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Characterization and Potential of Enhancing Bone Regeneration Using Raw Cuttlebone Aragonite Nanoparticles: In Vitro and In Vivo Studies

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Aim: This study aimed to examine the cuttlebone nanoparticles prepared by ball-milling top-down approach without any thermal treatments or chemical additives to investigate its constituents, chemical composition, surface topography, cytotoxicity, and its effect on osteoblasts alkaline phosphatase assay. Also, its effect on alveolar bone of ovariectomized rats was histologically evaluated.
Materials and methods: Cuttlebone nanoparticles were characterized by transmission and scanning electron microscopy. Both the elemental composition and its main compositional materials were measured using energy-dispersive X-ray and X-ray diffraction analyses, respectively. Moreover, we evaluated its cytotoxicity in dental pulp cells and examined its osteogenic effect on an osteoblast cell line for alkaline phosphatase activity. Histological examination was performed using a light microscope to investigate its effect on the ovariectomized alveolar bone of rats.
Results: The results revealed a nontoxic effect and a direct proportional relationship between the alkaline phosphatase absorption and cuttlebone concentration. Electron microscopy revealed a different regular spherical pattern. Elemental microanalysis detected high calcium and carbon levels, which was confirmed by X-ray diffraction and showed a high calcium carbonate composition. Histologically, normal alveolar bone architecture was noted after using cuttlebone nanoparticles in the ovariectomized group compared with the ovariectomized group only.
Conclusion: Raw cuttlebone nanoparticles can be used as biocompatible bone substitutes in bone regeneration.

Keywords: Cuttlebone fish, characterization, cytotoxicity, osteogenesis, alkaline phosphatase assay.

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Introduction

Biomaterial usage in the area of osseous regeneration as graft substitutes is becoming a necessity because of the several drawbacks of the traditional methods such as orthopaedic implants, allografts, and autografts. These drawbacks include the risk of infection, defective healing following invasive procedures, and insufficient bone donations to properly fill gaps. [1]

Calcium carbonate (CaCO3) has garnered attention in the domains of biomaterials and biomineralization as a prevalent biological mineral synthesized by living organisms. Utilized primarily for bone reconstruction, $CaCO_3$ serves as a biomaterial in the form of Biocoral®, a natural coral exoskeleton employed as a substitute for bone grafts.[2,3]

Anhydrous $CaCO_3$ exists in three polymorphic forms: aragonite, calcite, and vaterite. The most prevalent formations in nature that are more thermodynamically stable are aragonite and calcite, whereas vaterite is less frequently found in nature as it is in an aqueous solution and is the least stable thermodynamically polymorph, thus it can easily change into calcite and aragonite. According to experimental evidence, vaterite can change to calcite within 24h at ambient temperature and to aragonite within one hour at 60°C. [4]

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Aragonite, a rare biogenic polymorph of calcium carbonate ($CaCO_3$), is extensively employed in the repair of fractured bones, the development of advanced drug delivery systems, and construction of tissue scaffolds. Additionally, its density, which is higher than that of calcite, and its liability to be replaced by bone, renders it useful as an anticancer drug carrier, in tissue engineering, and in bone repairs. [4]

Different $CaCO_3$ polymorphs have been distinguished using several techniques such as X-ray diffraction, scanning electronic microscopy (SEM), and fourier transform infrared (FTIR) spectroscopy. However, SEM is doubtful for characterizing different CaCO3 polymorphs owing to the variation in the crystalline morphological shape, which could be altered because of the crystallization conditions. For instance, under specific crystallization conditions, the morphology of aragonite can alter from its typical "needle-like" appearance to forms resembling "flakes" or "cauliflowers." Moreover, as a significant biomaterial with potential applications in bone grafting, it is crucial to understand the surface topography and functionality of these polymorphic forms of CaCO3 to fully understand their biological characteristics. [3]

Cuttlefish bone represents an affordable, nontoxic, and worldwide available $CaCO_3$ source. [5] $CaCO_3$ is the primary ingredient in cuttlebone (CB) powder, with minor amounts of Al, Na, and Cl. [6] Numerous marine organisms employ chitin as the principal scaffold material in biomineralization processes, where it serves as an antibacterial, bioactive, and biodegradable polymer. [7]

Conversely, proteins in coral biominerals are a crucial component, serving as templates for the initial extracellular nucleation of nanosized aragonite crystals during the early stages of growth. Vida Cadez et al. [8] in 2017 conducted research on the role of soluble organic matrix (SOM) proteins in the development of nanosized biomineral structures in CB.

At the nanoparticle scale, CaCO3 is considered a chemically inert material and an excellent choice for biomedical and industrial

applications, owing to its remarkable characteristics such as biocompatibility, high surface-to-volume ratio, durability, simplicity of synthesis, potential for surface functionalization, and the ability to present in various polymorphs and morphologies. [9]

Extensive research has been conducted to develop techniques for the synthesis and use of aragonite nanoparticles. The bottom-up approach was developed using the precipitation process. However, this method did not produce the proper shapes and sizes necessary for pure aragonite nanoparticles because of its frequent blending with other polymorphs, such as calcite and vaterite. Another drawback is the necessity of adding numerous toxic impurities to the final product to produce crystalline calcium carbonate nanoparticles (CaCO3NP), which have negative effect on its final chemical composition. [1]

Accordingly, the top-down approach has been implemented, with ongoing improvements and developments. With this technique, bulk material is reduced to micron-and nano-sized particles. $CaCO_3$-aragonite nanoparticles created mechanically are biocompatible with cells and have minimal to no cytotoxic and immunogenic effects, which promotes their wide biomedical applications. [10]

Osteoblasts cultured with CaCO3 nanocrystals display increased extracellular calcium deposition, which is essential in bone formation. Osteocalcin is an important non-collagenous protein in bone secreted during osteoblast's late differentiation stage. Also, it acts as a regulator of bone formation as it controls osteoblast and osteoclast activity. [11,12]

This study seeks to delineate the dimensions and surface features of untreated cuttle bone nanoparticles (CBN) utilizing scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Additionally, it aims to analyse the chemical composition of CBN through energy dispersive x-ray analysis (EDX) and x-ray diffraction (XRD). Moreover, to evaluate its cytotoxicity on dental pulp cells and examine its osteogenic effect on osteoblast cell lines for alkaline phosphatase activity, in addition, its functionality was studied by evaluating its effect on the alveolar bones of ovariectomized rats.

Material and methods

This investigation adhered to the ethical guidelines for scientific research recommended by the Ethical Committee of the Faculty of Dentistry at Suez Canal University, which are based on "WHO-2011" standards.

Sample size calculation

The sample size for this study was determined using the G*Power software for statistical analysis (version 3.1.9.7, Heinrich Heine University Düsseldorf, Düsseldorf, Germany). For the in vitro component, 40 cuttlebone nanoparticles (CBN) samples were used and divided into groups of eight. In the in vivo experiments, the projected minimum sample size was 24 adult female albino rats, weighing 200-250 grams each, divided into groups of eight. These animals were used to assess the impact of CBN on the histological characteristics of osteoporotic alveolar bone in OVX rats that underwent ovariectomy. This study was designed to achieve an effect size of 0.4, 80% power (1-β), and a significance level (α) of 0.05.

Random allocation

Animal models in the in vivo study, were randomly assigned into five and three equal groups, respectively, using computer-assisted software. This randomization involved the use of computer-generated number sequences to allocate treatment conditions, thereby minimizing potential biases. In addition, all personnel conducting

the experiments were blinded to the treatment assignments to further reduce the risk of error.

Regarding the in vivo study, the three groups were divided as: Group I (Control group): Eight rats were sham -operated, in which the ovaries were not removed. Group II (OVX group): Eight rats were subjected to bilateral ovariectomy. Group III (OVX + CBN): Eight ovariectomized rats were administered CBN starting on the day of surgery.

Methods:

Specimen preparation

CB was purchased from a local food supplier. Forty samples were taken from the lamellar part of cuttlefish bone, which turned into nanosized powder using mortar. The dried powder was ground using a Planetary Ball Mill PM 400 machine. This process lasted for eight hours at a rotational speed of 350 rpm, with breaks occurring every three minutes. [13] The size of the CBN was approximately 30 nm.

Cuttlefish bones nanoparticles characterization

Viability assay

CBN powder was placed in 12-well plates, each populated at a density of 10,000 cells/cm² using Kaighn's modified Ham's F-12 medium (F12K) enriched with 20% fetal calf serum (FCS) and 100 µg/g streptomycin. The media in each well were replaced weekly, and cell development was monitored at predetermined intervals, with nine wells dedicated to each sample. MTT reagent was added to the wells at one day, one week, two weeks, and three weeks post-seeding. After a four-hour incubation period in the dark at 37°C, the cell medium was discarded, and the cells were lysed using 0.004 N HCl in isopropanol. The lysates were then centrifuged, and the supernatants were dispensed in triplicate into a 96-well plate for absorbance readings at 570 and 630 nm on a Synergy HT microplate reader (Bio-Tek, Bad Friedrichshall, Germany). Furthermore, we eliminated any potential influence of the material on the assay results and assessed the cellular response. Cellular morphology was evaluated using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany, Type 090–135.002) equipped with a Ds-Fi1 digital camera (Nikon, Düsseldorf, Germany).

Determination of cytotoxicity in cells (MTT protocol) [14].

1. MC3T3-E1 cells treated with 31.25, 62.5, 125, 250, 500, or 1000 (CBN) powder were introduced into a 96-well tissue culture plate at a concentration of 1 x 10 5 cells/ml (100 µl/well) and incubated at 37°C for 24 h to form a complete monolayer.
2. Upon reaching confluence, the growth medium was removed from the wells, and the monolayer was washed twice using wash medium.
3. The test sample was diluted twofold in RPMI medium supplemented with 2% serum, which was used as the maintenance medium.
4. A volume of 0.1 ml (about 0 oz) from each dilution series was added to various wells for analysis, reserving three wells to receive only the maintenance medium as controls.
5. The plate was inspected post- incubation at 37°C to evaluate the signs of cytotoxicity, which included complete or partial disruption of the monolayer, cell rounding, shrinkage, or granulation.
6. An MTT solution (5 mg/ml in phosphate-buffered saline) was prepared using BIO BASIC CANADA INC.
7. Each well was treated with 20 µL of MTT solution, and the plate was agitated at 150 rpm for five minutes on a shaker to ensure thorough mixing of MTT with the media.
8. MTT was allowed to metabolize for 1–5 h in an incubator set at 37°C with 5% CO2.

9. The media were then discarded, and a paper towel was used to dry the plate and remove any residues.
10. Formazan, a metabolic byproduct of MTT, was resuspended in 200 µL DMSO, and the mixture was agitated at 150 rpm for five minutes on a shaker to ensure proper dissolution.
11. The background was subtracted at 620 nm, and the optical density was measured at 560 nm, with an expected close correlation between optical density and cell quantity.

Alkaline phosphatase (ALP) activity assay. [15]

The Senso Lyte pNPP alkaline phosphatase assay (AnaSpec, Fremont, CA) was used on days 1, 3, 7, and 14 to monitor alterations in the differentiation behaviour of osteoblasts. For osteogenic induction, the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) enhanced with 10% fetal calf serum (FCS), low glucose, L-glutamine, 100 µg/g streptomycin, 100 U/ml penicillin, 0.005 mM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 µM dexamethasone. Nine wells/specimen were assessed at various intervals during the study. The cell medium was refreshed every week. Following the experiment, cells were cleaned and preserved at -80°C. DNA quantification in thawed cells was performed using the PicoGreen dsDNA quantitation assay (Invitrogen, Eugene, OR). Cells were lysed with 1% Triton X-100 in phosphate-buffered saline. After centrifugation, the supernatants were mixed with the PicoGreen® working solution in a 96-well plate. the samples were excited at 485 nm, and the fluorescence emission intensities were measured at 528 nm. For specific assays, the supernatants were diluted in buffer and coated with alkaline phosphatase, and absorbance was measured at 405 nm. This quantification was correlated with absolute cell counts.

Initial cell passages were cultured in 96-well plates at a density of 1.3 x 10^4 cells per well. Alkaline phosphatase (ALP) concentration was measured in 20 mL of the culture supernatant using an enzyme-linked immunoassay (Sigma ALP kit 104). This assay involves coating a strip with a monoclonal antibody specific to bone-derived ALP. Each experiment was conducted in triplicate and the mean values and standard deviations documented. The procedures were performed in accordance with the NIVA protocol (catalog number In-Hu0075).

Transmission electron microscopy (TEM)

CBN powder was placed on chamber slides (Nalge Nunc International, Rochester, NY) and initially fixed for 30 minutes using a solution of 2% glutaraldehyde, 0.02% picric acid, and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2–7.4). Subsequently, they underwent a 20-minute fixation in 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer (pH 7.2–7.4). Following drying, the specimens were embedded in Epon. Ultrathin sections ranging from 80 to 100 nm were cut using collodion-coated copper grids. Imaging was conducted using a JEOL JEM-2100 high-resolution transmission electron microscope at an accelerating voltage of 200 kV. [16]

Scanning Electron Microscope (SEM) and Energy Dispersive X-ray analysis (EDX)

CBN powder was examined under SEM to assess morphological characteristics and subjected to EDX analysis for elemental analysis (Quattros-Thermo Scientific, Netherland). [16]

X- Ray Diffraction XRD

The crystalline structure of CBN powder was evaluated using X-ray diffraction (XRD) using an Empyrean system (Malvern, Netherlands). The CBN powder

was compressed into a holder and subjected to continuous scanning on a PANalytical diffractometer system (X'Pert PRO), that utilized a copper tube. Diffraction intensities were measured in the range of 20 - 70 Å. [17]

In vivo animal study

Study design

This study involved 24 adult female albino rats, each weighing approximately 200-250 grams. The rats were randomly assigned to three groups, with eight rats in each group. Rats in groups II and III (OVX, OVX+CBN) underwent bilateral ovariectomy via a vertical dorsal incision. The ovaries were ligated and removed under sterile conditions. [18] In the third group (OVX + CBN), the OVX rats received CBN injections daily for 14 days starting from the day of surgery. The CBN was administered intravenously through the lateral tail vein at a dosage of 30 mg/kg. [19]

Rat Euthanasia and histological sample preparation:

Two months after ovariectomy, the rats were euthanized by cervical dislocation and mandibles were dissected. The right halves of the mandibles were prepared for histological examination under a light microscope. The mandibles were preserved in 10% neutral- buffered formalin, decalcified, cleansed, dehydrated, and subsequently embedded in paraffin. Sections with a thickness of 5 microns were serially sliced and stained using Hematoxylin and Eosin (H&E) as well as Gomori's Trichrome staining techniques.

Results

Cuttlefish bones nanoparticles characterization

1.MTT Assay, cell viability/ cytotoxicity

The cytotoxic effects of the nanoaragonite powder were evaluated using the UMR106 dental pulp cell line. The CBN samples showed no toxic effects at concentrations up to 125 µg/ml but demonstrated significant stimulatory activity at concentrations as high as 250 µg/ml. Cell viability was measured using the MTT assay, which indicated an increase in cytotoxicity of CBN at concentrations above 250 µg/ml. The most substantial reduction in cell viability (86.1%) was recorded at a concentration of 1000 µg/ml (Fig. 1).

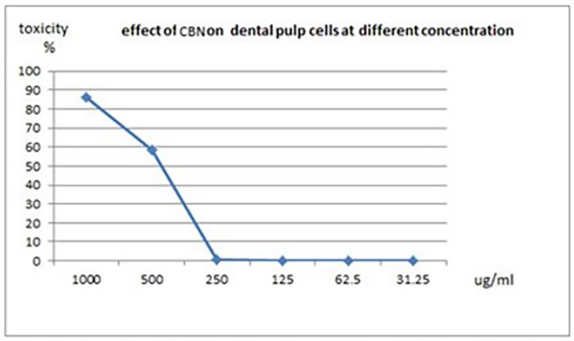

Figure 1: Graph showing dental pulp cells vitality at different CBN concentrations.

Furthermore, cells treated with concentrations of 125 µg/ml and 250 µg/ml displayed normal vital histological characteristics, as shown in Fig. 2, panels c and d. Conversely, cells exposed to 500 µg/ml and 1000 µg/ml (Fig. 2a & 2b) displayed clear signs of toxicity and degeneration, with a significant departure from the regular histological structure seen in the control dental pulp cells (Fig 2e).

2.Alkaline phosphatase (ALP) activity assay

The effect of CBN on the ALP activity of human hFOB1.19 osteoblasts was studied by treating cells with different CBN concentrations (0-36 µg/ml) for 14 days. ALP activity of hFOB1.19 cells was directly proportional with the CBN concentrations, reaching a maximum level of ALP activity at 36 µg/ml (Table 1 &2). The curve shows the linearity of the absorbance with respect to the ALP concentration, where a linear response was observed with increasing ALP

concentration (Fig.3). The Original concentration was calculated by multiplying the dilution factor by five.

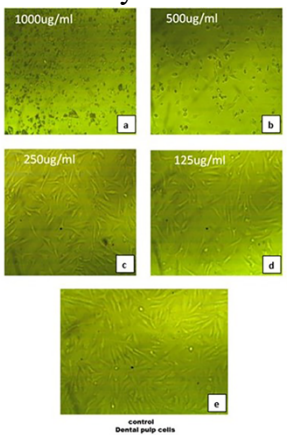

Figure 2: The results of the MTT assay after culture of dental pulp stem cells on CBN powder with different concentrations (a-d) after 3, 7, and 10 days, compared to the untreated control group(e).

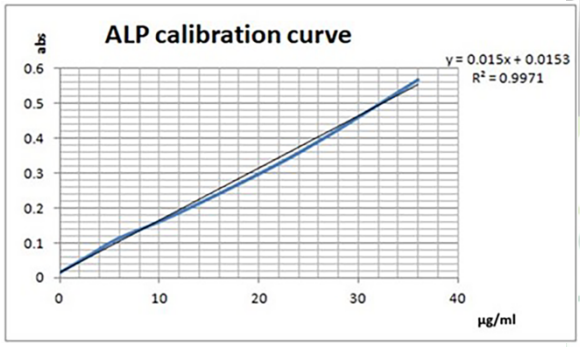

Figure 3: ALP assay: linear curve of the enhanced absorbance to activity of ALP.

3.Transmission, Scanning electron microscopes TEM, SEM and Energy Dispersive X ray (EDX):

TEM micrographs of the CBN (Fig. 4 (A, B, C, D) revealed relatively regular spherical particles with fairly uniform sizes, with an average particle size of 25±5 nm. CBN sizes were calculated as 20.19nm, 20.70nm, 22.29nm, 23.67nm, 24.04nm, 29.54nm and 30.91nm derived from the TEM micrographs using Image software (Fig. D). Moreover, some-scattered and others clustered together were clear as seen in the Fig C. SEM micrograph (Fig. 4 (E, F)) revealed varying surface morphologies and diverse forms of 'rod shaped' and 'spherical' patterns. Most of the crystals agglomerated and clumped. EDX analysis was performed to determine the elemental composition of CBN. Briefly, essential elements (O_2, Ca, and C) were observed as shown in (Table 3 and Fig 4G) by weight percentage: oxygen, calcium, carbon (58.26, 21.93 and 16.21 respectively), besides, some trace percentages of chloride and sodium.

Table 1: Results of the tested CBN samples at different concentrations for ALP activity.

Absorbance	Concentration µg/ml
0.015	0
0.065	3
0.114	6
0.186	12
0.357	24
0.566	36

Table 2: Expression of levels of ALP activity in osteoblast cell lines treated with different concentrations of CBN after 1,3,7 and 14 days of culture.

Day	Absorbance	µg/ml	Original concentration
Day 1	0.016	0.046667	0.233333
	0.016	0.046667	0.233333
	0.019	0.246667	1.233333
Day 3	0.018	0.18	0.9
	0.019	0.246667	1.233333
	0.018	0.18	0.9
Day 7	0.024	0.58	2.9
	0.032	1.113333	5.566667
	0.029	0.913333	4.566667
Day 14	0.037	1.446667	7.233333
	0.044	1.913333	9.566667
	0.032	1.113333	5.566667

Table 3: Semi quantitative chemical composition of CBN

Element	Weight %	Atomic %	Error %
CK	16.21	23.75	9.84
OK	58.26	64.09	10.89
Nak	2.75	2.11	20.1
ClK	0.85	0.42	15.42
CaK	21.93	9.63	1.82

4.X-Ray diffraction (XRD)

To determine the main crystalline phases in the bones, XRD data was analysed using Match software and the COD Inorganic 2011.06.14 database. Fig. 5 shows the XRD patterns for the CBN. $CaCO_3$ are detected by

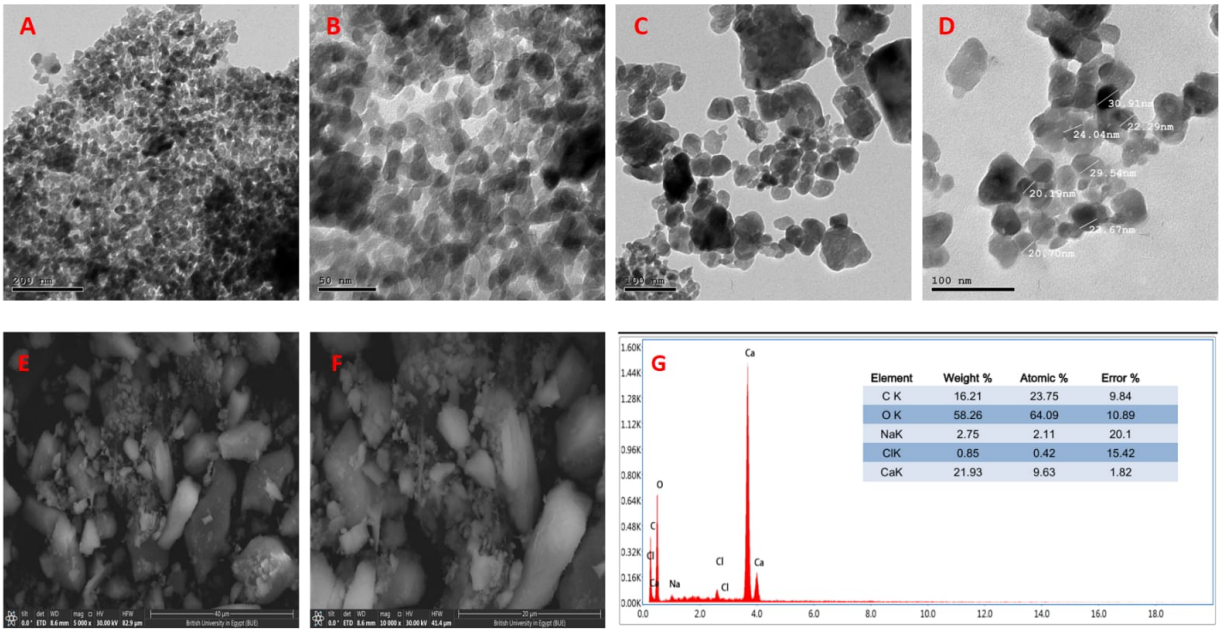

Figure 4: TEM images of prepared CBN crystal at different magnifications (A, B, C, and D) spherical regular appearance with average size 25±5 nm of CBN crystals was observed. SEM images of CBN (E, F) showing variable shapes of crystals, and corresponding EDX analysis (G).

the main peak at 2θ =25. This revealed that aragonite $CaCO_3$ in CB retains its crystallinity throughout the procedure.

To determine the main crystalline phases in the bones, XRD data was analysed using Match software and the COD Inorganic 2011.06.14 database. Fig. 5 shows the XRD patterns for the CBN. CaCO3 are detected by the main peak at 2θ =25. This revealed that aragonite CaCO3 in CB retains its crystallinity throughout the procedure.

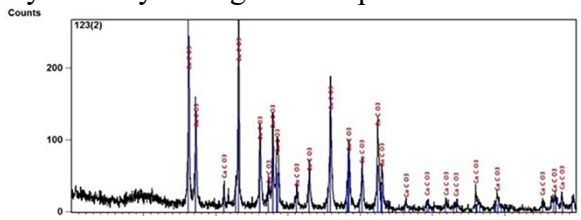

Figure 5: X-ray diffraction pattern of powdered CBN crystalline peaks corresponding to aragonite

CBN In vivo study results

Light microscopic results of CBN on OVX rats' alveolar bone:

Light microscopic pictures confirmed the previous results by showing significant improvement in the OVX+CBN group (Fig. 6c & 6f) compared to the OVX group (Fig. 6b & 6e) with much resemblance with the control group (Fig. 6a & 6d). Both control and OVX+CBN groups showed thick bone trabeculae with regular osteocytes embedded within. OVX+CBN specifically revealed clear reversal lines interpreting new bone formation (black arrows). However, the OVX group showed thinning and destruction of the bone trabeculae (yellow arrows) with widening in the marrow spaces. (Fig.6)

Discussion

Several invention methods have been conducted to synthesise aragonite nanoparticles with appropriate sizes and shapes utilising bottom-up techniques. Thus, the ball milling top-down technique was used in this study as it has been proven to be an efficient method to produce biogenic nanoparticles from biominerals. [20]

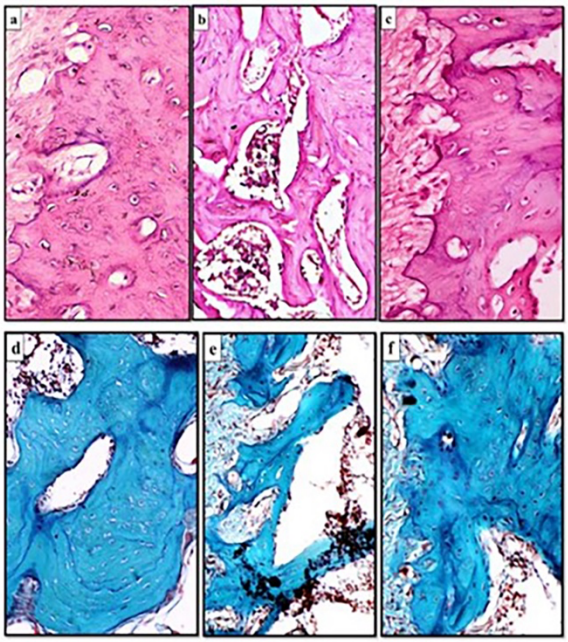

Figure 6: LM of showing (a-c H&E); control group with normal alveolar bone histology, OVX group revealing resorbed alveolar bone, OVX+CBN group showing relative regaining of normal histological feature of alveolar bone. (d-f Trichrome); control group showing normal thickness of bone trabeculae with regular osteocytes in lacunae, OVX group showing thinning in bone trabeculae with widening in marrow spaces, OVX+CBN showing relative normal thickness and histology of bone trabecular with regular marrow spaces.

The CBN has been chosen in the present study for the exceptional physicochemical properties which was established in the current study by grinding. This was in accordance with Ahmadi R, et al which demonstrated that, grinding of the initial powder for 2 hours yielded nanoparticles diameter 44 nm. Additionally, the increase in the grinding time is directly proportional with reducing the size of the nanoparticles thus, CB may be employed in industry to produce biocompatible nanoparticles. [21] The lamellar matrix of CB used in the present study consists of aragonite encircled in a layer of organic material formed mainly of β-chitin. This has been proven previously by a study done to examine the characterization of CB and other marine organisms using XRD spectra and the corresponding quantitative phase analysis and showed CB are composed of 100% aragonite. [22]

In this study, CBN showed no cytotoxic effects on cells at concentrations between 31.25 µg/ml and 250 µg/ml. Toxicity increased dramatically, with cell mortality rates reaching 59% and 89% at concentrations of 500 µg/ml and 1000 µg/ml, respectively. These results align with earlier studies on the MC3T3-E1 osteoblast cell line, which demonstrated that cell viability decreased from 107.52% ± 11.03% to 92.48% ± 5.60% when CB powder concentrations increased from 0.5 µg/ml to 200 µg/ml [23]. Additionally, Irianto K A and colleagues confirmed the high viability of human mesenchymal stem cells when exposed to CB, thereby underscoring its potential safety for human applications. [24]

Although some heavy metals in small amounts have been speculated to be found in CB, as mercury, copper, zinc, lead, and cadmium, they showed no cytotoxic effect on cell culture of human mesenchymal stem cells. Moreover, CB allowed the growth and attachment of these cells and maintained their regular fibroblast-like shape. [25] Heavy metals level in cuttlefish mantle were less than the maximum allowable levels determined by various authorities and organization and thus they represent no risk. [26] Moreover, $CaCO_3$ nanoparticles from cockle shell have been investigated earlier, and rats were used to test the safety of subcutaneous dosages of these biogenic nanoparticles. Doses of 59 mg/m^2 and 590 mg/m^2 were considered safe as they led to minimal lesions and no fatalities were observed. However, at elevated doses of 5900 mg/m^2 and 29,500 mg/m^2, multiple clinical symptoms and histopathological changes were noted. [27] Additionally, another investigation demonstrated that repeated intravenous doses of Dox-loaded $CSCaCO_3$NP up to a cumulative 150 mg/m^2 in dogs did not produce adverse effects, indicating a significant safety threshold at 30

mg/m^2. [28] Further studies have shown that the use of CB as a filler in acrylic bone cements can promote osseointegration without inducing secondary infections when applied in vivo in rabbits. [29,30] Moreover, existing research suggests that CS$CaCO_3$NP may enhance cellular differentiation, thereby promoting osteoblast cell proliferation. [31]

Alkaline phosphatase is an essential marker for osteoblast differentiation and osteogenesis. Previous study has shown that ALP levels rise when human mesenchymal stem cells are cultured on dorsal shield and lamellar blocks, suggesting that CB could potentially serve as a bone substitute and a scaffold for osteoblast differentiation. [26] All these previous results are in accordance with present study results that showed regular increase in the ALP calibration curve, indicating increase in alkaline phosphatase level release from an osteoblast cell line starting from day1, 3, 7 and 14.

The SEM and TEM results of the present study showed variable patterns of the CB crystals varying between spherical and rod shaped. Variations in the surface morphology of the CB is attributed to several factors as health, environment, and growth of parts of the CB during different seasons. [32] According to the present result, the EDX analysis of CB revealed high percentages of oxygen, calcium, and carbon (58.26%, 21.93% and 16.21% by weight respectively) accompanied by small percentages of sodium and chloride. These results align with the findings of Hemmatti et al., who reported that CB, being a natural substance rich in CaCO3, functions as a marine-derived antipyretic agent. [33] The high calcium content in CB observed in the EDX results of the present study agree with prior study that showed that the calcium content of CB is high enough to be utilised in foodstuffs and as calcium precursors in the formation of hydroxyapatite with excellent biocompatibility, bioactivity, and osteoconductive qualities. [34]

XRD results of the current study further confirmed the previous characterization test results where it showed high percentage of $CaCO_3$ as the main component of the CBN. This result agrees with a previous study that assured that the XRD pattern of CB powder exactly matches with the aragonite crystal structure of calcium carbonate. [5]

The Food and Drug Administration (FDA) has approved the ovariectomized (OVX) rat model as a preclinical model for investigating postmenopausal osteoporosis as a result of the ovaries' decreased production of endogenous oestrogen during menopause and the potential for interventions to maintain bone metabolism in this condition. [35] Sixty days duration after ovariectomy was in accordance with a prior study that showed that this duration caused noticeable detrimental effects on rats' alveolar bone. The volume and number of trabeculae in the bone of the OVX group were markedly reduced compared to the baseline and Sham groups. In the OVX group, numerous osteoclasts could be seen along the degraded, uneven surface that faced the bone marrow. These results agree with our light microscopic results that revealed thin, disorganized alveolar bone trabeculae with multiple osteoclasts, accompanied with discontinuous osteoblasts lining bone surface. [36] On the other hand, OVX + CBN group revealed significant improvement regarding the increase in thickness of bone trabeculae interpreted by increasing in the bone regenerative potential. This result is in accordance with a previous result that showed that rats subjected to the administration of cuttlefish bone powder showed significant increase in serum calcium and phosphorus and this could be attributed to an expanded osteoblastic movement, consequently improving bone development.[37] The increase in the bone formation and healing process due to

cuttlebone extract application has been confirmed previously in a study that measured osteoblasts formation by histochemical examination using Hematoxylin and eosin staining and proved that the higher production of osteoblasts revealed their higher proliferative activity. [38]

Relating all the characterization tests for CBN especially the cytotoxicity and ALP activity can act as a fundamental interpretation basic for the histological findings of the experimental study. These results are in accordance with a recent study that focused on the cuttlebone great effectiveness and suitability as a bone graft for human bone tissue engineering. Where it showed that cuttlebone can be used directly on osseous defect areas due to its chemical, biocompatibility, and crystalline properties. The later study further mentioned cuttlebone's ability to be used as xenograft for bone defects treatment in male rabbits. [39]

Conclusion

Due to the unique characteristic features of the cuttlebone fish, it has been considered as an important biomaterial in various medical field. It has shown great biocompatibility and effective osteogenic properties thus can be an effective agent in preventing osteoporotic changes especially in alveolar bone. Cuttlebone fish nanoparticles are a promising natural, affordable, and totally safe and nontoxic preventive measure for inhibiting the risk of alveolar bone resorption associated with osteoporosis.

List of abbreviations

Scanning electronic microscopy: SEM
Fourier transform infrared: FTIR
Cuttlebone nanoparticles: CBN
Soluble organic matrix: SOM
Calcium carbonate nanoparticles:$CaCO_3$NP
Alkaline phosphatase: ALP
Mesenchymal stem cells: MSCs
Vascular endothelial growth factor: VEGF
Transforming growth factor β: TGFβ
Hydroxyapatite: HA
Transmission electron microscopy: TEM
Energy Dispersive X-ray analysis: EDX
X-ray diffraction: XRD
Ovariectomized: OVX

Declarations

Ethics approval and Consent to Participate:

The current study has received approval from the Research Ethics Committee (REC) of the Faculty of Dentistry at Suez Canal University, established in accordance with the 'WHO-2011' standards, under serial number 537/2022. Consent to participate is not required.

ARRIVE guidelines:

The ARRIVE guidelines for the documentation of in vivo studies in animal research were followed in the conduction of this work.

Availability of data and materials:

All data generated and analysed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Huda Ahmed Amin El Gendi and Nihal Tawfik El Kazzaz. The first draft of the manuscript was written by Nihal Tawfik El Kazzaz and Amel M Ezzat Abdel Hamid

confirmed the authenticity of all the raw data and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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