate stimulates osteoblast maturation and also reduces the RANKL (receptor activator of nuclear factor kappa-B ligand) expression by the osteoblasts [16]. Previously conducted studies have shown that strontium can positively impact bone cells even if incorporated into HA. Nanocrystals of strontium substituted HA (SrHA) with different extents of calcium substituted by strontium has been prepared and shown to stimulate osteogenesis of osteoblast cells and inhibit osteoclast proliferation [17,18].

We have previously shown that HA facilitates the bioactivity of osteoblasts even if bisphosphonate like ZOL or risedronate are adsorbed on HA nanoparticles, to provide quicker bone regeneration in a rodent model of established osteoporosis [19,20]. Recent observation shows that HA containing strontium content of 10 at% was indicated to promote peri-implant bone healing in osteoporotic rats previously treated with ZOL when compared to HA [21]. Boanini et al., reported that presence of strontium and ZOL in HA nanocrystals has additional inhibitory effect on osteoclast activity and also enhances osteoblastic activation [22], but the information about the bone response to ZOL loaded with SrHA in experimental model of osteoporosis was still limited. As osteoporosis involves both diminished bone formation and elevated bone resorption, it appears obvious to target osteoporosis with a combined anti-resorptive and bone anabolic therapeutic intervention, such as ZOL and SrHA nanoparticles. Therefore, the aim of the present study was to research the efficacy of bone anabolic SrHA and anti-resorptive agent ZOL, and the combination of SrHA/ZOL nanoparticles in the treatment of ovariectomy induced osteoporosis in rats. Owing to the different mechanism of action of SrHA and ZOL, our hypothesis was that the simultaneous presence of strontium and ZOL in HA would facilitate greater improvements in bone properties.

## 2. Materials and methods

### 2.1. Materials

ZOL was obtained as a gift from Naprod Life Sciences Pvt. Ltd., District Thane, City Maharastra, India. Ethicon chromic sutures-3/0, and Ethicon mersilk sutures-3/0 were purchased from Johnson & Johnson Ltd. (Baddi, Himachal Pradesh, India). Povidone ointment was purchased from Cipla (India). The following analytical-grade chemicals were purchased from commercial sources and used without further purification: Calcium nitrate tetrahydrate, strontium nitrate, urea, ammonia solution, diammonium hydrogen phosphate, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, 4-nitrophenyl phosphate, sodium acetate, sodium tartrate, hydrochloric acid and xylene from S.D. Fine Chemicals (Mumbai, India); ketamine from Neon Pharma (Mumbai, India); xylazine from Indian Immunologicals Ltd. (Hyderabad, India).

### 2.2. Fabrication of ZOL functionalized SrHA nanoparticles

SrHA nanoparticles were synthesized in $N_2$ atmosphere according to the method previously reported with small modifications [18]. Briefly, nanoparticles of SrHA were synthesized from the initial molar ratio of (calcium + strontium)/phosphate at 1.67 with the 10 at% initial strontium concentrations to the total (calcium + strontium). The obtained gel was then dried at 340 °C for 3 h under ambient static air. The gel was subsequently subjected to 900 °C calcinations from 300 to 900 °C at 10 °C/min, holding at 900 °C for 3 h, followed by cooling the products from 900 to 200 °C at 10 °C/min and then air-cooling to room temperature. The resultant solid of white powder was crushed using a mortar and pestle into a fine powder. For field-emission scanning electron microscopy (FESEM) measurements, nanoparticles were mounted on metal stubs using double-sided adhesive tape, dried in a vacuum chamber, sputter-coated with a gold layer of 10 nm thick and viewed under high resolution FESEM (Ultra 55, Karl Zeiss Microscopy, Germany). Fourier transform infrared (FTIR) spectroscopy analysis (Nicolet-Nexus 670 FTIR spectrometer, Nicolet Instrument Corporation, Madison, USA) was carried out to identify the functional groups. The spectrum was recorded in the 4000–400 $cm^{-1}$ region with 2 $cm^{-1}$ resolution. The crystallographic structural analysis was carried out by XRD method using a Bruker D8 Advance X-Ray powder diffractometer (Bruker, Karlsruhe, Germany) with monochromatic Cu Kα radiation over the 2θ range of 0–80° at a scan rate of 0.02°/min.

ZOL is highly soluble in 0.1 N NaOH, therefore, NaOH was selected as drug loading medium. 100 mL of 0.1 N NaOH was taken in three 200 mL volumetric flasks. Three different ratios of SrHA to ZOL were taken i.e. 0.75:1, 0.50:1 and 0.25:1, respectively. Each SrHA/ZOL ratio was added into separate volumetric flask containing 0.1 N NaOH. These different ratios of SrHA/ZOL in 0.1 NaOH were stirred using the planetary mixer for 7 h, and the supernatant was analyzed every hour for ZOL by high performance liquid chromatography (HPLC) analysis [23]. The planetary mixer is well suited to this operation since it is equipped with a scraper bar that scrapes material off the sidewalls of the vessel. After stirring, the ZOL-loaded SrHA nanoparticles were separated from drug loading medium by filtration and dried.

### 2.3. Determination of drug-loading capacity

For estimation of ZOL loading on SrHA, around 10 mg of nanoparticles were taken in 5 mL of 0.1 N NaOH and ultrasonicated for 10 min. The supernatant was centrifuged for 10 min at 8000 rpm. The ZOL content in the formulation was determined by HPLC [23] and the drug loading efficiency was calculated [24].

### 2.4. In-vitro release study

About 100 mg of SrHA/ZOL based drug formulation was transferred to a 100 mL beaker containing 50 mL of phosphate buffer saline (PBS) pH of 7.4 and subjected to continuous stirring at 100 rpm 37 °C. Samples were withdrawn at various different time intervals from the beaker and the ZOL content was measured by HPLC analysis [23]. An equal volume of PBS replaced the samples that were withdrawn.

### 2.5. Animals

In-house laboratory bred healthy Wistar rats aged 12 weeks were used in this study. Animals were maintained under controlled temperature at 25 °C ± 2 °C with 12 h light/dark cycle with food and water provided ad libitum. Ethical clearance was obtained from the Institutional Animal Ethical Committee (IAEC) of Al-Ameen College of Pharmacy (Approval Number: AACP/IAEC/NOV2011/07).

### 2.6. Pre-clinical study design

An osteoporotic animal model was established using female rats as described previously [25]. SHAM operations were performed by exteriorizing the ovaries. Twelve weeks post OVX or SHAM (age: 24 weeks), rats were divided into 7 groups (8 rats/group). The SHAM group and one OVX group were administered once intravenously with saline. The SHAM group was used as a negative control and the OVX group treated with saline (vehicle) was used as a positive control. Other five OVX groups were treated with single intravenous dose of SrHA/ZOL (100, 50 and 25 μg/kg); ZOL (100 μg/kg); SrHA (100 μg/kg).