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Development and characterization of supramolecular calcitonin assembly and assessment of its interactions with the bone remodelling process

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ABSTRACT

Osteoporosis is the most common metabolic bone disease, which poses an immense socio-economic burden on the society. Human calcitonin, though safe, is not considered as a therapeutic option because of its high tendency to self-associate to form amyloid fibrils thereby affecting its potency. To circumvent this issue we harnessed the inherent capacity of aggregation and developed an assemblage of human calcitonin monomers, [Supramolecular Calcitonin Assembly (SCA−I)], which releases biologically active calcitonin monomers in a sustained manner for a period of at least three weeks. AFM and FT-IR analysis showed that SCA-I is amorphous aggregates of calcitonin monomers. Both SCA-I and monomer released from it demonstrated superior anti-osteoclast activity and proteolytic stability *in-vitro*. SCA-I upon single injection significantly improved bone formation markers and reduced bone resorption markers in ovariectomized (OVX) rat model of postmenopausal osteoporosis. Micro-CT analysis revealed that calcitonin released from SCA-I exhibits its beneficial effect on cortical bone more profoundly compared to trabecular bone. This study demonstrates that SCA-I is more effective compared to the human calcitonin monomers on osteoclasts and has site-specific effect on bone in a model of post-menopausal osteoporosis. This approach opens up an innovative way to use and study the function of human calcitonin.

1. Introduction

Osteoporosis, a most common disorder of bone remodelling, is growing as a major public health problem in the elderly as well as young population [1]. Osteoporosis is common in both men and women but post-menopausal women are more susceptible to bone loss [2]. Till date several therapeutic interventions have been approved for treatment of the disease. Most of them are anti-resorptive agents that target osteoclast activity which include bisphosphonates, estrogen replacement therapies, selective estrogen receptor modulators (SERMs), calcitonin and strontium ranelate [3]. Calcitonin has been approved as an alternative to hormone replacement therapy or alendronate for patients who have found other treatments unsuccessful or difficult to tolerate [4,5].

Calcitonin is a 32 amino acid peptide hormone secreted by C-cells of thyroid gland. It is a hypocalcemic peptide involved in calcium homeostasis and exerts its effects by antagonizing the effects of PTH [6]. Salmon calcitonin is preferred for clinical use because it is reported as 50 times more potent than human calcitonin and possesses superior analgesic effects [7,8]. Several modes of administration have been developed such as injectable, nasal spray and oral formulations. Nasal and oral routes are not preferred due to poor absorption, high variability and rapid degradation by proteases and enzymes [9,10]. Subcutaneous and intramuscular injections are associated with the drawbacks of poor patient compliance due to the need of multiple injections. Though research on the anti-resorptive potential of human calcitonin has been initiated in 1960s but still remained challenging due to the inherent tendency of the hormone to associate, fibrillize and lose potency [11]. Bioavailability and stability of human calcitonin prevents its way of being the first line therapy to osteoporosis. Therefore, a novel mode of drug delivery is prerequisite which could eliminate the shortcomings associated with bioavailability, potency and multiple injections.

The inherent capacity of many peptides to fibrillize and form amyloids evoked a lot of attention among researchers and realizes that some peptides and hormones can form protein supramolecular assemblies of various forms and functionality [12,13]. Amyloids are now

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considered as a new generic structure characterized by cross-beta-sheet formation, and this structure can be achieved by many peptides under suitable conditions [14]. During amyloid formation amorphous aggregates are generated as a prelude to gaining some new properties such as enhanced stability, protease resistance, self-propagation, longer shelf life, highly organized structure which lacks soluble precursor protein and serve as a supramolecular structure made up of compact source of pure molecules [15].

Here, we hypothesized that whether the generic ability of human calcitonin to fibrillize could be utilized for formation of supramolecular structures, a state that can harbor monomers in its native state. This property has been harnessed by our group previously to develop a sustained releasing supramolecular form of insulin and IL-1RA [16,17]. Herein we developed the supramolecular protein assembly of a hormone whose pharmacological effect on the bone is still less studied. In this study we primarily describe the formation of supramolecular calcitonin, its stability and ability to act as a sustained release form *in vitro* and *in vivo*. Apart from this, we employed supramolecular calcitonin assembly as a tool to evaluate the actual role of native human calcitonin monomers on the bone and also to shed light on how human calcitonin affects bone mass accrual. Thus, in present work we showed the characterization of supramolecular calcitonin, its potency to reduce osteoclast activity and evaluated its effect on both trabecular and cortical bone compartments following a single injection in a rat ovariectomy model of bone loss.

2. Materials and methods

2.1. Calcitonin fibril formation

For aggregation experiment, human/salmon calcitonin (AnaSpec, USA) was used at a concentration of 2 mg/ml solubilized in 1× PBS (pH 7.4) and incubated at 37 °C on thermal block for 100 h. No agitation is required for the aggregation of calcitonin. The kinetics of amyloid fibril formation was monitored by measuring the turbidity of solution at various time intervals using spectrophotometer (UV-2450 Shimadzu, Japan) at 600 nm and 340 nm for human and salmon calcitonin respectively.

2.2. SCA-I formation and in vitro release assay

Human calcitonin (2 mg/ml each) in 1× PBS (pH 7.4) was incubated at 37 °C for 4–6 h. When turbidity of solution reached between 0.8 and 1.0 OD at 600 nm, the reaction was stopped by keeping the solution at 4 °C and the solution was centrifuged at 6000 rpm for 30 min at 4 °C. Protein concentration of supernatant (SCA−I) was measured spectrophotometrically at 280 nm. The amount of calcitonin in SCA-I formed was calculated by deducting the amount of protein in supernatant from initial protein amount taken for aggregation.

In vitro release of calcitonin monomers from SCA-I was assessed as previously described method in our laboratory with slight modifications [16]. To examine the release of calcitonin, SCA-I was re-suspended in 100 µl of 2% mannitol in a 1.5 ml microfuge tube, the cap was removed and covered with 10 kDa dialysis membrane. The microfuge tube containing SCA-I was suspended in invert position inside 50 ml falcon tube containing 2% mannitol. In this manner, SCA-I was dialyzed at 37 °C with constant agitation (Supplementary Fig. 1). The absorbance of mannitol in the falcon was regularly monitored at 280 nm using Spectrophotometer (UV-2450, Shimadzu, Japan). Thus, kinetics of calcitonin release into mannitol was calculated by the amount of protein released in the 2% mannitol.

2.3. Atomic force microscopy

Samples were diluted 20-folds with ultrapure water and 5 µl of solution was deposited onto the freshly cleaved mica surface and left to dry for 2 min. After that each sample was rinsed with nanopure water and dried under a flow of nitrogen. Samples were imaged in air in non-contact acoustic AC mode using Atomic force microscope 5500 (Agilent Technologies, USA). AC cantilever (normal spring constant of cantilever) was used. The topographic images of all samples were used for analysis using PicoView software.

2.4. Fourier transform infrared spectroscopy

IR spectra were recorded using Bruker Tensor 27 bench top FT-IR spectrophotometer equipped with liquid nitrogen cooled mercury cadmium telluride detector. SCA-I was analyzed on Bio-ATR and 256 interferograms were recorded at room temperature with a resolution of 2 cm^{-1}.

2.5. Proteinase K digestion assay

Proteinase K digestion assay was performed as described previously [17]. SCA-I was incubated with Proteinase K (2 mg/ml; 1:1000 dilution) at 37 °C. Samples were drawn at 15 min and 30 min of incubation and electrophoresed on 20% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

2.6. Cell viability assay

RAW 264.7 cells were seeded to 96 well microtiter plates with starting density of 2.5×10^4 cells for each well. After 24 h cells were treated with PBS, mannitol (50 nM), human calcitonin (10 nM), SCA-I (25 µg) and dialysate (10 nM) of SCA-I for 24 h and 48 h. In each well, 10 µl of MTT was added from the stock (5 mg/ml) and incubated at 37 °C in humidified CO_2 incubator for 4 h. After incubation, medium was removed carefully and 100 µl of DMSO was added in each well to dissolve the formazan crystals. After gentle shanking for 2 min the absorbance was measured for each well at 560 nm using microplate reader (Tecan Infinite M200) with background correction at 690 nm. % survival was calculated by [Absorbance(treatment) × 100] / Absorbance (control).

2.7. Annexin V-FITC and PI staining and flow cytometry

RAW 264.7 cells were plated in 24-well plate with starting density of 5×10^4 and allowed to grow at 37 °C. After 24 h, cells were treated with PBS, mannitol (50 nM), human calcitonin (10 nM), SCA-I (25 µg) and dialysate (10 nM) of SCA-I. After 48 h, cells were harvested with trypsin EDTA and centrifuged at 800 rpm at 4 °C. After washing with ice cold PBS three times and cells were re-suspended in 100 µl Annexin V binding buffer. 5 µl of FITC conjugated Annexin V along with 1 µl Propidium iodide (100 µg/ml) was added to the cells and incubated for 15 min at 37 °C protecting from light. Final volume of the reaction was made up to 500 µl with Annexin V binding buffer. Cells were then placed on ice immediately after gentle mixing the cell suspension. The cells were analyzed immediately with flow-cytometry (BD FACS Canto II). 10,000 events were collected for each sample.

2.8. Primary bone marrow cells

Primary bone marrow cells were isolated and differentiated into osteoclast as described previously [18]. To study the effect of SCA-I mediated inhibition of osteoclast activity in primary bone marrow derived osteoclast cells, bone marrow was flushed using 18G needle into the media from the bones of SD rats. Cells were cultured for 2 days in αMEM media supplemented with 10% FBS, 2 mM glutamine and 1% anti-anti for removing non-adherent hematopoietic bone marrow cells in order to obtain characteristics of adherent bone marrow cells. Next, bone marrow mononuclear cells were seeded in αMEM supplemented with 10% FBS, 2 mM glutamine, 1% anti-anti and 50 ng/ml M-CSF in

35 mm culture dishes. After 3 days of M-CSF treatment, RANKL at a concentration of 50 ng/ml was supplemented in the media followed by treatment with Human calcitonin, SCA-I or dialysate for 4 days. Cells were then harvested for RNA isolation followed by RT-PCR for TRAP5b.

2.9. Osteoblast cell culture and differentiation

MC3T3-E1, a clonal preosteoblastic cell line derived from new born mouse calvaria, was purchased from the American Type Culture Collection (CRL-2593) Cells were cultured and differentiated as described previously [19]. Briefly cells were cultured in α-MEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. After attaining 70% confluency the cells were differentiated in presence of 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate.

2.10. Western blot analysis

Cells were treated with PBS, human calcitonin (10 nM), SCA-I (25 μg) and dialysate (10 nM) under differentiating condition. After treatment cells were rapidly washed with ice–cold PBS buffer lysed with RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich). Equal amount of protein for each sample was electrophoresed on SDS-PAGE and then transferred on to nitrocellulose membrane. After blocking, blots were incubated with primary antibody – RUNX2 (CST# 8486), Osteocalcin (sc-365797), and β-Actin (sc-47778) at 4 °C for overnight. Next day blots were washed and incubated with secondary antibody with HRP at room temperature for 1 h. Blots were then developed with ECL substrate (Bio-Rad) visualized on LAS 4000 (Fujifilm).

2.11. Ovariectomy in rats and development of post-menopausal animal model

All animal experiments were carried out accordance with the guideline of Animal ethics committee of National Institute of Immunology, New Delhi. Three month (12 week) old virgin female Sprague-Dawley rats weighing 220 ± 10 g were housed in commercially available polypropylene cages and maintained under controlled temperature conditions on a 12-h light-dark cycle and allowed *ad libitum* access to food and water. For performing ovariectomy, rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (50 mg/kg body weight) and xylazine (10 mg/kg body weight). A single transverse incision was then made in abdomen and both the ovaries were exteriorised along with distal uterine horns. The fallopian tubes were ligated with non-dissolvable sterile sutures and ovaries were removed. The uterine horns were placed back into the peritoneal cavity and wound was sealed with absorbable sterile sutures (muscle and skin separately). Post-surgery, care was taken to prevent hypothermia.

For dose dependent studies, animals ($n = 6$ per group) were administered intradermally SCA-I at doses 75, 150, 300 and 600 μg/kg. Body weights were recorded at different intervals and the results were compared with OVX control. Once the dose was standardized, similar set of experiments were conducted to evaluate the effect of SCA-I against human calcitonin (4 μg/kg), salmon calcitonin (4 μg/kg) and intermediates isolated at 24 h from salmon calcitonin aggregation reaction. Final set of experiments included Sham, OVX, OVX + human calcitonin and OVX + SCA-I groups.

Group 1 - Sham operated rats (Sham group).

Group 2 - Ovariectomized rats (OVX group).

Group 3 - OVX rats administered with Human calcitonin (4 μg/kg body weight, alternate days, Intradermal injection) (Hum CAL group).

Group 4 - OVX rats administered with single dose of SCA-I (75 μg/kg body weight, single dose, Intradermal injection) (SCA-I group).

SCA-I was suspended in 2% mannitol for administration to animals. Routine blood sampling was also conducted for assay of various biochemical parameters. At the end of the experiments bones were harvested for molecular, histomorphometric and micro-CT analyses.

2.12. Biochemical assays

Cross-linked C-telopeptides of bone type I collagen (CTX—I) in serum was measured using Rat Laps EIA kit (Immunodiagnostic Systems Inc., USA). Tartrate Resistant Acid Phosphatase (TRAP5b) activity in serum was assayed using Rat TRAP assay kit (Immunodiagnostic Systems Inc., USA). Osteoprotegerin (OPG), and RANKL levels were examined using ELISA kit from (USCN, China) IL-Iα and Adrenocorticotropic hormone (ACTH) levels in serum were assayed using multiplex assay kits (Millipore, USA) on Bio-Plex 200 (BioRad, USA). Serum Osteocalcin (Takara, USA), ALP (Biovision, California, USA), Calcium (Abcam, UK) and Phosphorous (Abcam, UK) were examined in the serum. The level of human calcitonin in rat serum was measured using human calcitonin ELISA kit (MD Biosciences, Switzerland). All the Elisa kits were used according to manufacturer's instruction in the manual provided.

2.13. Real time PCR

Total RNA was extracted from cells or femur of experimental rats using Trizol method. 1 μg of RNA was used to synthesize c-DNA from each sample using Verso c-DNA synthesis kit according to manufacturer's protocol. 10 ng of cDNA was amplified employing gene specific primers (Table 1) on Light Cycler 480 system (Roche) using SYBR Green Master Mix I (Roche). The PCR was carried out as follows: initial denaturation (94 °C, 4 min), amplification for 40 cycles (94 °C for 15 s; 60 °C for 20 s; and 72 °C for 20 s) followed by melt curve. Ct values were determined for all the genes and fold change were calculated using $2^{-\Delta\Delta Ct}$ method using β-actin as a reference gene as analyzed previously [20].

2.14. Micro-CT analysis

Distal femur of bone samples were scanned by cone beam microCT (μCT 100, SCANCO Medical AG, Brüttisellen, Switzerland) with source voltage of 70 kV, current of 85 μA and 10.0 μm voxel size resolution. The 3D reconstruction of mineralised bone was performed as described previously with modifications [21]. The starting point of the evaluation was defined 400 slices (4.0 mm) above the reference point. The evaluation was covering a height of 300 slices. The bone was automatically segmented, based on its gray scale value in the CT slices. The region of

Table 1
List of primer sequences used in real time quantitative PCR.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Accession no.
OPG	CACAGCTCGCAAGAGCAAAC	TCCATCAAGATGCGGAGCTG	NM_012870
RANKL	CGACTCTGGAGAGCGAAGAC	TGGCCCCACAATGTGTTGTA	NM_057149
Osteocalcin	CTCTCTGCTCACTCTGCTGG	GGGGCTCCAAGTCCATTGTT	NM_013414
Alkaline Phosphatase	GGACCCTGCCTTACCAACTC	GTGGAGACGCCCATACCATC	NM_013059
TRAP5b	GTGTGGGCTATGTGCTGAGT	TCCACGTATGTGAAGCCACC	NM_001270889
β-Actin	AAGGCCAACCGTGAAAAGAT	GGTACGACCAGAGGCATACA	NM_031144

interest (trabecular bone structure) was then selected by a contour. The volume of interest was selected as the 300 slices were trabecular bone was present in all samples. Evaluation parameters for trabecular (sigma - 0.7, support – 1, threshold – 240) and cortical (sigma – 1, support – 2, threshold – 280) were fixed. Before measurement a scout view was taken to ensure the exact position to be scanned. For each analysis the estimated mineral density of bone tissue was determined based on the linear correlation between CT attenuation coefficient and bone mineral density. The samples were segmented based on its gray scale values in the CT slices. The pore diameters were computed from the inverted segmented samples using the maximum fitted spheres methods After 3D reconstruction, following bone structural parameters were studied: relative bone volume (BV/TV), connectivity density (Conn. D), trabecular number, thickness and separation. For cortical bone analysis, BV/TV, cortical thickness and cortical spacing were considered.

2.15. Histomorphometry

Histomorphometric analyses were performed on femurs at the end of the experiment after fixation followed by dehydration in graded concentrations of ethanol and finally embedding in methyl methacrylate resin and sectioning as described previously [22]. Static histomorphometric analysis was performed using the Osteomeasure Analysis System (Osteometrics, Atlanta, USA) according to standard protocols.

2.16. Statistical analysis

Data are expressed as mean ± SEM (Standard Error of Mean) or mean ± SD (Standard Deviation). Two tailed, unpaired Student's *t*-test was employed to evaluate the differences in the mean. For more than two groups one way ANOVA followed by Duncan *post hoc* test was performed. Differences in mean with P value < 0.05 were considered significant.

3. Results

3.1. Development of supramolecular calcitonin assembly and its biophysical characterization

First, we examined how human calcitonin monomers aggregated in PBS (pH 7.4) and the nature of intermediates formed during the process of aggregation. For that we incubated human calcitonin at a concentration of 2 mg/ml in PBS (pH 7.4) at 37 °C for 100 h and observed the kinetics of aggregation by measuring turbidity at 600 nm. The result clearly demonstrates the aggregation kinetics of human calcitonin at different time points as evidenced by turbidity (Fig. 1A). We observed that there was a progressive increase in turbidity till 24 h. Note that the turbidity of solution is directly proportional to the process of oligomerisation (*i.e.* till a distinct OD value). Simultaneously we compared the aggregation kinetics of human calcitonin with a potent variant *viz* salmon calcitonin. We observed that salmon calcitonin displays considerable variation in aggregation behaviour in terms of lag phase (Fig. 1B). While aggregation of salmon calcitonin proceeded very slowly with a lag phase of 10–12 h, human calcitonin aggregation was rapid and proceeded without any lag phase (Fig. 1A and B).

Next, we evaluated whether intermediates formed during the course of this aggregation process yielded any products that exhibited features of '*Sustained Release Forms*'. To assess this, intermediates of human calcitonin reaction mixture were isolated at 4–6 h and 8–10 h (these are supramolecular calcitonin assemblies which is abbreviated as SCA-I and SCA-II) and dialyzed against 2% mannitol for 168 h *i.e.* 7 d (Fig. 1C). The release profiles of intermediate forms (isolated between 4 and 10 h) were then analyzed by measuring absorbance of dialysates at 275 nm. As compared to intermediates isolated at 8–10 h, the intermediates isolated at early time point's *viz.* 4 to 6 h displayed a sustained release profile of calcitonin monomers for a period of 7 d. Interestingly, the monomers released from SCA-I were intact and did not degrade as confirmed by mass spectrometry. The result illustrates that MALDI-TOF ESI MS peaks at 3419 by linear mode for released calcitonin that relates to the hormone's theoretical molecular weight of human calcitonin *i.e.* 3417.9 (Fig. 1D).

We then moved on to visualize and analyze these SCA−I, SCA-II and amyloid using AFM imaging. We noticed that SCA-I possessed heterogeneous oligomeric morphology mostly with height of 8.0 ± 1.0 nm while SCA-II adopted a linear arrangement of the globular oligomeric entities with height of 35.0 ± 3.0 nm (Fig. 2A). AFM images clearly showed that SCA-I was oligomeric and hydrated in nature, a characteristic of supramolecular entities. As anticipated, human calcitonin fibrillar aggregate isolated at 24 h acquired a highly ordered fibrillar form with height of 1.0 ± 0.2 nm. SCA−I, formed during 4–6 h time period during calcitonin aggregation, showed preferable features for further *in vitro* and *in vivo* studies and hence used for all the further experiments.

To further characterize SCA−I, Fourier Transform Infrared Spectroscopy (FT-IR) was performed. FT-IR data showed two Amide *I* peak at 1655 and 1647 cm^{-1}. The peak centred at 1647 cm^{-1} must be attributed by random coil and the other at 1655 cm^{-1} attributed to α-helices, indicating that SCA-I is primarily α-helical and unstructured in contrast to the amyloid form that showed peaks at 1637 cm^{-1} (Amide *I* band indicative of β-sheet secondary structure) and 1685 cm^{-1} (Amide *I* band indicative of β sheet or β-turn) (Fig. 2B).

Our next objective was to demonstrate the stability of SCA-I over human calcitonin for which we conducted the proteinase K digestion assay. Proteinase K is a potent endopeptidase that degrades a wide range of peptidic substrates and dictates various regulated cascades in biological processes. Interestingly, our result illustrates that how human calcitonin easily degrades upon proteinase K digestion while SCA-I remains resistant to proteolytic cleavage even after extended period of digestion (Fig. 2C). Since SCA-I was stable in proteolytic environments, thus it was clear that the release of monomers from SCA-I assembly was not because of any degradation of supramolecular assembly but a reversible association of monomers within the supramolecular assembly and the release of monomers from SCA-I was dependent on monomeric concentration in *milieu*.

3.2. SCA-I inhibits osteoclast activity in vitro and in vivo in a rat bone loss model

Apart from sustain release and stability, it was equally important to reveal functionality of SCA−I. First RAW 264.7 murine cells were used to assess the effect of SCA-I and released monomers from SCA-I on cell viability. The treatment of either SCA-I or dialysate (monomers released from SCA−I) did not affect the cell viability of these cells indicating the non-toxic nature of SCA-I and its released monomers (Fig. 3A). Furthermore apoptosis in SCA-I or dialysate treated cells was analyzed by flow cytometry (FITC-Annexin V and PI). SCA-I or dialysate treatment did not induced apoptosis as there are almost no early or late apoptotic cells (Fig. 3B, Supplementary Fig. 2). These results clearly demonstrated that SCA-I or calcitonin monomers released from SCA-I are nontoxic in nature and does not cause cell apoptosis thus corroborating the results of MTT assay. Additionally, these results provide the suitable concentration of SCA-I that can be employed to primary osteoclasts and also 2% mannitol (vehicle) does not affect the cell viability. Furthermore, to determine the effect of SCA-I on TRAP5b expression in primary bone marrow cells Real-Time PCR was performed. SCA-I and dialysate significantly reduced the TRAP5b mRNA levels in bone marrow cells confirming the inhibitory effect of released calcitonin monomers on osteoclast activity (Fig. 3C). Moreover, the biological activity of SCA-I and the monomers released from it were tested by evaluating its effect on osteoclast mediated pit formation assay upon bone slices. Herein primary osteoclasts were first induced with pro-osteoclastogenic cytokines like M-CSF and RANKL and their activity was evidenced by the

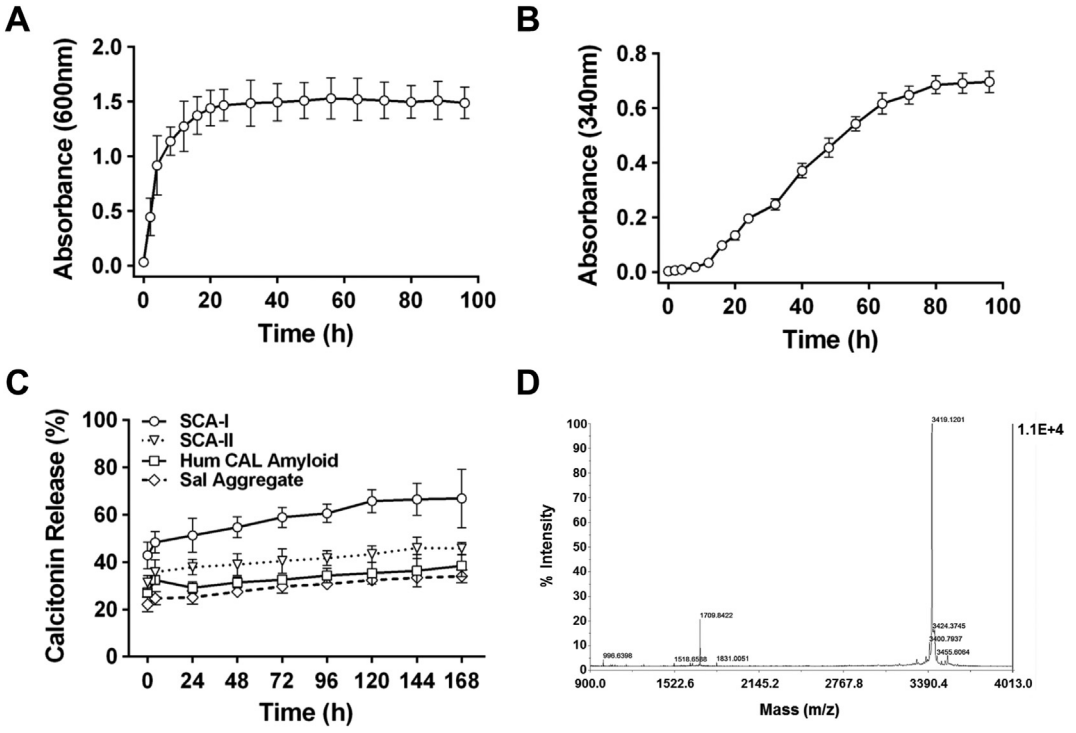

Fig. 1. Formation of supramolecular calcitonin assembly. (A) Time course changes in turbidity of human calcitonin (2 mg/ml) at 37 °C for 100 h monitored at 600 nm. (B) Time course changes in turbidity of salmon calcitonin (2 mg/ml) at 37 °C for 100 h monitored at 340 nm. (C) Release kinetics of supramolecular forms of human calcitonin *viz.* SCA-I (isolated from reaction mixture between 4 and 6 h), SCA-II (isolated from reaction mixture at 8–10 h), amyloid of human calcitonin (Hum CAL Amyloid) and Salmon calcitonin aggregate were observed by dialyzing against 2% mannitol through 10 kDa membrane and monitored spectrophotometrically at 280 nm. Human calcitonin amyloid is isolated from reaction mixture at 48 h. Sal aggregate is an isolate from salmon calcitonin reaction mixture at 24 h. (D) Representative MS spectra of released calcitonin in dialysates acquired on MALDI TOF ESI MS. Values are Mean ± SEM of 5 independent experiments.

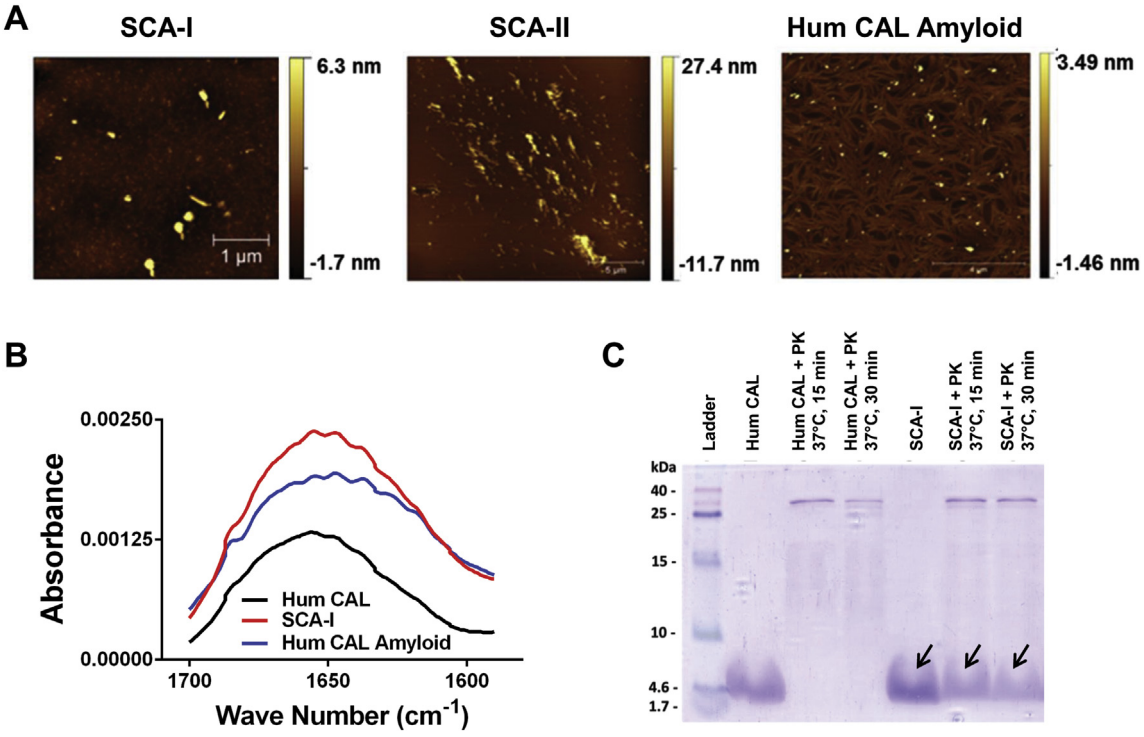

Fig. 2. Characterization and functionality of SCA—I. (A) Representative AFM images of SCA—I, SCA-II and amyloid of human calcitonin. (B) Representative FT-IR spectra of human calcitonin (Hum CAL), SCA-I and amyloid of human calcitonin (Hum CAL Amyloid). (C) Representative image of 20% SDS-PAGE gel stained with Coomassie Brilliant Blue. SCA-I and human calcitonin was incubated with proteinase K at 37 °C for 15 and 30 min and the digested material was electrophoresed by SDS-PAGE. Experiments were conducted at least 3 times and representative images and values have been presented.

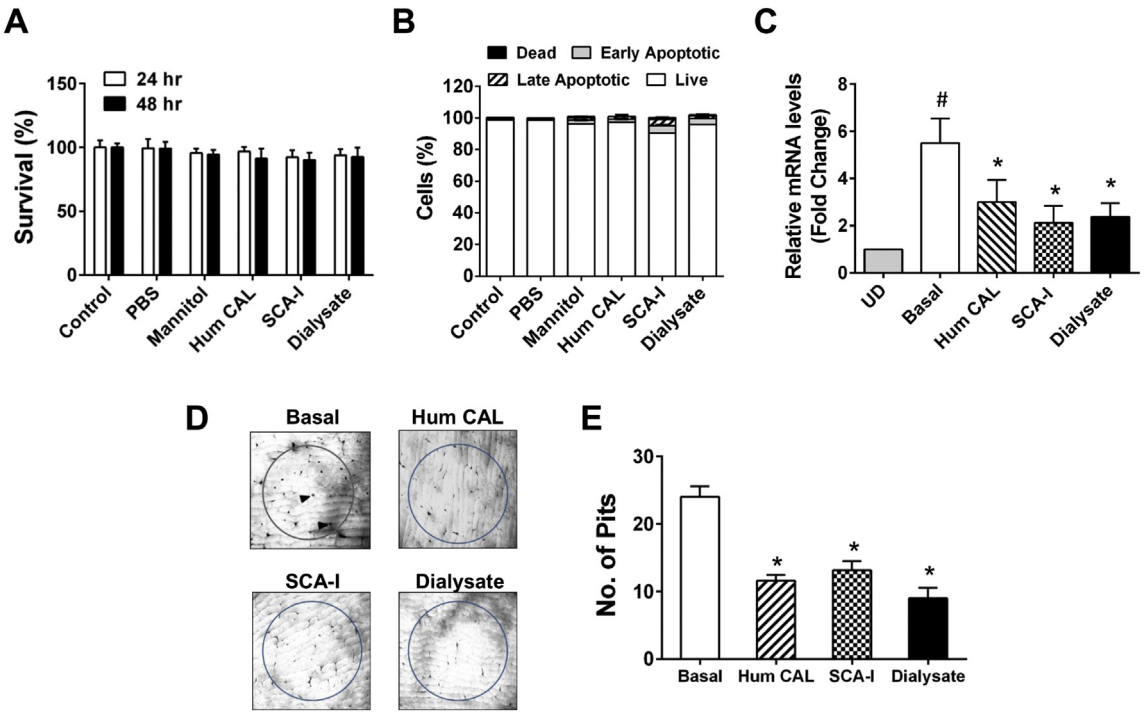

Fig. 3. *In vitro* evaluation of SCA−I. (A) Cell viability assay. Percentage cell survival of RAW 264.7 murine cells incubated with PBS, mannitol (50 nM), human calcitonin (10 nM), SCA-I (25 μg) and dialysate (10 nM) of SCA-I for 24 h and 48 h. (B) Flow cytometric analysis of RAW 264.7 cells. FITC conjugated annexin V and PI staining of cells incubated with PBS, mannitol (50 nM), human calcitonin (10 nM), SCA-I (25 μg) and dialysate (10 nM) of SCA-I for 48 h. percentage of total cell population was plotted in consolidated manner for (i) live, (ii) early apoptotic, (iii) late apoptotic and (iv) dead. (C) Quantitative Real Time PCR analysis of expression of TRAP5b gene in primary bone marrow cells treated with Hum CAL (10 nM), SCA-I (25 μg) and dialysates (from release kinetics experiment; 10 nM). (D) Representative images of osteoclast pits after pit formation assay. Primary osteoclasts were matured using M-CSF (50 ng/ml) and RANKL (100 ng/ml) for 7 d and transferred to 96 well plates containing bone slices. Cells were treated with human calcitonin (10 nM), SCA-I (25 μg) and dialysates (from release kinetics experiment; 10 nM) for 7 d. Bone slices were then cleaned, sonicated and stained with Mayer's Hematoxylin to visualize osteoclast pits. The number of pits counted in a given area. (E) Number of pits formed on different bone slices by osteoclasts when treated with Hum CAL, SCA-I and Dialysate (from SCA−I). Values are mean ± SEM of 3 independent experiments. #Statistical significance with respect to UD (undifferentiated) ($P \leq 0.05$), *Statistical significance with respect to basal ($P \leq 0.05$).

formation of pits on bone slices. The results show that co-incubation of bone slices with SCA-I reduced the activity of osteoclasts as evidenced by reduced number of pits in a given area of the bone slice. More importantly the released monomers from SCA-I (dialysate) significantly reduced pits formed by osteoclasts in the given area indicating its functionality (Fig. 3D and E). In conjunction with release profile data (Fig. 1C) and MALDI-TOF data (Fig. 1D) it became clear that SCA-I released calcitonin is in biological active form.

In the light of the success of our approach, we expanded the applications of SCA-I *in vivo* to study the effect of SCA-I in regulating bone mass accrual for which an ovariectomized rat model was employed. Phenotypically rats upon estrogen deficit following removal of ovaries gain immense body weight and this increase in body weight is exploited by researchers to primarily assess whether anti-osteoclastic drug under study has any potency *per se* [23–27]. In the present study food and water was provided *ad libitum* to the experimental rats. We observed an increase in daily food intake upon ovariectomy but at lower levels. However, treatment of human calcitonin or SCA-I did not alter the daily food intake showing regular eating behaviour by experimental OVX rats (Supplementary Fig. 3). Herein for dose standardization of SCA−I, we harnessed this parameter. SCA-I at doses 75 to 600 μg/kg body weight in 2% mannitol were administered intradermally to ovariectomized rats (Fig. 4B). We examined the effect of single intradermal injections of SCA-I at doses 75 and 150 μg/kg body weight in OVX rats for 42 d (6 weeks). The effect of SCA-I was prominent from second week post injection and lasted for the period of 42 d. At higher doses of 300 and 600 μg/kg, SCA-I significantly decreased body weight and hence was not considered for any further prospective studies (Fig. 4B). Further, human calcitonin (4 μg/kg, injections on alternate days) was employed as a positive control and compared with the single dose administration with SCA-I (Fig. 4C). Note the effectiveness of SCA-I (75 μg/kg) in regulating body weight till day 56 as compared to human calcitonin. We also injected an intermediate of salmon calcitonin aggregation (isolated from aggregating mixture at 20 to 24 h) into OVX rats and found the effect akin to that rendered by human calcitonin (intermittent injections) indicating that human calcitonin (SCA−I) gained functionality during the first few hours of aggregation in PBS while salmon calcitonin lost its biological activity by assuming protein structures of different conformation and of less biological relevance (Supplementary Fig. 4).

Next we determined the release profile of SCA-I *in vivo*. Employing a specific indirect EIA kit, we found that from day 14–21, rats that received 75 μg/kg body weight of SCA-I in a single dose exhibited serum human calcitonin level in the physiological range of 15–20 pg/ml (Fig. 4D). Indeed, the human calcitonin level in OVX rats treated with 150 μg/kg body weight of SCA-I was found above the physiological range therefore this dose was not considered for further experiments (Fig. 4D). Next the effect of SCA-I on osteoclast activity was analyzed. In mammals bone osteoclastic activity is characterized by high acid phosphatase activity and particularly tartarate resistance acid phosphatase 5b (TRAP5b) and as collagen degradation end product (C-terminal telopeptides, CTX−I) in serum. These two parameters are reliable markers for bone resorption. In our study we noticed that after one month of ovariectomy, OVX rats showed significant rise in TRAP5b and CTX-I that progressively increased in the next 4 weeks (Fig. 4E and F). The result shown Day 0 in Fig. 4E and F represents TRAP5b activity and CTX-I level in OVX rats post one month of ovariectomy and a day before the injection of SCA−I. It was observed that there was a

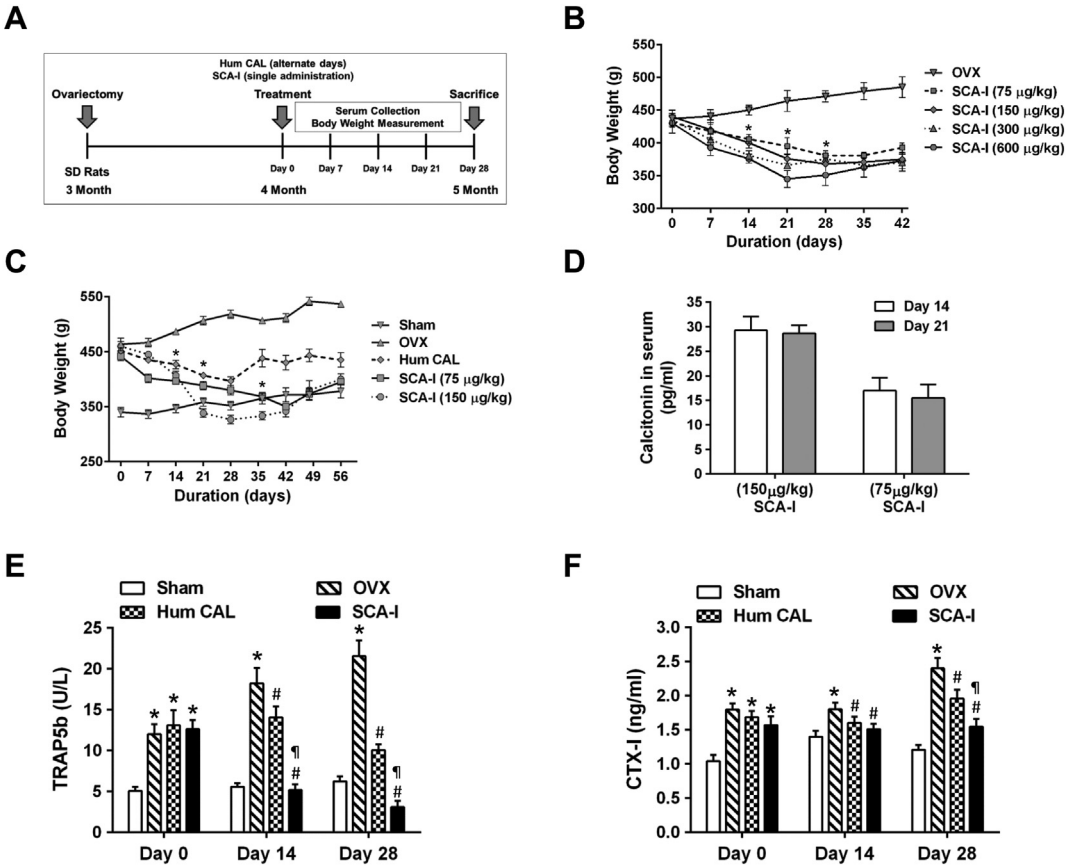

Fig. 4. *In vivo* evaluation of SCA-I in rat bone loss model. (A) Schematic representation of experimental study followed on rats. (B) Dose response of SCA−I: Changes in body weight profile of Sham and OVX rats administered with SCA-I (75–600 μg/kg, id, single injection) for 8 weeks. (C) Changes in body weight profile of Sham and OVX rats administered with Hum CAL (4 μg/kg, id, alternate days injection) and OVX rats administered with SCA-I (75 and 150 μg/kg, id, single injection) for 8 weeks. (D) Release of human calcitonin from SCA-I (75 and 150 μg/kg, id, single injection) into circulation was measured using Human Calcitonin ELISA. (E) Changes in TRAP5b activity in serum of OVX and OVX rats administered Hum CAL and SCA-I by ELISA. (F) Changes in CTX-I levels in serum of OVX and OVX rats administered Hum CAL and SCA-I by ELISA. Values are mean ± SEM of 4 independent experiments with 6 animals in each group. *Statistical significance with respect to Sham group ($P \leq 0.05$); #Statistical significance with respect to OVX group ($P \leq 0.05$); ¶Statistical significance with respect to Hum CAL group ($P \leq 0.05$).

significant decline in the activity of TRAP5b within 14 d of SCA-I administration and this effect was persistent till 28 d (Fig. 4E). Likewise, CTX-I levels in SCA-I treated OVX rats also showed a similar response from day 14–28 (Fig. 4F). Next, the serum markers critical in regulation of bone remodelling were measured. Serum levels of RANKL were significantly higher whereas serum OPG were lower in OVX rats compared to sham animals (Fig. 5A and B). As a result OPG/RANKL ratio was lower in OVX rats than in the sham rats (Fig. 5C). After 3 week of treatment, SCA-I significantly increased OPG levels, decreased RANKL concentration and hence increased OPG/RANKL ratio favouring increased bone formation (Fig. 5A–C). However rats treated with human calcitonin (Hum CAL) did not show significant improvement in RANKL concentration and OPG/RANKL ratio (Fig. 5B and C). To determine the effect of SCA-I treatment on bone metabolism, distinct serum marker of bone formation – osteocalcin (OC) and alkaline phosphatase (ALP) were examined. The serum levels of OC and ALP in OVX rats were significantly increased compared with sham. SCA-I treatment caused decline in serum OC and ALP levels compared with OVX rats (Fig. 5D and E).

The serum calcium and phosphorous levels were significantly declined in OVX rats than in sham rats. OVX rats treated with SCA-I demonstrated a significant increase in calcium levels accompanied with non-significant change in serum phosphorous level when compared to OVX rats (Fig. 5F and G). We also observed that the level of a pro-inflammatory cytokine with pro-osteoclastogenic effect IL-1α was also reduced in SCA-I treated rats (Supplementary Fig. 5A). Collectively, these results indicated that OVX rats exhibit a dramatic biochemical response to SCA-I showing reduced TRAP5b activity and CTX-I level as compared to the control group of animals that could be possible indication of a sustained period of biochemical remission. After 28 d following SCA-I injection the drug was well tolerated as evidenced by normalized body weight and ordinary eating pattern. ACTH, a marker to evaluate stress level, was found to be within normal range in SCA-I treated OVX rats (Supplementary Fig. 5B).

3.3. SCA-I as a tool to study the effect of human calcitonin in a postmenopausal model

In the next part of the study, we evaluated the effect of SCA-I in a rat OVX model employing Micro-CT, histomorphometric analysis and gene expression analysis. OVX + salmon calcitonin group was included as control in Micro-CT analysis experiment. We isolated bone samples on 28 d, the same primary time point when normalization of both serum TRAP5b activity and CTX-I level were observed.

We generated an overall 3D architecture of these bones by micro-CT and analyzed trabecular parameters. OVX rats displayed lower trabecular mineral density, relative bone volume (BV/TV), connectivity density and trabecular number and showed increased trabecular spacing as compared to Sham control (Fig. 6C and D). Surprisingly, we observed that administration of SCA-I to OVX rats aggravated the response of ovariectomy by further decreasing relative bone volume, trabecular number and connectivity density and increasing trabecular

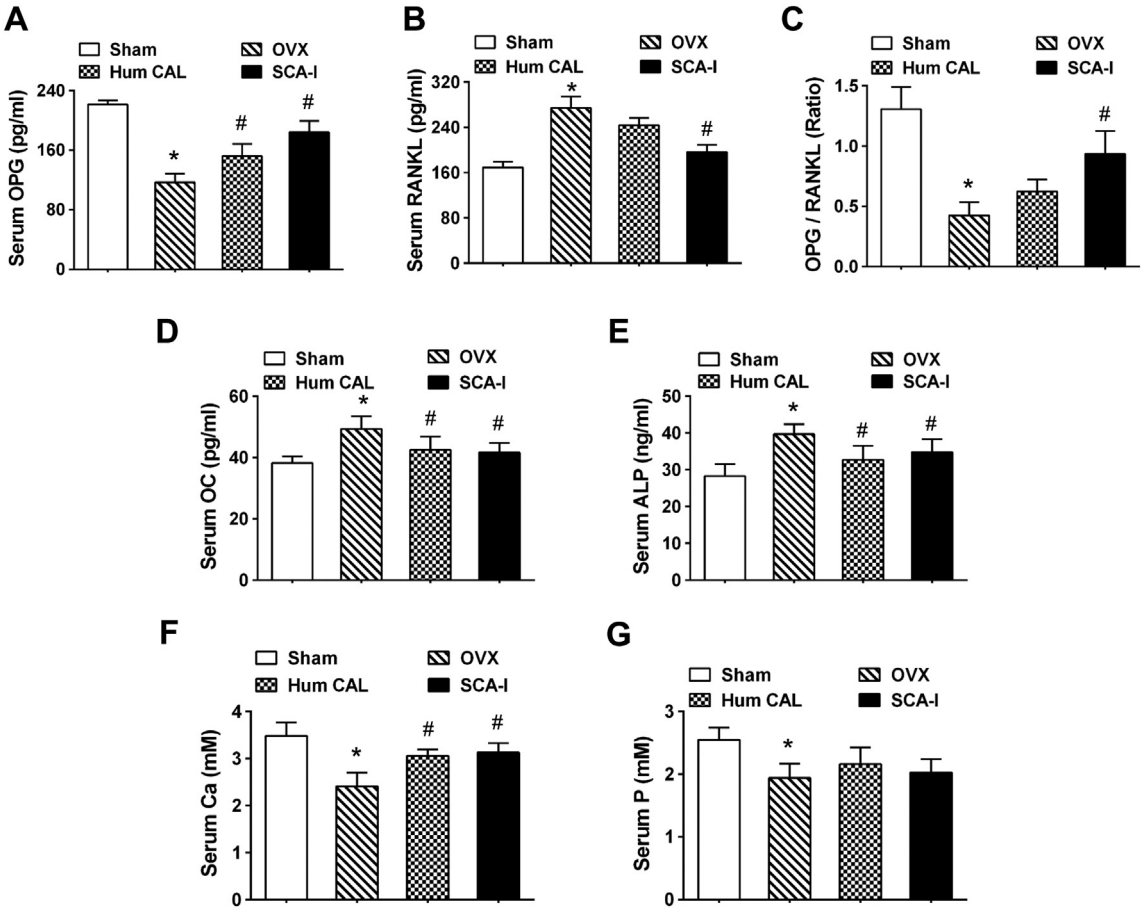

Fig. 5. Serum parameters of bone remodelling and bone formation. (A and B) Changes in OPG and RANKL in serum of OVX and OVX rats administered Hum CAL and SCA-I measured by ELISA. (C) serum OPG/RANKL ratio in OVX and OVX rats administered Hum CAL and SCA—I. Changes in (D) osteocalcin, (E) Alkaline phosphate (ALP), (F) calcium and (G) phosphorous in serum of OVX and OVX rats administered Hum CAL and SCA-I measured by ELISA. Values are mean ± SEM of 4 independent experiments with 6 animals in each group. *Statistical significance with respect to Sham group ($P \leq 0.05$); #Statistical significance with respect to OVX group ($P \leq 0.05$);

spacing (Fig. 6D). Careful evaluation of these results demonstrated that such effects were not due to any pitfalls in the formulation of SCA-I as OVX rats that received intermittent injection of human calcitonin also showed similar response. It could be as in mature bone endosteal surface has both osteoclast and osteoblast hence more active surface for bone resorption/remodelling. On other hand periosteal surface has osteoblast, hence responsible for appositional growth. As significant increase in bone resorption occurs in a week time compared to bone formation that takes 2–3 months to complete and increased osteoblast number at endosteal surface indicates that the process of the bone formation had initiated however, complete mineralization has not occurred at the time point bones were isolated for analysis. Hence, the spatial and temporal difference in osteoblast and osteoclast activity could be the reason that increased loss of trabeculae that occurred due to OVX has not shown significant increase at the time point when bones were isolated for studies. It could also be hypothesized that loss of trabeculae may have occurred as a counter protective mechanism to stabilize other compartments of the bone *viz.* the cortical region of femora. We therefore moved to analyze the cortical compartment of normal and experimental rats. Interestingly, SCA-I treated rats showed an increase in relative cortical volume and cortical thickening with normalized cortical spacing (Fig. 6B). Visual inspection of the images showed that bone formation occurred uniformly over the surface was more due to periosteal apposition than endosteal (Fig. 6B). Since we observed a beneficial effect of SCA-I on the cortical thickness we next analyzed cortical porosity that together with the cortical thickness determines cortical mechanics. As expected ovariectomy increased cortical porosity. Human calcitonin treatment of OVX animals further increased their cortical porosity. In contrast, SCA-I treated OVX animals had a cortical porosity that was even less than the sham animals. Together the result, increased cortical thickness and deceased cortical porosity in the OVX animals treated with SCA-I as compared to OVX animals suggest an improved cortical architecture and mechanics upon SCA-I treatment.

Next, histomorphometric analysis of distal region of the femur of experimental rats revealed increased number of osteoblasts in the endosteum of bone of SCA-I treated rats corroborating the fact that SCA-I have marked effect on bone formation by increasing the osteoblast number thereby improving the bone deposition in the cortex (Supplementary Fig. 6). Since SCA-I administration significantly improved the TRAP5b and CTX-I levels in the serum of OVX rats, it became imperative to check the effect of SCA-I on determinants of bone remodelling such as OPG, RANKL, osteocalcin and alkaline phosphatase (ALP) in the femur of experimental rats. For this purpose, quantitative real time PCR analysis has been performed to examine the status of mRNA expression of these important molecules in the femur of experimental rats. OVX rats showed significant reduction in expression of OPG mRNA as compared to sham while significant increase in RANKL mRNA expression (Fig. 7A). SCA-I administration significantly improved OPG expression (Fig. 7A). On the other hand, SCA-I administration significantly reduced the expression of RANKL in the femur of experimental rats (Fig. 7A). Thus, single dose of SCA-I administration improved the OPG to RANKL ratio (index of osteoclastogenic activity) towards reduced bone resorption. Concomitantly, the increase in the

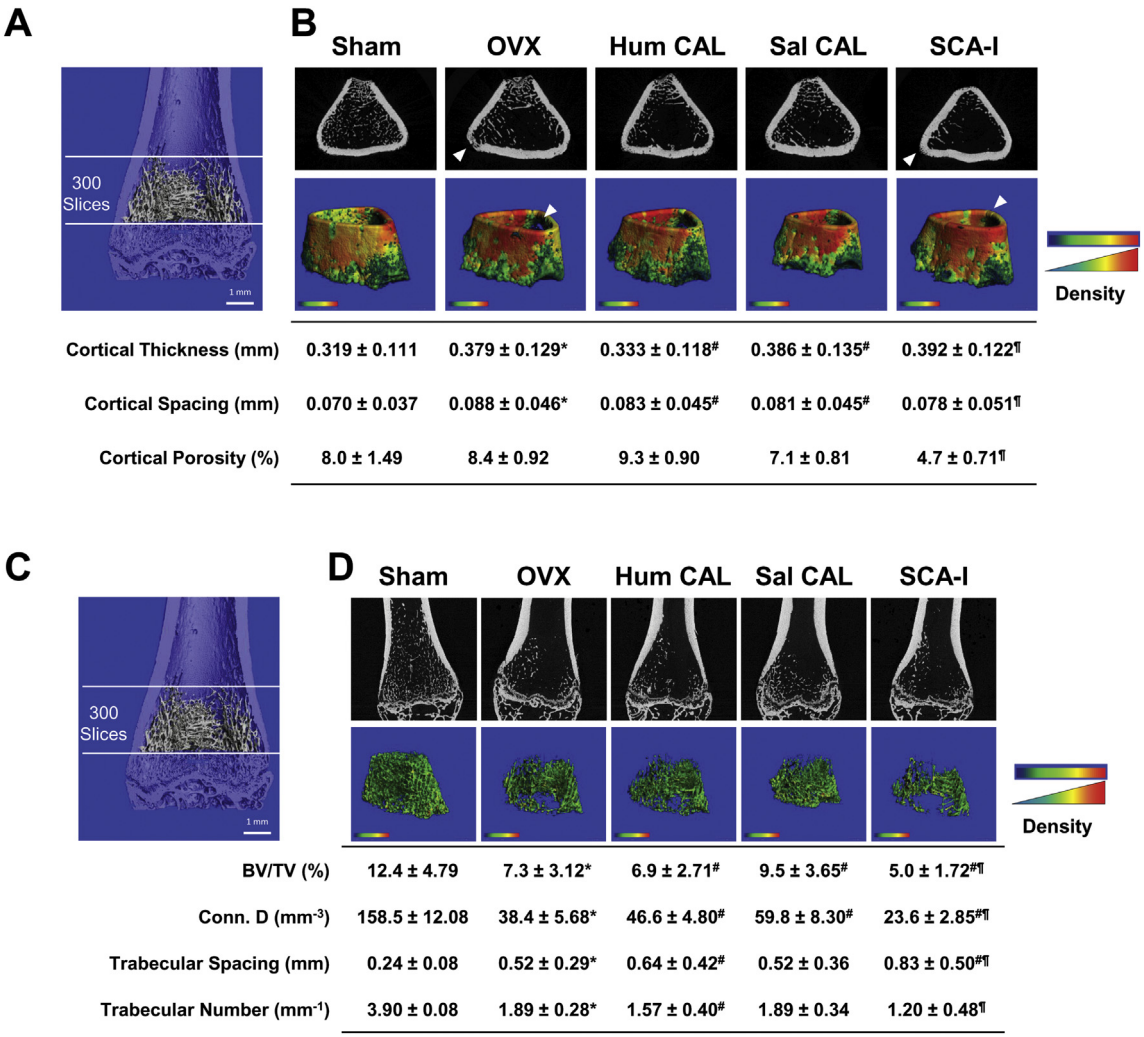

Fig. 6. Micro CT analysis of femur of SCA-I administered rats. (A) Region of interest chosen for analysis for cortical compartment of distal femoral metaphyseal. (B) Representative images of segmented and colour coded 3D images showing changes in thickness of cortical region and changes in cortical parameters (Cortical Thickness, Cortical Spacing and Cortical Porosity) of distal femoral region of OVX rats and rats administered with Hum CAL, Sal CAL, SCA-I by micro-CT analysis. Cortical porosity = [1-(BV/TV)]. (C) Region of interest chosen for analysis for trabecular compartment of distal femoral metaphyseal. (D) Representative images of longitudinal section of bone and colour coded 3D images of trabecular region and changes in trabecular parameters (BV/TV, Connectivity density, Trabecular spacing and Trabecular Number) of distal femoral region of OVX rats and rats administered with Hum CAL, Sal CAL, SCA-I by micro-CT analysis. Values are mean ± SEM of 4 independent experiments with 6 animals in each group. *Statistical significance with respect to Sham group ($P \leq 0.05$); #Statistical significance with respect to OVX group ($P \leq 0.05$); ¶Statistical significance with respect to Hum CAL group ($P \leq 0.05$). Density of the bone is represented on a heat map scale where red and green colour depicting the more dense and less dense areas respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of bone anabolic markers – osteocalcin and alkaline phosphatase (ALP) induced by ovariectomy was reinstated in SCA-I administrated rats, showing improvement in bone turnover rate (Fig. 7A). These results clearly suggest that single dose of SCA-I administration has beneficial protective effect on the cortex while at the same time accelerated bone loss in the trabecular compartment was observed upon ovariectomy. We hypothesized that as the treatment duration *viz.* 28 days might not sufficient to observe bone formation, hence, it is difficult to extrapolate effect of SCA-I on trabecular bone.

Further, since SCA-I demonstrated the beneficial effect on bone formation markers and improved cortical bone microarchitecture in the femur of experimental rats, we employed SCA-I and dialysate on MC3T3-E1 pre-osteoblast cells to assess the effect of SCA-I on bone forming cells. Calcitonin monomers released from SCA-I *i.e.* dialysate increased the expression of RUNX2 (Fig. 7B), early osteoblast differentiation marker and osteocalcin (Fig. 7C) protein expression in differentiating MC3T3-E1 cells after 7 days. These results indicate that monomers released from SCA-I could induce osteogenic differentiation of osteoblast cells and in the same manner could promote bone formation *in vivo*.

4. Discussion

The use of peptides and proteins for therapeutic purposes are marred by the disadvantages such as aggregation, proteolytic cleavage, short half-lives, immunogenicity, multiple injections *etc.* Aggregation and formation of amyloids remain the major challenge in the field of peptide therapeutics. One innovative way to overcome this problem is to develop macro-molecular cluster of monomers that can release biologically active monomers in a sustained manner. Herein we have advanced the calcitonin therapy by regulating the aggregation of human calcitonin and developed conglomerate of monomers which upon single injection releases biologically active monomers in a sustained manner for 3 weeks and significantly improves bone quality of estrogen deficient OVX rats.

Besides parathyroid hormone and vitamin D, estrogen and

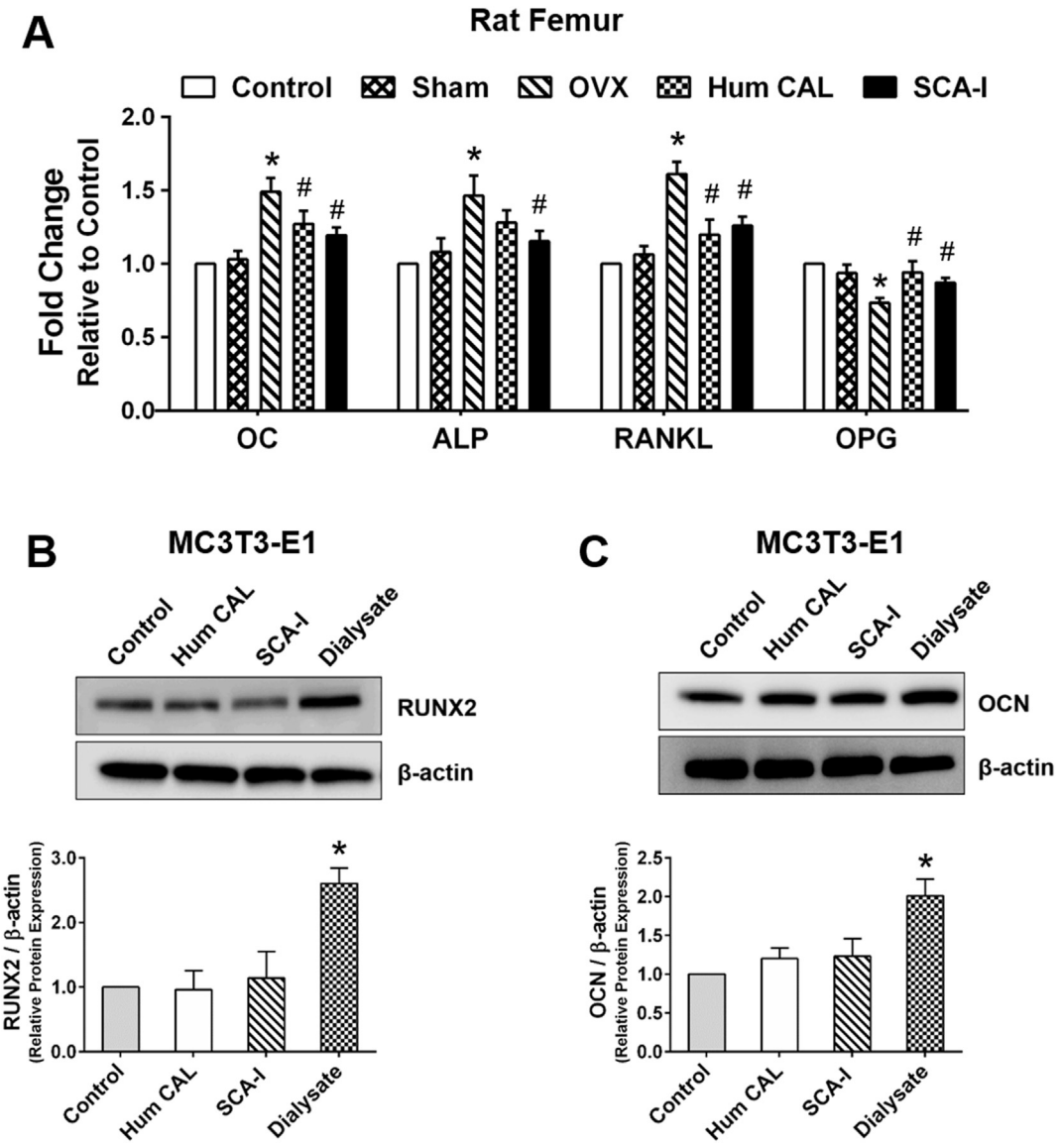

Fig. 7. (A) Fold change in mRNA levels of Osteocalcin (OC), Alkaline Phosphatase (ALP), RANKL and OPG in femur of experimental rats administered with Hum CAL, Sal CAL, SCA-I relative to control group. Gene expression was analyzed by real time quantitative PCR. Values are mean ± SEM of 3 independent experiments with 6 animals in each group. *Statistical significance with respect to control group ($P \leq 0.05$); #Statistical significance with respect to OVX group ($P \leq 0.05$). (B and C) Effect of calcitonin on osteoblast differentiation by immuno blot analysis of bone formation markers in MC3T3-E1 cell line. MC3T3-E1 cells were grown in differentiating medium and treated with Hum CAL (10 nM), SCA-I (25 μg) and dialysates (from release kinetics experiment; 10 nM) followed by western blotting and probed with RUNX2, Osteocalcin (OCN) and β-Actin. Representative western blot images and densitometric analysis of (B) RUNX2 and (C) Osteocalcin (OCN). Values are mean ± SEM of 3 independent experiments. *Statistical significance with respect to control group ($P \leq 0.05$).

calcitonin are the major hormonal regulators of osteoclastic bone resorption. Estrogen inhibits bone resorption by inhibiting RANKL induced osteoclast differentiation and stimulating osteoclast apoptosis [28,29]. On the other hand calcitonin suppresses bone resorption by inhibiting osteoclast activity. Recently it has been shown that calcitonin increases proliferation of osteoblasts [30] and prevents apoptosis of osteoblasts and osteocytes [31]. Calcitonin secretion in postmenopausal women may be regulated by estrogen and hence is considered to be a moderator of estrogen action on bone [32]. In estrogen deficit condition osteoclasts become more viable and active which leads to accelerated bone loss [33]. Increased activity of osteoclasts can be regulated by inhibitory action of calcitonin [34]. Therefore calcitonin is considered as a one of the promising therapy for postmenopausal osteoporosis. Despite of in-depth understanding of bone biology, till date the therapeutic use of drugs to treat osteoporosis is lacking due to adverse side effects and inadequate long-term compliance including complexity of dosing. Calcitonin derived from salmon is one among such therapies which is approved by FDA for use in the treatment of postmenopausal osteoporosis. Unfortunately, human calcitonin was never employed as frontline treatment for osteoporosis because of its lower potency and higher propensity to aggregate while being less immunogenic compared to salmon calcitonin. Cudd et al. have demonstrated that in an *in vitro* resorption assay, EC50 of human calcitonin is 2–4 times higher in magnitude when fibrillation is minimised by using acetic acid solution in spite of buffers along with high salt content [35]. In order to improve the therapeutic efficacy and bioavailability of human calcitonin by increasing its systemic persistence, we devised to cluster monomers of calcitonin to form a Supramolecular Calcitonin Assembly (SCA—I) by utilising the intrinsic property of protein to form amorphous aggregates. The formation of SCA-I from human calcitonin was easier as it easily undergoes fibrillation process at physiological conditions. The off pathway intermediates formed during the aggregation process *i.e.*

between 4 and 6 h showed significant release of calcitonin. The intermediates obtained during the initial time-points of log phase were termed as SCA-I and biophysically characterized.

AFM studies indicate that SCA-I is merely a cluster of monomers while amyloid is fibrillar in nature. SCA-I is devoid of any definite and repetitive organisation of monomers indicating its amorphous and heterogeneous nature. FTIR analysis further corroborates the finding that SCA lacks the cross-β structures, a characteristic feature of amyloid structure. Resistance to cleavage by proteinase K demonstrates the stability of monomers in SCA-I and substantiates its higher order oligomeric state unlike calcitonin that gets cleaved by proteinase K. The biological activity of released calcitonin monomers were substantiated by pit formation assay. The inhibitory action of released calcitonin monomers on osteoclasts was confirmed by reduction in the number of pits on bone slices. At the same time, MALDI-TOF analysis of released calcitonin did not show any fragmentation of calcitonin molecules released from SCA−I. Further pit formation assay showed that SCA-I and calcitonin released from it had inhibited the primary ostoclast activity. Hence, these experiments proved that the released calcitonin monomers from SCA-I does not disintegrate and remain biologically active. The same strategy was employed to develop oligomers from salmon calcitonin as with human calcitonin. However, salmon calcitonin oligomers failed to show a sustained release of monomers *in vitro* as well as *in vivo*. We assume that this discrepancy arises due to the different aggregation kinetics of both the calcitonins wherein salmon calcitonin has a low propensity to aggregate than human calcitonin [6].

To assess the *in vivo* efficacy of SCA−I, rat model of postmenopausal osteoporosis was developed which is well documented and accepted model to evaluate the effects of new drugs and therapeutic interventions on bone structure and bone turnover [36]. Since this model is deficit in circulating estrogen levels, an increase in body weight in the early months of post ovariectomy appears to be a potential phenotypic marker for studying antiresorptive formulations [24]. The gain in body weight after ovariectomy is associated with higher food intake *i.e.* hyperphagia under *ad libitum* conditions. Nevertheless, increased food intake due to estrogen deficiency does not affect bone mineral density of experimental OVX rats [37]. Treatment with calcitonin either intermittently or continuously (with Alzet osmotic pump) decreases the body weight of OVX rats significantly compared to vehicle treated rats [38]. In mammals bone resorption due to increased osteoclastic activity is characterized by high acid phosphatase activity, particularly tartarate resistance acid phosphatase 5b (TRAP5b) and CTX-I(C-terminal telopeptides, CTX−I) as collagen degradation end product in serum. Earlier reports suggest that calcitonin inhibits TRAP5b in terms of both synthesis and release by inhibiting Na^+-K^+-ATPase activity, H^+ATPase and carbonic anhydrase [39]. Changes in BV/TV, trabecular number and trabecular spacing seen in OVX rats were in concordance with that reported previously [40]. The demineralization observed in OVX rats must be an attempt to stabilize normocalcemia which is preferred over skeletal mineral conservation [41]. Monier-Faugere et al., reported that human calcitonin significantly reduces bone mineralization in ovariohysterectomized dogs [42]. Analysis of the cortical region of OVX rats showed that ovariectomy increases cortical thickness and cortical spacing, an adaptive mechanism normally observed in young OVX rats [43]. Formerly it has been shown that human calcitonin possessed significant effect on lamellar bone substantiating the efficacy of SCA-I [44]. Controls used for this study *viz.* human and salmon calcitonin also showed positive effect on cortical spacing but its effects were less than SCA−I. So far no calcitonin receptors have been found to be present on osteoblasts, the hormone has been demonstrated to exert its effects on bone formation *via* alternate routes *i.e.* inhibition of sphingosine-1 phosphate release from osteoclasts [45]. The significance of calcitonin action on bone remodelling in context to OPG-RANKL-RANK system is not yet established. Nevertheless, the changes in OPG and RANKL mRNA expression in reduced bone resorption upon treatment of salmon calcitonin along with aspirin in OVX rats has been recently demonstrated [46]. Our study reports that SCA-I improves bone remodelling in favour of bone formation by upregulating OPG expression and downregulating RANKL expression. Although significant improvement in bone formation in trabecular region was not observed which could be due the study duration chosen *i.e.* 28 days, not sufficient to observe visible bone formation. However, studies on pre-osteoblast cells showed that SCA-I and calcitonin released, had induced proliferation and differentiation of osteoblast. This was further substantiated by histomorphometric analysis where increased number of osteoblasts were observed at endosteal surface and in line with the reports [30,47]. The positive role of Eel calcitonin has been previously shown in osteoinduction by rHBMP-2 suggesting anabolic role of calcitonin [48].

Till date, human calcitonin and its pharmacological effects are less studied due to its inherent propensity to self-assemble and form long fibrils [49]. The physiological role of calcitonin in regulation of bone turnover and calcium homeostasis has been debated for long [50]. Thus calcitonin and its antagonistic hormone PTH continues to exist as a conundrum for more than half a century since its discovery [51,52]. Recently, use of genetically engineered mouse model has led the advancement in the role of calcitonin and its action through calcitonin receptor [53]. However, the precise mechanism of action of calcitonin in bone remodelling and its physiological relevance remains unanswered. This warrants much detailed and long term animal experiment using SCA-I as a tool to understand the effect of human calcitonin on overall bone formation/remodelling.

In summary, we have improved the efficacy of Human calcitonin by developing supramolecular calcitonin assembly (SCA−I) without any chemical intervention. SCA-I is a cluster of near native human calcitonin monomers in their near α-helical conformation with physical attributes like amorphous nature, stability against proteolysis and sustained release when placed in a *milieu* of low monomeric concentration. Upon *in vivo* administration, SCA-I releases calcitonin monomers in continuous manner for more than three weeks. Studies conducted in OVX rats showed that SCA-I releases human calcitonin in its active form as its pharmacological attributes like anti-osteoclast activity was found to be improved than intermittent injections of human calcitonin. SCA-I in the absence of exogenous supplementation of calcium and vitamin D increases lamellar periosteal bone formation. On one hand while human calcitonin demonstrates an anti-osteoclast activity, the other hand it increases cortical bone accrual irrespective of estrogen deficit and increasing the overall bone density. To conclude, the results of this study demonstrate how a hormone (human calcitonin) that was once side-lined owing to increased fibrillization tendency when fastened into an amorphous substance (SCA−I) can be employed for better understanding of the mode of action of human calcitonin.

5. Conclusions

Thus, human calcitonin without any chemical intervention is coerced into a supramolecular assembly stage; so that a distinct active form of the hormone is maintained to perform its function which was previously remained unexplored. A single dose administration of SCA-I forms a depot of calcitonin which releases biologically active monomers in a sustained manner. The outcomes of our study not only open up an innovative way to use human calcitonin but also eliminate its limitations. Such supramolecular oligomeric forms could hold enormous potential for treating a number of diseases where proteins and peptides are used as bio-therapeutics and avoid the need of multiple injections. SCA-I facilitated to understand the differential effect of human calcitonin on cortical and trabecular compartments of bone. Thus, this study also advances our understanding about the effect of human calcitonin on bone remodelling process that is rarely used for basic and therapeutic studies.

Authors' contributions

Concept and study was designed by AS and SG. The work was conducted by KM, SG, CP, MK, PS, SC and VV. Data analysis and interpretation by AS, SG, KM, CP, VV, PS and VKY. SG, KM and VV wrote the final form of manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.02.019.

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