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Research Article
Ameliorative Effects of *Brachidontes variabilis* Calcium Carbonate Against Bone Loss in Ovariectomized Rats

[1]Wagdy Khalil Bassaly Khalil, [1]Hoda Fahim Booles, [1]Naglaa Abd El-Maksoud Hafiz and [2]Gehan El-Tabie El-Bassyouni

[1]National Research Centre, Department of Cell Biology, 33-Bohouth St., 12622 Dokki, Giza, Egypt
[2]National Research Centre, Department of Ceramics, Refractories and Building Materials, 33-Bohouth St., 12622 Dokki, Giza, Egypt

Abstract

Background and Objective: Marine products rich in calcium became promising supplements used in combination with medication for osteoporosis treatment. This study aimed to use natural products extracted from marine organisms in osteoporosis treatment as novel approach in drug discovery. Therefore, calcium carbonate isolated from *Brachidontes variabilis* (*B. variabilis*) shells were used against bone loss in ovariectomized rats. **Materials and Methods:** Ovariectomized (OVX) rat model was used to test treatment strategy for osteoporosis. Bivalve, *Brachidontes variabilis,* is a biomineral lamellar composite of calcite and/or aragonite ($CaCO_3$) embedded within an organic framework. Efficaciously advanced novel class of $CaCO_3$ particles isolated from *Brachidontes variabilis* shells (BVC) were used against bone loss in ovariectomized rats. All obtained data were analyzed using the general liner models (GLM) technique of statistical analysis system. **Results:** The present study demonstrates that both the X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR) could be feasible methods for structural identifications of the $CaCO_3$. The present study showed that treatment of OVX-rats with low and high doses of BVC revealed significant $p<0.05$ increase in the proximal, distal and total bone mineral density (BMD), elevation in the serum osteoprotegerin (OPG) level and reduction in serum receptor activator of nuclear factor-κB ligand (RANKL) and β2-microglobulin levels. Results revealed that treatment of OVX-rats with low and high doses of BVC prevented significantly the expression alterations of bone resorption and bone formation genes compared with those in OVX-rats. **Conclusion:** These results suggested that $CaCO_3$ isolated from *B. variabilis* shells may indeed have a promising significant potential in clinical translation, which then are exploited as a carrier for efficient loading of different types of therapeutic strategies.

Key words: Osteoporosis, ovariectomized rats, *Brachidontes variabilis*, bone resorption and formation genes, 8-OHdG/2-dG

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Corresponding Author: Wagdy Khalil Bassaly Khalil, National Research Centre, Department of Cell Biology, 33-Bohouth St., 12622 Dokki, Giza, Egypt
Tel: +202-33353498/+2012-27410600 Fax: +202-33370931

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Calcium is an important structural component of bone and teeth and is required for the normal function of all muscles and nerves. Calcium carbonate is widely used medicinally as an inexpensive dietary calcium supplement. Calcium carbonate is composed of three important elements that are present in almost all organic and inorganic materials carbon, oxygen and calcium. It exists in three types of crystalline form and this phenomenon is called polymorphism. The crystalline forms of $CaCO_3$ are calcite, aragonite and vaterite. Calcite is the primary constituent of many marine organisms like plankton, sponges and parts of the shells of some bivalves[1]. The skeleton of corals is commonly believed to be composed entirely of aragonite due to the current Mg/Ca molar ratio of the seawater, which thermodynamically favors the deposition of this polymorph of calcium carbonate ($CaCO_3$)[2]. $CaCO_3$ is one of most abundant biominerals in nature, it has been considered as a potential inorganic precursor to induce the formation of bone minerals such as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$][3].

Pathologically, osteoporosis is defined as a skeletal disorder characterized by absolute decrease in the amount of bone, leading to fractures after minimal trauma[4]. During menopause, osteoporosis is a most important age related health problem for women who often have a negative calcium balance. Decline of calcium is most probably due to decreased intestinal calcium absorption, insufficient dietary calcium intake, as well as increased urinary calcium loss associated with estrogen deficiency[5]. As a result of bone loss, the main underlying cause of fractures in osteoporosis increased the fragility of bone. Fracture risk is determined by absolute bone mineral density (BMD), regardless of age. The most important risk factor that can cause osteoporosis is the low BMD[6].

Arjmandi *et al.*[7], used the OVX rat as a model for studying postmenopausal bone loss. There are many matches between bone loss in rats after ovariectomy and postmenopausal bone loss in women, like (1) Bone resorption exceeding bone formation, (2) Improved bone loss virtually followed by slower bone formation and (3) Higher loss of cancellous bone than cortical bone[7]. The most mutual animals that are used for experimental studies are rodents, mainly rats and mice. Ovariectomized (OVX) rats were used to study postmenopausal osteoporosis[8].

In humans, there are 3 types osteoporosis, Type I: Postmenopausal osteoporosis, Type II: Age-related osteoporosis and Type III: Secondary osteoporosis. Type I typically affects women by estrogen deficiency within 15-20 years after menopause. Wilkosz *et al.*[9] indicated the fact that the decline of ovarian function is more important than age. Therefore, postmenopausal administration of estrogen decreases the occurrence of fractures associated with osteoporosis by about one half.

The treatment of osteoporosis using drugs can be classified into two groups, anti-resorptive or stimulators of bone formation. Antiresorptive drugs include calcium, estrogen, calcitonin and bisphosphonates[10,11]. Also plant estrogen has been used for prevention of bone resorption. Stoppage of estrogen administration primes to the recurrence of bone resorption and the degree is analogous to the bone resorption after ovariectomy. However, recent findings suggest that bisphosphonates prompt osteonecrosis of the jaw, atrial fibrillation, raising safety and ethical concerns around the use of bisphosphonates[12]. Therefore, development of new drugs was needed for the prevention of osteoporosis, particularly in postmenopausal women[13]. Excessive bone degradation in osteoporosis result from an increased activity of resorptive osteoclasts, which express proteases such as metalloproteinase (MMP-9) and cathepsin K. Cathepsin K, is considered as a key player in the process of bone resorption[14]. Regulation of the osteoclast differentiation and function was obtained by the receptor activator of nuclear factor kappa B ligand (RANKL)[15].

Sigma anti-bonding molecule calcium carbonate (SAC; activated ionic calcium) as isolated from oyster shells, this calcium has a weak bonding force with other molecules, radicals or atoms[16]. In addition, SAC has been used for treatment against bone loss in ovariectomized rats. The aim of the present work was designed to find the best and cheaper source for calcium carbonate. Calcium carbonate isolated from *Brachidontes variabilis* shells, was used against bone loss in ovariectomized rats. These calcium molecules are expected to increase the animal metabolic system by being absorbed into the cell. Moreover, the present work is conducted to compare between the effect of SAC and BVC as natural products against bone loss in rats model for osteoporosis.

MATERIALS AND METHODS

Characterization

Phase analysis: This study has been conducted at Ceramics, Refractories and Building Materials Department, National Research Centre, Egypt, during August-December, 2015, where the isolation of calcium carbonate from *Brachidontes variabilis* was carried out. Structural identifications have been confirmed using both the X-ray diffraction (XRD) analysis and

fourier transform infrared spectroscopy analyses (FT-IR)[17-20]. Functional nature of the samples was obtained in a Diano X-ray diffractometer. Fourier transform infrared spectroscopy analyses (FT-IR) was attained using [FT/IR-4600 type A (JASCO, USA) and detector TGS][21-24].

Drugs and chemicals: Sigma anti-bonding molecule calcium carbonate (SAC; activated ionic calcium) was purchased from Sigma-Aldrich Corporation, USA. TRIzol reagent was bought from Invitrogen (Germany). The reverse transcription and PCR kits were obtained from Fermentas (USA). SYBR Green Mix was purchased from Stratagene, USA.

Preparation of *Brachidontes variabilis* calcium carbonate: For administration of the bivalve calcium carbonate, *Brachidontes variabilis* were collected from the coast of Port Said, Egypt. Bivalve shell, the exoskeleton was finely grounded and kept under -20°C until use.

Experimental animals: Sixty adult albino female rats, weight 250±13 g and aged 14-15 weeks were purchased from the Animal House Colony, Giza, Egypt and maintained on standard laboratory diet and water *ad libitum* at the Animal House Laboratory, National Research Center (NRC), Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were divided into 6 groups (10 rats/group) and housed individually in filter-top polycarbonate cages. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

Experimental design: The acclimatized rats underwent either bilateral laparotomy (sham group, n = 10) or bilateral ovariectomy (OVX, n = 50). Three weeks after recovering from surgery, the OVX rats were randomly allocated into 5 groups as following: Vehicle-treated (OVX, n = 10): SAC1-treated (OVX+SAC1, n = 10): Animals were given drinking water containing 0.0012% SAC for 12 weeks following 3 weeks post-operation, SAC2-treated (OVX+SAC2, n = 10): Animals were given drinking water containing 0.0024% SAC for 12 weeks, BVC1-treated (OVX+BVC1, n = 10): Animals were given drinking water containing 0.0012% BVC for 12 weeks and BVC2-treated (OVX+BVC2, n = 10): Animals were given drinking water containing 0.0024% BVC for 12 weeks.

Sample collections: At the end of the experimental period, blood samples from fasting rats were withdrawn from retro-orbital venous plexus under diethyl ether [$(C_2H_5)_2O$] anesthesia in dry clean centrifuge. Blood samples were centrifuged and clear sera were separated and immediately stored at -20°C until analyses. The animals were then rapidly sacrificed and the right femurs were harvested. Each right femur bone was carefully cleaned, length and weight were recorded and then stored in formalin buffer 10% for dual energy X-ray absorptiometry (DEXA) [a means of measuring bone mineral density (BMD)]. Bone mineral density of each right femur were measured by DEXA using Norland XR46, version 3.9.6/2.3.1 instrument (Norland X-R-46 version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. Such technique provided an integrated measure of right femur proximal, distal and total areas.

Analytical determinations: Serum osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) levels were determined via enzyme linked immunosorbent assay (ELISA) technique as described by O'Brien *et al.*[25] and Teng *et al.*[26], respectively. Serum β2-microglobulin level was assayed by ELISA procedure using International Immuno-Diagnostics kit (Orgentec Diagnostika GmbH, Mainz, Germany) as described by Khalil *et al.*[27].

Isolation of total RNA: Total RNA was extracted from the bone samples obtained from the intertrochanteric region of the proximal femurof female rats by the standard TRIzol® Reagent extraction method (Invitrogen, Germany). Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Aliquots were used immediately for reverse transcription (RT).

Reverse transcription (RT) reaction: The complete Poly(A)$^+$ RNA isolated from female rat bone samples was reverse transcribed into cDNA in a total volume of 20 µL using RevertAidTM first strand cDNA Synthesis Kit (MBI Fermentas, Germany). The synthesized cDNA containing tubes were flash-cooled in an ice chamber immediately after reaction termination and stored under -20°C until being used for DNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

Quantitative real time-polymerase chain reaction (qRT-PCR): The PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1×SYBR® Premix Ex TaqTM

Table 1: Primer sequences of bone resorption and formation related genes

	Sequence 5' to 3	
Genes	Forward primer	Reverse primer
RANKL	GGACGGTGTTGCAGCAGAT	GCAGTCTGAGTTCCAGTGGTA
MMP9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
BGLAP2	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG
COL1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTGCTG

(TaKaRa, Biotech. Co. Ltd., Germany), 0.5 μL 0.2 μM sense primers, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water and 5 μL of cDNA template. Each experiment included a distilled water control[28].

The quantitative values of RT-PCR (qRT-PCR) of bone resorption (receptor activator of nuclear factor-κB, RANKL and Matrix metallopeptidase 9, MMP9) and bone formation (Bone gamma-carboxyglutamate protein 2/osteocalcin, BGLAP2 and Collagen type I alpha 1, COL1a1) genes were normalized on the bases of β-actin expression (Table 1). The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method[29].

HPLC measurement of 8-Hydroxy-2-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG): The DNA was extracted from female rat bone marrow by homogenization in buffer containing 1% sodium dodecyl sulphate, 10 mM Tris, 1 mM EDTA (pH 7.4) and an overnight incubation in 0.5 mg mL^{-1} proteinase K at 55°C. Homogenates were analyzed according to Patel *et al.*[30] and Mazlumoglu *et al.*[31].

Statistical analysis: All data were analyzed using the general liner models (GLM) procedure of statistical analysis system[32] followed by Scheffé-test to assess significant differences between groups. The values are expressed as Mean±SEM. All statements of significance were based on probability of $p<0.05$.

RESULTS

Phase analysis of the shells calcium carbonate: The infrared (FT-IR) signal positions for CO_2^{-3} ions and their assignments are shown in Fig. 1. Characteristic carbonate bands of aragonite were shown at wave number of 1082 cm^{-1} (υ_1) and 860 cm^{-1} (υ_2). The broad band positioned at 1473 cm^{-1} (υ_3) was assigned to asymmetric stretching mode of CO_2^{-3} ions whereas, the peak at about 712 cm^{-1} (υ_4) was unique indications of the calcite crystals. Moreover, the results showed extra IR frequencies corresponding to another chemical species in addition to carbonate ions.

Figure 2 shows the XRD pattern, the calcite robust peak was shown at $2\theta = 29.494 \approx 30^\circ$ of intensity 100%. However, such band indicated the existence of calcite and comparatively small other peaks at $2\theta = 36.094^\circ$, 45.098° and 48.46°. Aragonite peak was shown at $2\theta = 33^\circ$ of intensity 28% and several minor peaks at $2\theta = 45.908^\circ$ of intensity 32.1% and at $2\theta = 26.283$ of intensity 49.4%. The calcite peak is typically larger than that of aragonite peaks.

Levels of serum OPG, RANKL and β2-microglobulin in female rats: The supplementation effect of Brachidontes variabilis calcium carbonate (BVC) and Sigma anti-bonding molecule calcium carbonate (SAC) in ovariectomized rats (OVX) on serum osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) and beta 2-microglobulin (β2-microglobulin) levels are summarized in Table 2. The results revealed that OVX rats exhibited alteration in levels of different bone related markers compared with sham rats. The OPG level in OVX rats decreased significantly ($p<0.05$), while levels of serum RANKL and β2-microglobulin increased significantly ($p<0.05$) in comparison with the sham rats. In contrary, treatment of OVX-rats with SAC exhibited increase in serum OPG level and change in the other two parameters as compared with OVX rats but this effect was significant only ($p<0.05$) with the high dose of SAC. Furthermore, supplementation of OVX-rats with low and high doses of BVC revealed significant increase ($p<0.01$) in serum OPG level and decrease ($p<0.01$) in serum RANKL and β2-microglobulin levels as compared with OVX-rats.

Levels of bone mineral density (BMD) in female rats: Table 3 shows the results of bone mineral density levels in OVX-rats supplemented with BVC and SAC. The results found that OVX-rats showed significant $p<0.05$ decrease in bone mineral density of proximal (BMD- proximal), distal (BMD-distal) and total (BMD- total) areas of femur bones in comparison with the sham rats. In contrast, OVX-rats treated with low dose of BVC increased slightly the three measurements of BMD compared with OVX-rats. While, OVX-rats treated with high dose of SAC induced significant $p<0.05$

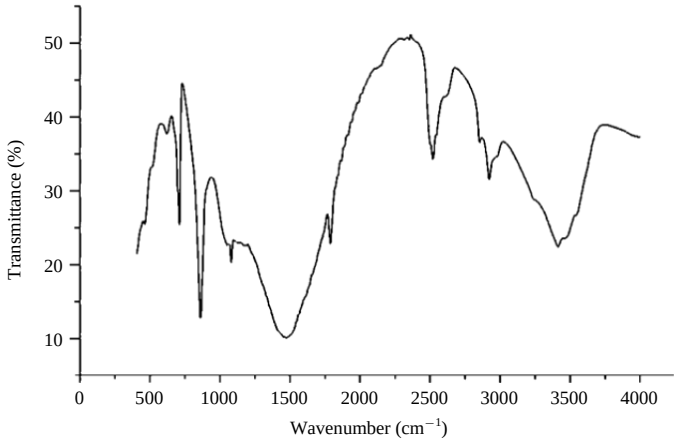

Fig. 1: FTIR spectra of *Brachidontes variabilis* explained a common phase of the mineral calcium carbonate

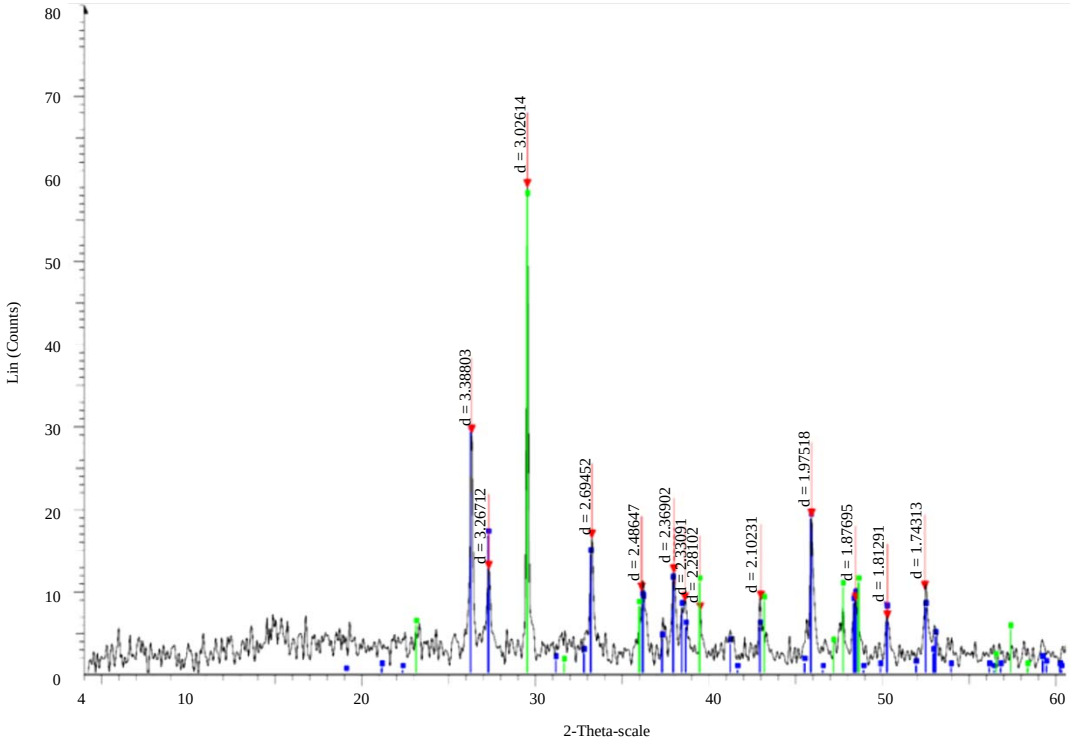

Fig. 2: X-ray diffraction (XRD) of the of polymorphs of $CaCO_3$ of *Brachidontes variabilis*

Table 2: Levels of serum OPG, RANKL and β2-microglobulin in female rats treated with different doses of SAC and BVC

Treatments	OPG (ng mL^{-1})	RANKL (pg mL^{-1})	β2-microglobulin (μg mL^{-1})
Sham	4.11±0.68^{a}	62.21±12.16^{e}	0.21±0.04^{b}
OVX	1.22±0.27^{c}	183.17±15.12^{a}	0.43±0.06^{a}
OVX+SAC1	1.64±0.42^{c}	151.62±13.16^{b}	0.38±0.02^{a}
OVX+SAC2	2.23±0.39^{b}	117.28±14.36^{c}	0.32±0.03^{b}
OVX+BVC1	2.49±0.31^{b}	102.15±6.81^{cd}	0.29±0.04^{b}
OVX+BVC2	3.25±0.73^{ab}	92.73±8.19^{d}	0.24±0.05^{b}

^{a,b,c,d}Mean values within column with unlike superscript letters were significantly different ($^{a}p<0.01$, $^{b,c,d}p<0.05$, Scheffé-test), OPG: Osteoprotegerin, RANKL: Receptor activator of nuclear factor-κB ligand, OVX: Ovariectomized, SAC: Sigma anti-bonding molecule calcium carbonate, BVC: Bivalve calcium carbonate, results are expressed as Mean±SEM

Table 3: Bone mineral density (BMD) in proximal, distal and total areas of femur bones of female rats treated with different doses of SAC and BVC

Treatments	BMD-proximal (mg cm^{-2})	BMD-distal (mg cm^{-2})	BMD-total (mg cm^{-2})
Sham	123.1±12.3[a]	121.2±11.3[a]	122.8±10.6[a]
OVX	67.2±5.2[b]	68.1±4.2[b]	67.8±5.3[b]
OVX+SAC1	74.1±4.1[b]	73.5±5.6[b]	72.9±6.4[b]
OVX+SAC2	98.7±8.3[a]	96.8±9.4[a]	97.6±8.2[a]
OVX+BVC1	94.3±9.2[a]	96.1±7.1[a]	94.3±7.6[a]
OVX+BVC2	118.7±10.41[a]	117.8±11.2[a]	119.4±9.7[a]

[a,b]Mean values within column with unlike superscript letters were significantly different ([a]p<0.01, [b]p<0.05, Scheffé-test), OVX: Ovariectomized, SAC: Sigma anti-bonding molecule calcium carbonate, BVC: Bivalve calcium carbonate, results are expressed as Mean±SEM

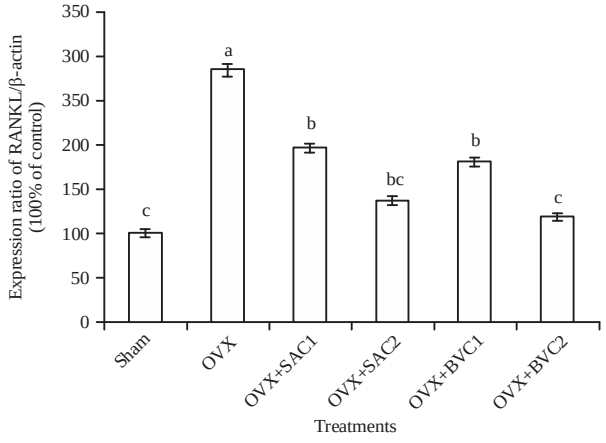

Fig. 3: Alterations of RANKL mRNA in bone tissues isolated from female rats treated with different doses of SAC and BVC

[a,b,c]Percentage values within tissue with unlike superscript letters were significantly different ([a]p<0.01, [b,c]p<0.05), results are expressed as Mean±SEM of data from at least 10 samples

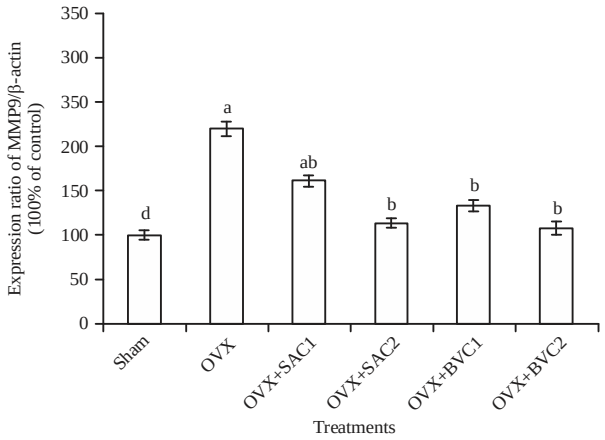

Fig. 4: Alterations of MMP9 mRNA in bone tissues isolated from female rats treated with different doses of SAC and BVC

[a,b,c]Percentage values within tissue with unlike superscript letters were significantly different ([a]p<0.01, [b,c]p<0.05), results are expressed as Mean±SEM of data from at least 10 samples

increase in BMD of the three areas as compared with OVX-rats. On the other hand, treatment of OVX-rats with low and high doses of BVC SAC induced highly significant increase in BMD of the three areas as compared with OVX-rats.

Expression alteration in the genes encoding bone resorption and bone formation: The expression results of bone resorption (RANKL and MMP9) and bone formation (BGLAP2 and COL1a1) genes are presented in Fig. 3-6. The results showed that OVX-rats exhibited significantly p<0.05 higher expression values of bone resorption (RANKL and MMP9) genes and lower expression values of bone formation (BGLAP2 and COL1a1) genes in comparison to the sham female rats. However, OVX-rats treated with the low dose of SAC inhibited slightly the expression alterations of bone resorption and bone formation genes compared with those in OVX-rats. Moreover, treatment of OVX-rats with high dose of SAC suppressed significantly the expression alterations of bone resorption and bone formation genes compared with those in OVX-rats. Furthermore, treatment of OVX-rats with low and high doses of BVC prevented significantly the expression alterations of bone resorption and bone formation genes compared with those in OVX-rats.

Generation of 8-hydroxy-2-deoxyguanosine (8-OhdG): Determination the levels of "8-OHdG" generation in bone marrow cells of female rat genome following SAC and BVC treatment is summarized in Fig. 7.

The results indicated that generation of 8-OHdG/2-dG ratio in OVX female rats increased with highly significant in comparison to sham female rats. However, OVX-rats treated with low dose of SAC induced insignificant decrease in the -OHdG/2-dG ratio compared with OVX-rats. However, treatment of OVX-rats with high dose of SAC decreased p<0.05 significantly the ratio of 8-OHdG/2-dG generation compared with those in OVX-rats. Moreover, treatment of

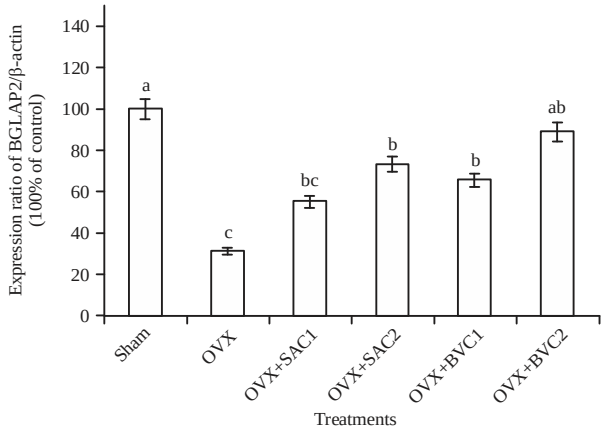

Fig. 5: Alterations of COL1a1 mRNA in bone tissues isolated from female rats treated with different doses of SAC and BVC

[a,b,c]Percentage values within tissue with unlike superscript letters were significantly different ([a]p<0.01, [b,c]p<0.05), results are expressed as Mean±SEM of data from at least 10 samples

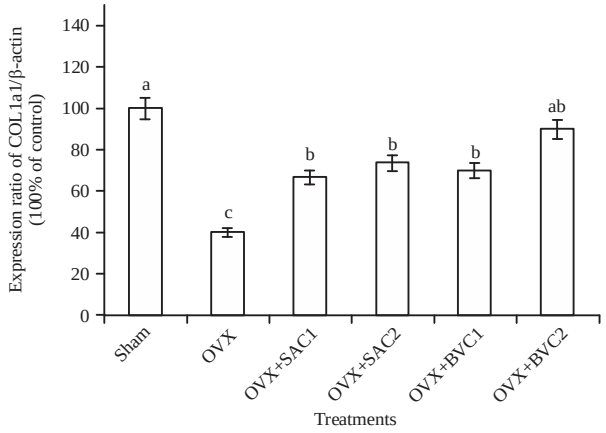

Fig. 6: Alterations of BGLAP2 mRNA in bone tissues isolated from female rats treated with different doses of SAC and BVC

[a,b,c]Percentage values within tissue with unlike superscript letters were significantly different ([a]p<0.01, [b,c]p<0.05), results are expressed as Mean±SEM of data from at least 10 samples

OVX-rats with low and high doses of BVC decreased with highly significant differences the ratio of 8-OhdG/2-dG generation compared with those in OVX-rats.

DISCUSSION

The physical measurements of *B. variabilis* calcium carbonate indicated that characteristic carbonate band of aragonite was shown at wavenumber of 1082 cm^{-1} (υ_1) and 860 cm^{-1} (υ_2) as they are infrared inactive for carbonate ions in

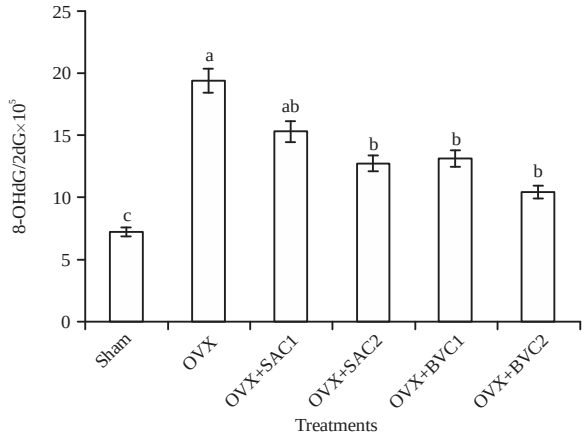

Fig. 7: Generation of 8-OHdG in rat bone marrow genome following SAC and BVC treatments. DNA damage was expressed as the ratio of oxidized DNA base (8-OHdG) to non-oxidized base (2-dG) in bone marrow DNA

Results are expressed as Mean±SEM of data from at least 10 samples, [a,b,c]Mean values within cell samples with unlike superscript letters were significantly different ([a]p<0.01, [b,c]p<0.05)

calcite structure. The broadband positioned at 1473 cm^{-1} (υ_3) was assigned to asymmetric stretching mode of CO_2^{-3} whereas, the peak at about 712 cm^{-1} (υ_4) was unique indications of the calcite crystals, in-plane bending mode, which may be attributed to a change in the local symmetry of carbonate ions[17]. Besides, a wide band was observed at high wave number related to OH at 3413 cm^{-1} correlated to the stretching vibrations of structural hydroxyl from the skeletal ring designating the presence of water. More importantly, authors have observed extra IR frequencies corresponding to another chemical species in addition to carbonate ions. IR band at about 2922 cm^{-1} was attributed to C-H stretching mode[18,19]. It was clearly found that the fresh specimen was made up of aragonite [a common phase of the mineral calcium carbonates[20]].

Furthermore, the XRD pattern indicated an easy way to distinguish between the two polymorphs of $CaCO_3$, aragonite and calcite. The calcite robust peak at $2\theta = 29.494^\circ \approx 30^\circ$ of intensity 100%, indicated the existence of calcite and comparatively small other peaks at $2\theta = 36.094^\circ$, 45.098° and 48.46°[21]. Aragonite peak at $2\theta = 33^\circ$ of intensity 28% and several minor peaks at $2\theta = 45.908^\circ$ of intensity 32.1% and at $2\theta = 26.283^\circ$ which represent intensity of 49.4%. Calcite peak was typically larger than that of aragonite peaks[22]. It's clear that calcite provides higher intensity in XRD than aragonite[23]. Overall, from the FT-IR and XRD results, it may be concluded that the components of these products were mainly calcium carbonate[24].

The biological study in this work was designed to understand the action mechanism of *Brachidontes variabilis* calcium carbonate (BVC) on bone tissue formation in OVX-rats. To get a good understanding for the action mechanism of BVC against bone loss in OVX-rats, a biological marker which has high susceptibility and possibility of applying is required to assess the bone transformation in osteoporosis. Therefore, several biological analyses were carried out to evaluate the effect of BVC against bone loss in OVX-rats. The results revealed that treatment of OVX-rats showed decrease in the proximal, distal and total bone mineral density (BMD), decline in the serum OPG level and increase in serum RANKL and β2-microglobulin levels. These findings were in consistent with other studies which reported that ovariectomized rats showed significant $p<0.05$ increase in serum RANKL and osteoclast surface and with decline of volumetric and areal BMD[33-35].

Several studies tried to explain the relationship between exclusion of the ovaries and bone loss in female laboratory animal model. Thus, the primary change in OVX rats after separation of the ovaries coincided with estrogen deficiency is an augment in the depletion of calcium from the skeleton[5]. In addition, as a result of the fast bone loss in OVX rats, a decrease in the parathyroid hormone secretion takes a place which induces a decline in the absorption of calcium in the intestine and resulting in the loss of calcium from the whole body and bone[36].

On the other hand, the present study showed that treatment of OVX-rats with low and high doses of BVC revealed significant $p<0.05$ increase in the proximal, distal and total BMD, elevation in the serum OPG level and decrease in serum RANKL and β2-microglobulin levels. However, similar effect on BMD, OPG level, RANKL and β2-microglobulin levels was observed with the high dose of SAC. In the same line with our results, Bae and Kim[37] reported that OVX rats administrated with a diet having sufficient calcium showed significantly $p<0.05$ higher lumbar spine BMC compared with OVX rats fed diet without calcium. They reported also that OVX rats showed higher OPG and RANKL levels compared with the control sham rats in which these findings concludes that bone transformation increases rapidly after isolation of ovaries in OVX rats.

Heaney *et al.*[38] reported that calcium carbonate and calcium citrate have been used widely in bone loss disease because they are considered a main ordinary calcium supplements due to well absorption when taken with food. Yang *et al.*[18] demonstrated that absorption of calcium can be secured by intake with food. Moreover, Wright *et al.*[39] reported that when calcium carbonate is taking in meal a well absorption was observed. Furthermore, Loke *et al.*[40] reported that administration of $CaCO_3$ in meal for more than 2 year in bone loss patients after menopause was more efficient in decreasing bone shortage.

Several studies investigated the effect of Ca on the activation and formation of bone tissues using molecular markers such as the mRNA levels[37]. Therefore, to understand the biological action of BVC on the molecular biology basis authors have used the mRNA expression tool for analysis the activity of genes related to bone resorption (RANKL and MMP9) and bone formation (BGLAP2 and COL1a1)[41]. The results revealed that treatment of OVX-rats with low and high doses of BVC prevented significantly the expression alterations of bone resorption and bone formation genes compared with those in OVX-rats. RANKL is considered to be one of molecules which regulate the bone metabolism. It is one of the cytokines belong to the TNF (tumor necrosis factor) which play an essential role in development and bone formation[42]. It is also reported that the main roles of RANKL are condensate on the biology of bone, in particular in bone metabolism such as osteoclastogenesis.

It has complicated role with M-CSF in the differentiation of mature osteoclasts starting of monocyte progenitor from the reservoir of the hematopoietic myeloid. RANKL activates the osteoclast in which it is the main responsible for bone resorption.

During the resorption of bone matrix numerous proteolytic enzymes play degradation role in this process. From these proteolytic enzymes are MMPs (matrix metalloproteinases) and cysteine proteases which considered as the most important proteases in the bone resorption process. Andersen *et al.*[43] reported that among the MMPs gene family, MMP-9 was showed at high expression level during bone resorption process. It has been found that the effect of MMP-9 during the bone resorption is considered as (a) removing the layers of collagen from the bone surface and then (b) demineralization from bone tissues[44]. Moreover, Logar *et al.*[45] suggested that the cysteine proteases degrade the collagenous layers and then the MMPs act on the bone resorption pits to clean it from the residual of collagen. In contrary, degradation of the bone tissue matrix was prevented in the existence of using MMP inhibitors[45].

Osteocalcin, namely bone gamma-carboxyglutamic acid protein 2 (BGLAP2) is produced only by specific bone cells called osteoblasts[46]. BGLAP2 is considered to play important role in building of bone and regulation of the biological processes associated with body metabolism[47]. In addition, BGLAP2 is coinciding with regulation of bone mineralization during the bone formation[48].

The chemical structure of collagen is a protein which supports and builds up several issues in the body[49]. In the present study have selected the COL1A1 gene which responsible for production of collagen from the type I, namely pro-a1 (I) chain[50]. The pro-a1 (I) chain is associated with several kinds of pro-chains such as pro-a1 (I) chain of another type and pro-a2 (I) chain (which coded by COL1A2 gene) to build a complex component of procollagen[51]. This complex molecule is responsible for formation of strong type of collagen fibers.

In the current study, evaluation the relationship between bone loss and oxidative stress as determined by generation of 8-OHdG was conducted. The results showed that generation of 8-OHdG in OVX female rats increased $p<0.05$ significantly in comparison to sham female rats. However, treatment of OVX-rats with low and high doses of BVC decreased significantly the ratio of 8-OHdG/2-dG generation compared with those in OVX-rats. For our knowledge, no available data are published concerning he genetic toxicity in OVX rats. However, Donida *et al.*[52] studied the correlation between 8-OHdG generation and RANKL to OPG ratio in women with bone loss. They found that high level of genetic toxicity (in the form of 8-OHdG generation) was correlated with increase of the ratio of RANKL to OPG in serum of women with osteoporosis. Thus, the mechanism of DNA damage in OVX rats may be correlated with the ratio of RANKL to OPG[53,54]. Further study should be conducted to investigate the relationship between the molecular osteoporosis markers such as ration of RANKL to OPG and oxidative stress inducing DNA damage.

CONCLUSION

This study exhibited positive results for osteoporosis treatment using *B. variabilis* $CaCO_3$ on the basis of bone mineral density parameters and on the expression of genes related to bone resorption and bone formation. These findings suggested that $CaCO_3$ isolated from *B. variabilis* shells may have a promising significant potential in clinical translation, which then are exploited as a carrier for efficient loading of different types of therapeutic strategies.

SIGNIFICANCE STATEMENTS

This study discovers the potential effective impact of *Brachidontes variabilis* calcium carbonate that can be beneficial for osteoporosis treatment. This study will assist the researchers in medicine to uncover natural compounds isolated from marine organisms as safer drugs which enhance the immune system in bone loss patients that many researchers were not able to explore. Thus, a new theory on the calcium carbonate molecules from *Brachidontes variabilis* as a novel compounds and possibly other components of marine organisms, may be arrived at new composition of drug discovery.

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REFERENCES

1. Ehrlich, H., P. Simon, W. Carrillo-Cabrera, V.V. Bazhenov and J.P. Botting *et al.*, 2010. Insights into chemistry of biological materials: Newly discovered silica-aragonite-chitin biocomposites in demosponges. Chem. Mater., 22: 1462-1471.
2. El-Bassyouni, G.T., N.R.E. Radwan and W.I. Abdel-Fattah, 2004. Assessment of thermal stability and characterization of Egyptian corals (*Favia stelligera* species). Ceramurgia, 34: 85-97.
3. Pujari-Palmer, S., S. Chen, S. Rubino, H. Weng and W. Xia *et al.*, 2016. *In vivo* and *in vitro* evaluation of hydroxyapatite nanoparticle morphology on the acute inflammatory response. Biomaterials, 90: 1-11.
4. Li, K.C., Y.H. Chang, C.L. Yeh and Y.C. Hu, 2016. Healing of osteoporotic bone defects by baculovirus-engineered bone marrow-derived MSCs expressing MicroRNA sponges. Biomaterials, 74: 155-166.
5. Kaplan, B. and M. Hirsch, 2004. Current approach to fracture prevention in postmenopausal osteoporosis. Clin. Exp Obstet. Gynecol., 31: 251-255.
6. Dang, M., A.J. Koh, X. Jin, L.K. McCauley and P.X. Ma, 2017. Local pulsatile PTH delivery regenerates bone defects via enhanced bone remodeling in a cell-free scaffold. Biomaterials, 114: 1-9.
7. Arjmandi, B.H., S.A. Johnson, S. Pourafshar, N. Navaei and K.S. George *et al.*, 2017. Bone-protective effects of dried plum in postmenopausal women: Efficacy and possible mechanisms. Nutrients, Vol. 9, No. 5. 10.3390/nu9050496.
8. McMillan, J., R.C. Kinney, D.M. Ranly, S. Fatehi-Sedeh, Z. Schwartz and B.D. Boyan, 2007. Osteoinductivity of demineralized bone matrix in immunocompromised mice and rats is decreased by ovariectomy and restored by estrogen replacement. Bone, 40: 111-121.

9. Wilkosz, P., G.D. Greggains, T.G. Tanbo and P. Fedorcsak, 2014. Female reproductive decline is determined by remaining ovarian reserve and age. PLoS ONE, Vol. 9. 10.1371/journal.pone.0108343.
10. Verron, E., O. Gauthier, P. Janvier, P. Pilet and J. Lesoeur *et al*., 2010. *In vivo* bone augmentation in an osteoporotic environment using bisphosphonate-loaded calcium deficient apatite. Biomaterials, 31: 7776-7784.
11. Fraser, L.A., K.N. Vogt, J.D. Adachi and L. Thabane, 2011. Fracture risk associated with continuation versus discontinuation of bisphosphonates after 5 years of therapy in patients with primary osteoporosis: A systematic review and meta-analysis. Therapeut. Clin. Risk Manage., 7: 157-166.
12. Muratore, M., E. Quarta, A. Grimaldi, F. Calcagnile and L. Quarta, 2011. Clinical utility of clodronate in the prevention and management of osteoporosis in patients intolerant of oral bisphosphonates. Drug Des. Dev. Ther., 5: 445-454.
13. Hopwood, B., A. Tsykin, D.M. Findlay and N.L. Fazzalari, 2009. Gene expression profile of the bone microenvironment in human fragility fracture bone. Bone, 44: 87-101.
14. Troen, B.R., 2004. The role of cathepsin K in normal bone resorption. Drug News Perspect., 17: 19-28.
15. Hwang, M.P., R. Subbiah, I.G. Kim, K.E. Lee, J. Park, S.H. Kim and K. Park, 2016. Approximating bone ECM: Crosslinking directs individual and coupled osteoblast/osteoclast behavior. Biomaterials, 103: 22-32.
16. Choi, S.Y., D. Park, G. Yang, S.H. Lee and D.K. Bae *et al*., 2011. Effects of sigma anti-bonding molecule calcium carbonate on bone turnover and calcium balance in ovariectomized rats. Lab. Anim. Res., 27: 301-307.
17. Vagenas, N.V., A. Gatsouli and C.G. Kontoyannis, 2003. Quantitative analysis of synthetic calcium carbonate polymorphs using FT-IR spectroscopy. Talanta, 59: 831-836.
18. Yang, Y.X., J.D. Lewis, S. Epstein and D.C. Metz, 2006. Long-term proton pump inhibitor therapy and risk of hip fracture. J. Am. Med. Assoc., 296: 2947-2953.
19. Ni, M. and B.D. Ratner, 2003. Nacre surface transformation to hydroxyapatite in a phosphate buffer solution. Biomaterials, 24: 4323-4331.
20. Goncalves, A.M.M., A.F. Mesquita, T. Verdelhos, J.A.P. Coutinho, J.C. Marques and F. Goncalves, 2016. Fatty acids' profiles as indicators of stress induced by of a common herbicide on two marine bivalves species: *Cerastoderma edule* (Linnaeus, 1758) and *Scrobicularia plana* (da Costa, 1778). Ecol. Indicat., 63: 209-218.
21. Xu, A.W., Y. Ma and H. Colfen, 2007. Biomimetic mineralization. J. Mater. Chem., 17: 415-449.
22. Ni, M. and B.D. Ratner, 2008. Differentiating calcium carbonate polymorphs by surface analysis techniques-an XPS and TOF-SIMS study. Surf. Interface Anal., 40: 1356-1361.
23. Kamitakahara, M., T. Nagamori, T. Yokoi and K. Ioku, 2015. Carbonate-containing hydroxyapatite synthesized by the hydrothermal treatment of different calcium carbonates in a phosphate-containing solution. J. Asian Ceram. Soc., 3: 287-291.
24. Yuan, G., X. Chen, X. Li, Q. Liang, G. Miao and B. Yuan, 2015. The synthesis of calcium carbonate microparticles with multiple morphologies through self-assembly method. Powder Technol., 284: 253-256.
25. O'Brien, E.A., J.H. Williams and M.J. Marshall, 2001. Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocranial membrane. Bone, 28: 208-214.
26. Teng, Y.T.A., H. Nguyen, X. Gao, Y.Y. Kong and R.M. Gorczynski *et al*., 2000. Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. J. Clin. Invest., 106: R59-R67.
27. Khalil, W.K.B., G.T. El-Bassyouni and H.F. Booles, 2016. Nano-encapsulated form of citrus medica for osteoporosis treatment in animal model. Int. J. Pharm. Clin. Res., 8: 49-59.
28. Assaf, N., A.B. Shalby, W.K.B. Khalil and H.H. Ahmed, 2012. Biochemical and genetic alterations of oxidant/antioxidant status of the brain in rats treated with dexamethasone: Protective roles of melatonin and acetyl-L-carnitine. J. Physiol. Biochem., 68: 77-90.
29. Hulme-Moir, K.L., E.N. Barker, A. Stonelake, C.R. Helps and S. Tasker, 2010. Use of real-time quantitative polymerase chain reaction to monitor antibiotic therapy in a dog with naturally acquired *Mycoplasma haemocanis* infection. J. Vet. Diagn. Invest., 22: 582-587.
30. Patel, M., L.P. Liang, I.I. Roberts and L. Jackson, 2001. Enhanced hippocampal F_2-isoprostane formation following kainite-induced seizures. J. Neurochem., 79: 1065-1069.
31. Mazlumoglu, M.R., O. Ozkan, H.H. Alp, E. Ozyildirim, F. Bingol, O. Yoruk and O. Kuduban, 2017. Measuring oxidative DNA damage with 8-hydroxy-2'-deoxyguanosine levels in patients with laryngeal cancer. Ann. Otol. Rhinol. Laryngol., 126: 103-109.
32. SAS., 2003. SAS User's Guide: Statistics. SAS Institute Inc., Cary, NC., USA.
33. Khan, A. and Z. Syed, 2006. Bone mineral density assessment in premenopausal women. Women's Health, 2: 639-645.
34. Hughes, J.M., K.L. Popp, R. Yanovich, M.L. Bouxsein and R.W. Matheny Jr., 2017. The role of adaptive bone formation in the etiology of stress fracture. Exp. Biol. Med., 242: 897-906.
35. Ominsky, M.S., X. Li, F.J. Asuncion, M. Barrero and K.S. Warmington *et al*., 2008. RANKL inhibition with osteoprotegerin increases bone strength by improving cortical and trabecular bone architecture in ovariectomized rats. J. Bone Miner. Res., 23: 672-682.

36. Mundy, G.R., 2006. Nutritional modulators of bone remodeling during aging. Am. J. Clin. Nutr., 83: 427S-430S.
37. Bae, Y.J. and M.H. Kim, 2010. Calcium and magnesium supplementation improves serum OPG/RANKL in calcium-deficient ovariectomized rats. Calcif. Tissue Int., 87: 365-372.
38. Heaney, R.P., M.S. Dowell, J. Bierman, C.A. Hale and A. Bendich, 2001. Absorbability and cost effectiveness in calcium supplementation. J. Am. Coll. Nutr., 20: 239-246.
39. Wright, M.J., R.R. Sullivan, E. Gaffney-Stomberg, D.M. Caseria and K.O. O'Brien *et al*., 2010. Inhibiting gastric acid production does not affect intestinal calcium absorption in young, healthy individuals: A randomized, crossover, controlled clinical trial. J. Bone Miner. Res., 25: 2205-2211.
40. Loke, Y.K., V. Jeevanantham and S. Singh, 2009. Bisphosphonates and atrial fibrillation: Systematic review and meta-analysis. Drug Saf., 32: 219-228.
41. Wu, J., S. Xu, Z. Qiu, P. Liu and H. Liu *et al*., 2016. Comparison of human mesenchymal stem cells proliferation and differentiation on poly(methyl methacrylate) bone cements with and without mineralized collagen incorporation. J. Biomater. Applic., 30: 722-731.
42. Kohli, S.S. and V.S. Kohli, 2011. Role of RANKL-RANK/osteoprotegerin molecular complex in bone remodeling and its immunopathologic implications. Indian J. Endocrinol. Metab., 15: 175-181.
43. Andersen, T.L., M. del Carmen Ovejero, T. Kirkegaard, T. Lenhard, N.T. Foged and J.M. Delaisse, 2004. A scrutiny of matrix metalloproteinases in osteoclasts: Evidence for heterogeneity and for the presence of MMPs synthesized by other cells. Bone, 35: 1107-1119.
44. Delaisse, J.M., M.T. Engsig, V. Everts, M. del Carmen Ovejero and M. Ferreras *et al*., 2000. Proteinases in bone resorption: Obvious and less obvious roles. Clin. Chim. Acta, 291: 223-234.
45. Logar, D.B., R. Komadina, J. Prezelj, B. Ostanek, Z. Trost and J. Marc, 2007. Expression of bone resorption genes in osteoarthritis and in osteoporosis. J. Bone Miner. Metab., 25: 219-225.
46. Hannon, R.A. and R. Eastell, 2003. Biochemical markers of bone turnover and fracture prediction. Br. Menopause Soc. J., 9: 10-16.
47. Lee, N.K., H. Sowa, E. Hinoi, M. Ferron and J.D. Ahn *et al*., 2007. Endocrine regulation of energy metabolism by the skeleton. Cell, 130: 456-469.
48. Karsenty, G. and F. Oury, 2014. Regulation of male fertility by the bone-derived hormone osteocalcin. Mol. Cell. Endocrinol., 382: 521-526.
49. Alvarez-Hernandez, D., M. Naves, J.B. Diaz-Lopez, C. Gomez, I. Santamaria and J.B. Cannata-Andia, 2003. Influence of polymorphisms in VDR and COLIA1 genes on the risk of osteoporotic fractures in aged men. Kidney Int., 63: S14-S18.
50. Eimar, H., F. Tamimi, J.M. Retrouvey, F. Rauch, J.E. Aubin and M.D. McKee, 2016. Craniofacial and dental defects in the *Col1a1*$^{Jrt/+}$ mouse model of osteogenesis imperfecta. J. Dent. Res., 95: 761-768.
51. Chamberlain, J.R., U. Schwarze, P.R. Wang, R.K. Hirata and K.D. Hankenson *et al*., 2004. Gene targeting in stem cells from individuals with osteogenesis imperfecta. Science, 303: 1198-1201.
52. Donida, B., D.P. Marchetti, C.E.D. Jacques, G. Ribas and M. Deon *et al*., 2017. Oxidative profile exhibited by Mucopolysaccharidosis type IVA patients at diagnosis: Increased keratan urinary levels. Mol. Genet. Metab. Rep., 11: 46-53.
53. Farombi, E.O., A.O. Abolaji, I.A. Adedara, I. Maduako and I. Omodanisi, 2015. Artemisinin induces hormonal imbalance and oxidative damage in the erythrocytes and uterus but not in the ovary of rats. Hum. Exp. Toxicol., 34: 83-92.
54. Dincel, G.C. and H.T. Atmaca, 2016. Role of oxidative stress in the pathophysiology of *Toxoplasma gondii* infection. Int. J. Immunopathol. Pharmacol., 29: 226-240.