

# Valorization of Bone Waste of Saudi Arabia by Synthesizing Hydroxyapatite

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**Abstract** At present, hydroxyapatite is being frequently used for diverse biomedical applications as it possesses excellent biocompatibility, osteoconductivity, and non-immunogenic characteristics. The aim of the present work was to recycle bone waste for synthesis of hydroxyapatite nanoparticles to be used as bone extracellular matrix. For this reason, we for the first time utilized bio-waste of cow bones of Albaha city. The residual bones were utilized for the extraction of natural bone precursor hydroxyapatite. A facile scientific technique has been used to synthesize hydroxyapatite nanoparticles through calcinations of wasted cow bones without further supplementation of chemicals/compounds. The obtained hydroxyapatite powder was ascertained using physicochemical techniques such as XRD, SEM, FTIR, and EDX. These analyses clearly show that hydroxyapatite from native cow bone wastes is biologically and physicochemically comparable to standard hydroxyapatite, commonly used for biomedical functions. The cell viability and proliferation over the prepared hydroxyapatite was confirmed with CCK-8 colorimetric assay. The morphology of the cells growing over the nano-hydroxyapatite shows that natural hydroxyapatite promotes cellular attachment and proliferation. Hence, the as-prepared nano-hydroxyapatite can be considered as cost-effective source of bone precursor hydroxyapatite for bone tissue engineering. Taking into account the projected demand for reliable bone implants, the present research work suggested using environment friendly methods to convert waste of Albaha city into nano-hydroxyapatite scaffolds. Therefore, besides being an initial step towards accomplishment of projected demands of bone implants in Saudi Arabia, our study will also help in reducing the environmental burden by recycling of bone wastes of Albaha city.

**Keywords** Implants · Hydroxyapatite · Native cow · Recycling · Bones

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## Introduction

Tissue engineering scaffolds which contribute as an extracellular matrix (ECM) to interact to living cells seem to be an excellent platform for regeneration and fixation of bone defects. It has been widely accepted that chemical composition and physical structural properties of scaffolds are matter of great concerns in fabricating ultimate scaffolds for tissue regeneration [1]. Hydroxyapatite [HAP,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ] has been recognized as the vital constituent of inorganic composition of bone in humans. HAP has attracted attention to a great extent due to its biocompatibility and its status as the main inorganic component in teeth and bones [2]. Werner in 1786 initially introduced the term apatite [3]. HAP is a classic biomineral that is plentiful in higher organisms. For this reason, it can be used as bone scaffolds [3] and luminescence materials [4]. Besides, it also possesses loads of essential applications in drug delivery [5, 6] and biomedical engineering [7] owing to its chemical and biological likeness with the mineral composition of human bones and teeth [2, 8, 9].

Inspired by intrinsic properties of HAP, the present research proposes to make use of unexplored waste of discarded bones of native cows of Albaha city. The bone waste has been considered for the extraction of natural bone precursor HAP. Up to now, a variety of HAP derivatives, for example, carbonated HA [10–12], strontium HA [13, 14], F-substituted HA [15], and HA-based nanocomposites [16] have been reported. Previous workers have utilized a number of approaches such as emulsion [17], hydrothermal [18] and solvothermal technique [19], sol–gel [20], biomimetic process [21], and microwave irradiation [22] for synthesis of HAP and its derivatives. In addition to the aforementioned techniques, recently, microbial method [23] has been utilized for synthesis of nano-hydroxyapatite. However, in the present study, we utilized a simple hydrothermal approach for synthesis of biologically preferable HAP. Extraction of HAP from Albaha bone wastes is biologically safe as no synthetic chemicals are required for the synthesis. Moreover, this is an economically desirable process as herein, the waste material is recycled to meet projected demand of HAP bio-ceramics which has enormously been increased in recent years throughout the world [24]. The natural HAP possesses a great potential for preparation of bone scaffolds. Given to understand the importance and need of efficient and cheap bone implants, the main aim of this research was to design a cost-effective strategy for the synthesis of biomimetic nanotextured HAP using cow bone wastes collected from Albaha city. HAP was extracted biochemically from disposed bones and their potential for cultivation of myoblasts and biomedical applications was studied.

## General Experimental Procedures

### Extraction of Bone Precursor HAP from Wasted Cow Bones of Albaha

For the preparation of natural HAP nanoparticles from waste bones of native Albaha cow, hydrothermal approach was adopted. To sum up the practical procedure, the discarded cow bones were collected from the Albaha city of Saudi Arabia and have been cut down into small pieces. These bone pieces were initially cleaned with water and then carefully washed with acetone to remove fats, connective tissue, and other contaminants. After removal of adulterants, the bones were again washed with distilled water 2–3 times. Then, the bones were dried at 160 °C for 2 days. The cleaned, dried bones were crushed into small particles which were then powdered by ball milling to further decrease the particle size.

The natural HAP was extracted from the cleaned bones following Barakat et al.'s procedure [24, 25]. All the chemicals were of analytical grade and used as received without further purification. All experiments were conducted under ambient conditions. In a typical process, the cleaned bones were kept in an open alumina crucible and heated in the furnace. The treated bones were subjected to heat treatment, (calcined at 850 °C at heating rate of 10 °C/min) for 2 h to produce natural HAP ceramics. At the end, the obtained ceramics were powdered to get particle size dimensions.

### Physicochemical Categorization

In order to determine the crystalline phase of waste-oriented HAP ceramics, X-ray diffraction analysis was carried out. The XRD analysis of as-synthesized cow bone HAP was recorded on a Rigaku/Max-3A X-ray diffractometer (XRD, Rigaku, Japan) with CuK radiation ( $\lambda = 1.5418 \text{ \AA}$ ) and operational voltage of 30 kV, and the current for analysis was kept at 40 mA. Fourier transform infrared (FTIR) spectrum was recorded as KBr pellets using Varian FTS 1000 FTIR, Mid-IR spectral range, cooled DTGS detector, Scimitar series, Varian Inc. The spectrum was analyzed by using Varian Resolution Pro version 4.0.5 from Varian. FTIR analysis of HAP powder from Albaha cow bone residues was carried out in the range of 4000–400  $\text{cm}^{-1}$  to detect the presence of various functional groups. The morphology of the natural HAP nanoparticles was determined by scanning electron microscopy. In order to check the microstructure, the natural HAP powder was homogeneously scattered on carbon tape and Pt coating was made for 10 s onto the as-synthesized HAP and SEM has been taken (SEM, JEOL JSM6700, Japan) at different resolutions.

### In Vitro Cytobiocompatibility Assay

The HAP nanoparticles derived from the natural bone residues have been decontaminated with UV radiation for 30 min in a biosafety chamber. Skeletal muscle precursor cells (ATCC-CRL 1772) purchased from American Type Culture Collection were cultivated on the HAP nanoparticles. The HAP nanoparticles were placed in the bottom of a 6-well tissue culture plates after UV exposure. The HAP nanoparticles were washed with autoclaved distilled water and muscle precursor cells were inoculated (concentration— $1 \times 10^6$  cells/well) with the help of a micropipette into the wells. The cells were propagated in Dulbecco's modified eagle medium (DMEM, pH 7.4) in which 10% fetal bovine serum and 1% penicillin–streptomycin were added to control microbial contamination. The culture plates were then held in a humidified incubator with 5%  $\text{CO}_2$  and 95% air. The incubation temperature was set at 37 °C. The exhausted culture medium was revived at days 3 and 5 respectively. Cell counting kit-8 (CCK-8) assay was carried out after 3 and 5 days to evaluate the viability of culture under in vitro conditions. In this study, CCK-8 assay was performed to evaluate cell viability and mitochondrial activity of skeletal muscle precursor cells seeded over the HAP. Ten microliters of WST-8 solution was added in each well and incubated for 4 h at 37 °C according to the standard protocol procured from company. At the end of experiment, absorbance was calculated at 450 nm by a microplate spectrophotometer (Bio-Rad: 680, Japan). Three parallel experiments were carried out for each sample and the average reading has been taken into consideration. In order to check the morphology of cells, the cells were grown as described in the earlier section. The unattached cells were washed away with PBS, and attached cells were fixed in 4%

formaldehyde in PBS. Finally, the cells were analyzed using a microscope and photos were taken by a preset camera.

## Results and Discussion

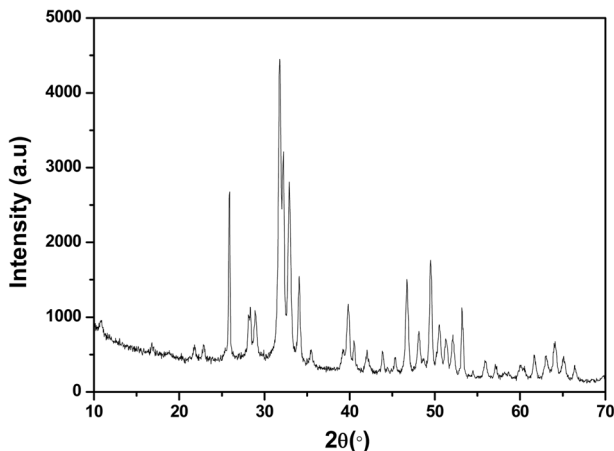
In the present study, the preparation of natural HAP nanoparticles was done following previous techniques with modifications [25]. Briefly, the leftover bones of Albaha cows were used for extraction of vital natural bone precursor HAP. The main organic compound present in the natural bone was collagen (95%). Additionally, there are other organic constituents such as hyaluronic acid, **chondroitin sulfate**, keratin sulfate, and lipids (e.g., phospholipids, triglycerides, fatty acids, cholesterol) which are present in small quantities [24]. Figure 1 demonstrated the stepwise methodology pursued in this study for recycling of bone wastes for the extraction of natural HAP.

### Characterization

The X-ray diffraction analysis of the natural-extracted HAP powder was carried out which consisted of a well-crystalline phase with a hexagonal structure (JCPDS no.-86-0740). Complete crystallization of the bone HAP powder has been confirmed due to sharp peak intensity and well-resolved peaks in XRD patterns of the samples obtained at high calcination temperature. The crystalline peaks of the bone waste originated HAP nanoparticles at  $2\theta = 25.7, 31.7, 32.9, 34.1, 39.7, 46.7,$  and  $49.6$  are evident in Fig. 2, thus confirming the presence of the HAP structure. The morphological characterizations of the natural HAP particles have been carried out by SEM taken at low and high magnifications. The micrographs of synthesized HAP particles depicted a porous morphology (Fig. 3) and the natural HAP particles were found



**Fig. 1** Stepwise methodology for this study



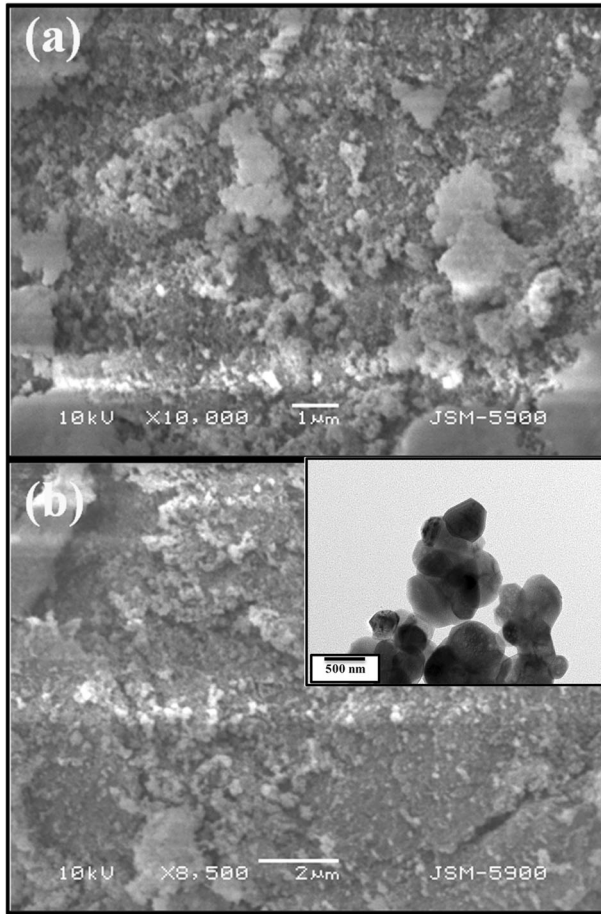
**Fig. 2** Crystalline peaks of the bone waste HAP nanoparticles

agglomerated. The size of pure HAP particles was estimated to be between 300 and 500 nm which has been further confirmed by TEM (Fig. 3a, b and inset TEM) analysis. EDX analysis confirmed the presence of Ca and P in the Albaha cow bone samples (Fig. 4), and besides, oxygen has also been detected. The molecular structure of the HAP sample was characterized by FTIR (Fig. 5). The  $\text{PO}_4^{3-}$  asymmetric stretching mode of vibration has been characterized by a broad and strong band in the range of  $1040\text{ cm}^{-1}$  and a sharp intensity peak at  $960\text{ cm}^{-1}$  that results from the symmetric stretching vibrations [26–30]. The sharp peaks between  $450$  and  $610\text{ cm}^{-1}$  confirm the presence of P–O stretching vibration [26–28]. The crystalline HAP also generates characteristic OH bands at about  $2930\text{ cm}^{-1}$ . Meanwhile, small peaks at  $1650\text{ cm}^{-1}$  indicated the existence of a Ca–O phase. On the basis of FTIR, the formation of bone precursor HAP from the bone wastes of Albaha was established.

### Investigation of Cellular Biocompatibility and Morphology over Natural HAP Nanoparticles

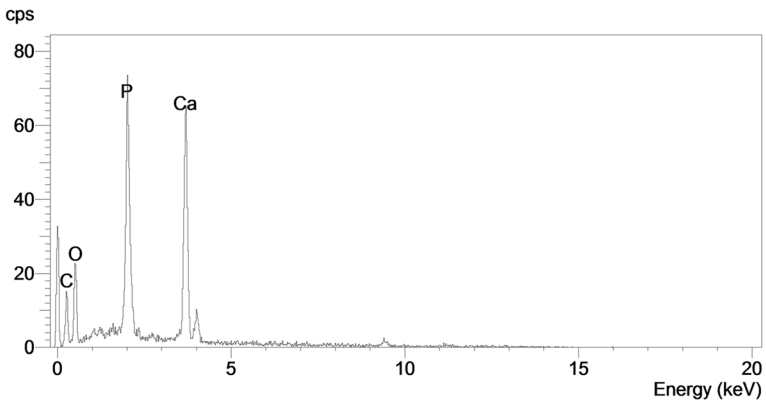
Cytobiocompatibility assay of the natural HAP nanoparticles (derived from waste cow bones of Albaha city) was performed on muscle precursor cells which have been regularly maintained in cell culture division of our laboratory. CCK-8 assay is a fine marker for evaluation of cell viability and proliferation. The test results are shown in Fig. 6a. This study (Fig. 6a) shows enhanced cellular viability on the fifth day in wells having HAP nanoparticles on bottom. Figure 6b shows the microscopic image of control cells with media attached in one of the 6-well culture plates. Similarly, Fig. 6c, d illustrates the growth of cells in wells having natural HAP. Furthermore, the microscopy analysis shows uniform distribution of cells over the HAP nanoparticles at day 3 (Fig. 6c) and day 5 (Fig. 6d) respectively. The prominent growth of cells over the natural HAP nanoparticles prepared from bone waste validated its prospective uses in biomedical fields.

In recent times, the HAP has been extensively utilized as coatings in orthopedic and dental implants and as novel fillers for the amplification of functions of dental adhesives. HAP is a bone precursor and is commonly employed in medical fields owing to its excellent biocompatibility and osteoconductive and non-inflammatory behavior [24]. Additionally, some researchers have utilized hydroxyapatite column and SDS-page for analysis of differentially expressed proteins in colorectal

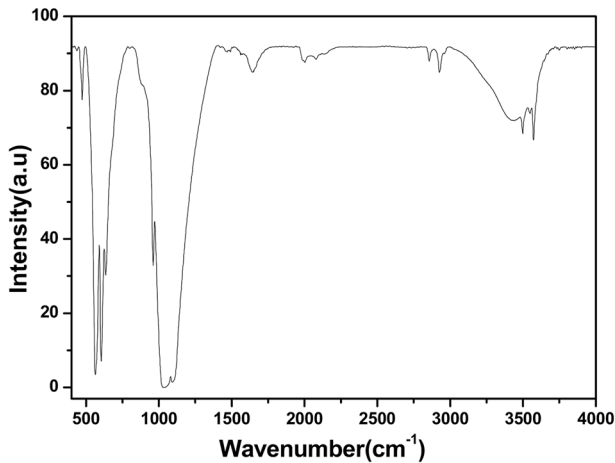


**Fig. 3** TEM image of the size of pure HAP particles and its porous morphology

cancer [31]. Bearing in mind the global-projected demand of bone implants and in particular in Saudi Arabia, there is great need of inexpensive source of bone precursor HAP. Consequently, our present

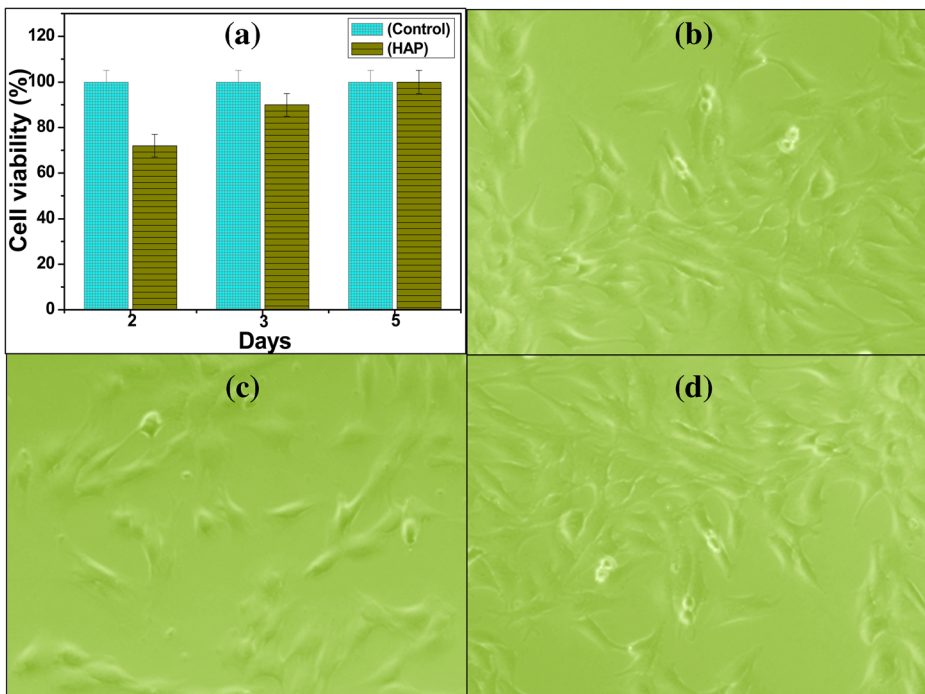


**Fig. 4** EDX analysis of the Albaha cow bone samples

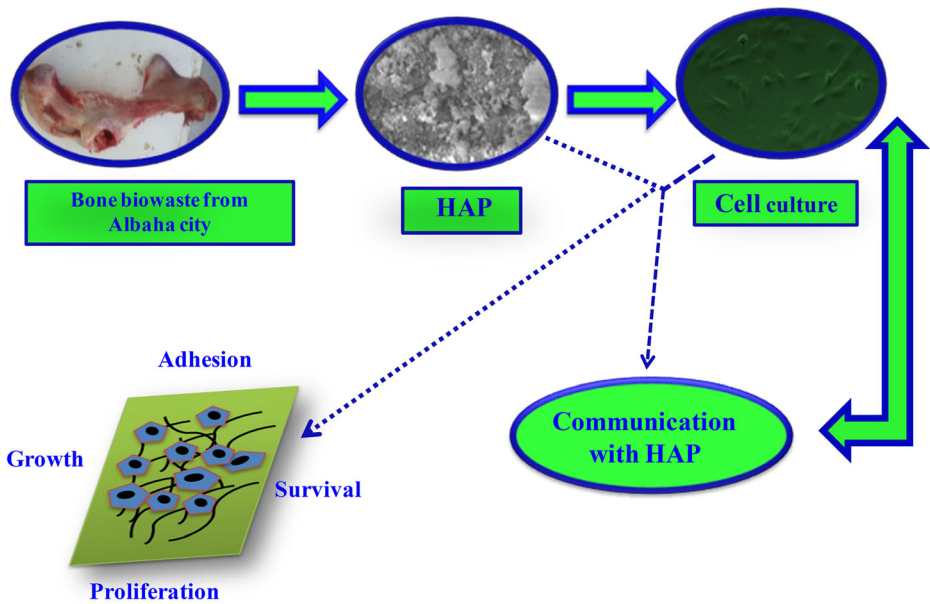


**Fig. 5** FTIR analysis of the molecular structure of the HAP sample

research recommends making use of the novel, cost-effective, and environment friendly method to convert the bone waste of Albaha city into nano-HAP. Earlier workers have adopted emulsification cross-linking approach [32] for in vitro biomineralization of chitosan–hydroxyapatite composite microparticles with the aim of orthopedic use. The whole experimental work has been demonstrated in Scheme 1. Besides other significant parameters, it has also been accounted now that the nature and accessibility of bio-wastes are essential criteria for commercial production of natural HAP. Various



**Fig. 6** Cytotoxicity assay test results of the natural HAP nanoparticles



**Scheme 1** Schematic illustration of plausible interaction of muscle precursor cells with natural bone precursor HAP

scientific research studies have shown production of useful products by utilization of different food wastes. Nevertheless, previous studies have shown the two-phase methanization of food wastes in a pilot scale; approximately 2.9 t of food organic was treated in order to produce about 230 m<sup>3</sup> of biogas with 70% (v/v) of methane and 80 kg of humus [33]. In other studies, mesophilic acidogenesis of food waste has been utilized for the recycling wastewater [34].

## Conclusion

To sum up, in our present work, HAP powder with nano-size has been prepared from waste cow bones by facile extraction and heat treatment method. The obtained HAP powder was characterized by various techniques. The synthesized HAP is cytobiocompatible. Generally sophisticated methods and expensive precursor are used for the preparation of bone implants. Herein, we report synthesis of biocompatible HAP from bones waste. Therefore, the prepared HAP can be regarded as valuable resource of hydroxyapatite for bone implants. Bone weakness hampers daily activities and results in poor quality of life. The treatments are very costly. In this work, we have used a very simple extraction method for isolation of natural HAP from native cow bones for the production of HAP-based implants in the Kingdom of Saudi Arabia.

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## Compliance with Ethical Standards

**Conflict of Interest** The author declares that he has no conflict of interest.



**Ethical Statement** In the present study, there is no use of experimental animals. All the experiments were done under in vitro conditions following standard scientific procedures and ethics.

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