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SYNTHESIS AND CHARACTERIZATION OF HYDROXYAPATITE NANOPARTICLES FROM BOVINE BONE BY THERMAL HEAT TREATMENT AND EFFECTS ON MACROPHAGES AND TNFA IN MICE

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Abstract:
Background: Due to the fact that its structure and function comparable to those of reel human bone hydroxyapatite (HA) is regarded as an essential bio-ceramic component that generated a wide range of applications in the field of biomaterials. Consequently, the primary purpose of this study to produce a pure nanoparticles powder of hydroxyapatite through calcination process while the bovine bone is subjected to heat treatment at 800℃.

Methods: X-ray diffraction (XRD), energy dispersive spectrometry (EDS), field emission scanning electron microscopy (FE-SEM), and Fourier transform infrared spectroscopy (FTIR) were the techniques that were used in order to examine the samples that were recovered.
Results: The findings demonstrated that the analyzed HA had a high level of purity, with Ca/P ratio= 1.7. This ratio is very close to the stoichiometric value of 1.67 of that are seen in normal human bone. In addition, to having a tiny particle size in the range of (140-200) nm, the HA that was produced had a number of morphologies including sphere and polygonal. In addition, the chemical structure of the synthesized HA is similar to the chemical structure of human bone. Furthermore, it demonstrated a high level of purity and crystallinity with thermal colloidal stability. The resulted white powder represented pure hydroxyapatite nanoparticles and did not include any other organic materials of the bone. On the other hand, it is worth noting that the activation of NF-kappa B, as well as, particle and pore size are inversely associated with the capacity of hydroxyapatite crystals to stimulate the production of TNFα by morphologies. It is worth noting that crystals with a size 3-5 micron are the most bioactive. Because of this, the plaque may be more susceptible to inflammation as a result tiny calcific deposits in early stages of atherosclerosis as opposed to deposits that are visible under microscope or by radiological examination in later stages of several lesions. When HA was administrated to obese mice, it

has a substantial impact on the production of pro-inflammatory cytokines, normally TNF-α in macrophages cells and serum.

Conclusion: In conclusion, the goal of this work is to synthesize pure hydroxyapatite nanoparticles and use them anti-inflammatory effects to treat macrophage cells and mouse serum for Nonetheless, the results showed that using a spectrophotometer set at 480 nm, has decreased concentration of TNFα in mouse serum and macrophage cells had decreased

Keywords: hydroxyapatite, biomaterial, macrophages, calcination process, TNFα, Obese mice.

Introduction

One of the major bioceramic biomaterials that have been utilized in biomedical research is Hydroxyapatite (HA). Since it is considered the main inorganic mineral component of vertebrate skeleton system and teeth [1]. Therefore there are great research exertions have been focused on the synthesis of hydroxyapatite to be used predominantly in orthopedics and dentistry applications [2], However, it was found that the composition can be changed due to the potential of anionic and cationic changes and the presence of diverse kinds of ion vacancies [3]. HAp is the most emerging calcium phosphate-based material in bone with a Ca/P molar ratio in the range of 0.5–2. Meanwhile, a 1.67 stoichiometric ratio is pure hydroxyapatite with excellent biocompatible and bioactive properties [4]. However, considering that the ratio of calcium to phosphorus is less than 1.67 and near to 1.5, the stoichiometric ratios for TCP may lead to the formation of a biphasic material known as HAp: β-TCP. Therefore, by changing the proportion either, we can either get biphasic material or pure calcium phosphate [5].

It is possible to generate HAP by co-precipitation pathway, which may be used to synthesis of HA. In this technique, disodium hydrogene phosphate, (calcium bis(2-Ethylhexyl) sulfosuccinate and calcium acetate were used as sources of calcium and Ca PO_4^{-3}. Additionally, hexamethylenetetramine (HMT) was utilized as a buffer in order to control the amount of OH^- ion that was present in the solution during the reaction. Considering that the generation of HAP is dependent on the acidity of the solution, which should be between 3 to 12 [6,7]. In addition, other properties that HA exhibited is osteoconductive, since it can simply provide porous structure with size and interconnectivity that permit bone ingrowth [8], Thus, it has been used as a bioactive coating in cementless implants to improve their osteointegration and increase osteoblast growth [8,9]. Moreover, the porous conformation of HA promotes to be used as a drug delivery system by forming a suspension and in situ polymerization for the sustained release of numerous drugs for example anti-inflammatory and antibiotics drugs [10,11]. Another usage of HA nanoparticles as smart delivery of antitumor agents is by offering high selectivity to cancerous cells through targeting proficiency and selectivity of chemotherapeutic drugs [11]. Additionally, HA is water insoluble with the biodegradable feature but with a very slow rate of degradation [12]. However, the synthetic HA showed a weakness in its mechanical

properties that led to a limit in its usage in low-load bearing applications, for instance in tooth root substitutes, contour and malformation defects, and nonunion of long bones [9]. Hydroxyapatite either it is derived from natural or synthetic source played a majored parts in bone graft. Since it identified as bioactive element resulted from the initiation of robust bond with living bone tissue. Besides, HA shows feature related to be not only bioactive but also osteoconductive, non-toxic and non-immunogenic [11]. Also, in restorative dentistry, nano-hydroxyapatite has been used to enhance material features such as its mechanical properties and provide high binding to dentin [13].

To the top of our knowledge, this would be the principal study to examine the therapeutic potential of the extracted HA composite with large animal sources on inflammatory response [14]. Macrophages have the potential to contribute to the vicious cycle of calcification and inflammation that occurs in the arterial wall. This is accomplished recognizing neointimal calcific deposits, which are mostly composed of hydroxyapatite and secreting tumor necrosis factor TNFα, which is also a vascular calcifying agent [15]. We conducted a study whether particle size influences hydroxyapatite crystals has an effect on their capacity to induce anti-inflammation responses in a group of mice. We also tested whether the nuclear factor NF-B pathway contributes to the TNFα response in macrophages.

Macrophage TNFα exudation was in reverse related to hydroxyapatite particle, as it relates NF-kappaB activation and particle and pore size are inversely correlated with the ability of HA crystals to promote macrophage TNFα secretion, with crystals with 3-5 microm being the most bioactive. The plaque may consequently be more at risk for inflammation from microscopic calcific deposits in early atherosclerosis than from macroscopically or radiologically evident deposits in later advanced lesions. HA reduced significantly the release pro-inflammatory cytokines TNF-α in lab [16].

Materials and Methods

Bovine bone preparation and calcination:

Bovine femur bone shaft was afforded from local meat shop. The bovine bone cuts into pieces and boiled in water for two hours to remove the extra meat and other tissues surrounded the bone. After that, the boiled bone washed several times with DI water. Next, the whole bone immersed in bowl of acetone for 1 hours in order to remove fat and other compounds found within the bone, then rinsed again with DI water where it turns bone to yellowish color. Later, drying the bone pieces in furnace under 160℃ temperature for 16 hours that process turns bone into ashes black color. For calcination process, a muffle furnace was used in order to calcined the bovine bone at temperature of 800 ℃ for 4 hours and the increment was kept constant at 10 °C/min for heating and cooling rate until the ashes turn into white color to produce HAp powder and grinded to small particles by using grinder.

Characterizations

The chemical structure of HAp was characterized using FTIR (Shimadzu AIM-8800, wave number 400-4000 cm^{-1}) to measured bond vibration frequencies of the functional group of

hydroxyapatites such as phosphate, hydroxyl, and carbonate groups. EDS analysis was used to detect the elements compositions of the atomic ratio Ca/P of the calcined bone. FE-SEM from ZEISS was used to detect the calcined bone powder surface morphology and topography and to determine particles dimensions. As well, X-ray diffraction (XRD) from Panalytical company- model X'Pert Pro was used to measure constitution and crystallinity of the calcined bone powder. XRD with copper filtered Cu at 40 kV and 40mA. The scan range (2θ) was 0°-80°.

HA particles effect on the synthesis and expression of TNF-α

In order to investigate the impact of various HA particles on the production of TNF-*α*, mice macrophages were subjected to an incubated for 18 h. When cells were not exposed to HA particles, the level of TNF-*α* mRNA expression was very low. Following a peak TNF-*α* mRNA expression at 10 h, which was six times greater than the control, the expression of TNF-*α* mRNA began to decrease at 18 hours. The expression of TNF-*α* was altered by the addition of HA particles, as determined by gene expression. Based on the physical feature of HA particles, it is shown in figure 1 that these particles have the ability to include mice macrophages to generate cytokines [17].

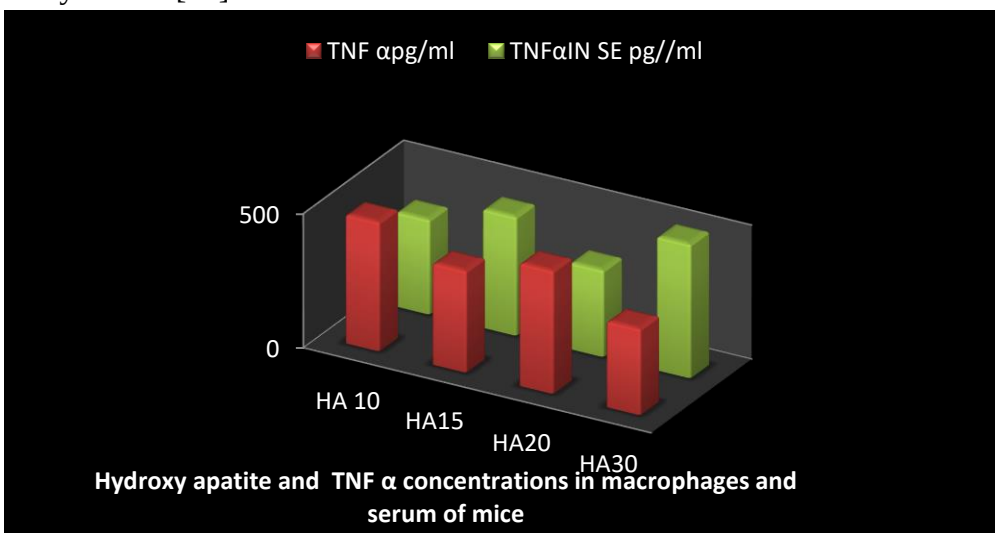

Fig. 1: Hydroxyapatite and TNF α concentrations in macrophages and serum of mice

Results and Discussion

Analysis of sample using FTIR technique

FTIR analysis of the calcined bovine bone powder showed that the presence of different peaks that represent the hydroxyapatite as shown in Figure 1, the hydroxyapatite powder has shown major sharp peaks, located at (1041.49-1095.49), 570.89, 601.75, 632.61 cm^{-1}. The FTIR spectrum showed the primary inorganic components of bone which are the phosphate, carbonate and hydroxyl groups. The large peak at (1041.49) cm^{-1} represent the bending mode of the phosphate group. Also, symmetric stretch mode of the phosphate group (PO^{-3}_4) at 964.34 peak. While the asymmetric stretch mode of (PO_4) shown in the sharp peaks of 570.89, 601.75

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and 1095.49 cm^{-1}. Besides, the peak at 632.61 cm^{-1} is denoted as a result of the vibrational of the hydroxyl group (OH) and stretching vibration of (OH) at 3570 cm^{-1}. Moreover, the carbonate group has been shown at the peak 879.48 cm^{-1}.

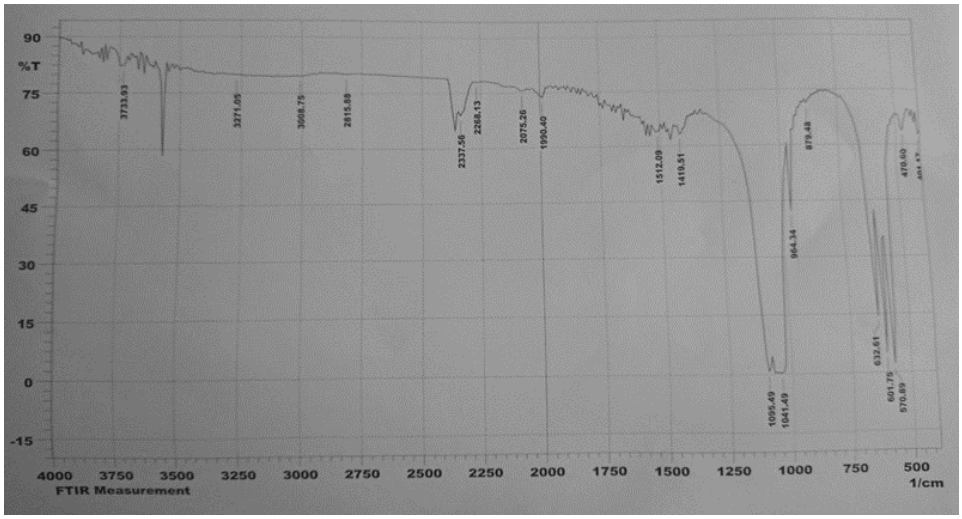

Figure 2: FTIR spectra for calcined bone powder at 800 ℃ calcination temperature

Analysis of sample using EDS technique

As shown in figure 3**,** the result of EDS analysis of the calcined bone at temperature of 800 ℃ to measure the Ca/p ratio was calculated to be 1.7, which is almost close to the stoichiometric value of 1.67. thus, the result indicated that the calcinated bone powder at 800℃ produced a pure hydroxyapatite similar to the natural hydroxyapatite in human bone. Also, the result showed that the major primary constituents are the Ca and P with some minor constituents such as Au. According to Piccirillo and co-authors explained that the presence of these minor constitutes during HAp synthesis could enhance biocompatibility and osteointegration [12].

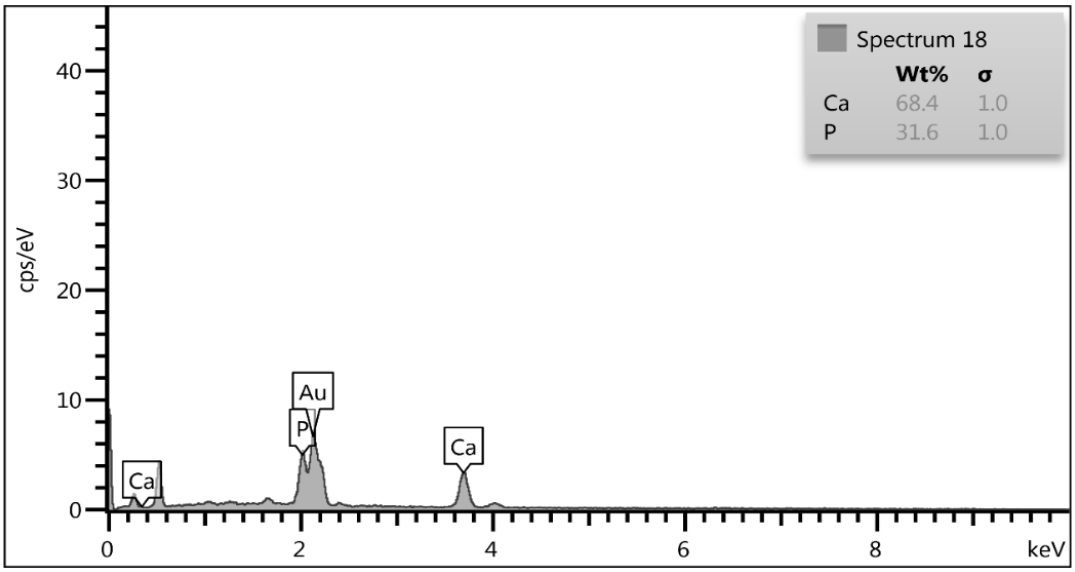

Figure 3: EDS spectrum for calcined bone powder at 800 ℃ calcination temperature

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Analysis of sample using FE-SEM technique

By using the FE-SEM to get the morphology of hydroxyapatite sample and size particles as shown in figure 4. The morphology of hydroxyapatite particles was in the form of agglomerates with irregular shape like spherical and polygonal as shown in Figure (4. a, b) Besides, the size ranged around (140- 200) nm. Thus, from the average sized of these particles showed that the hydroxyapatite prepared was in the dimensions of nanoparticles which could be used as substitute in the biomedical field.

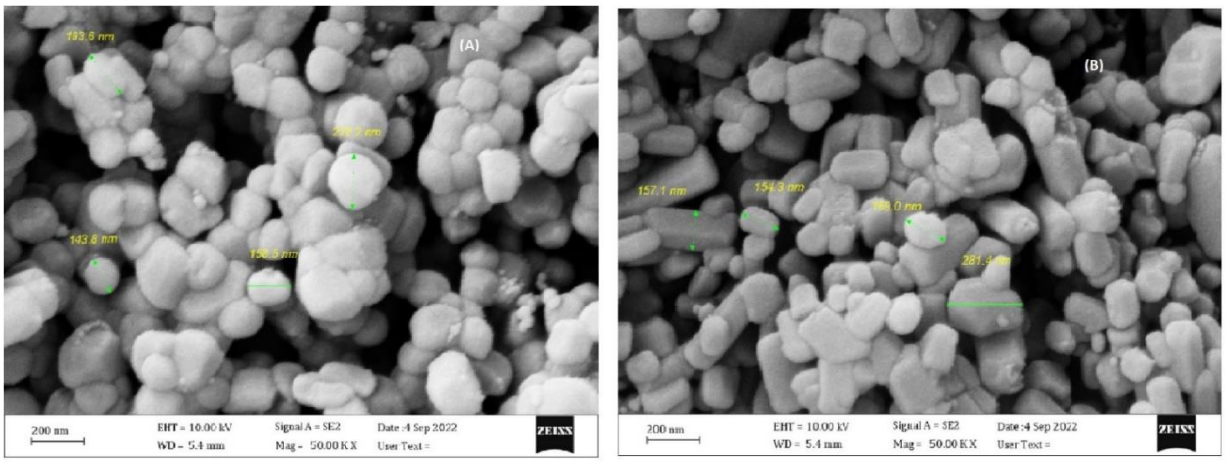

Fig. 4: A and B: FE-SEM images of HAp with scale equal to 200 nm

Analysis of sample using XRD technique

The result of XRD spectrum was compared according to XRD peaks of hydroxyapatite (based on ICDD 9–432) [1,13] as shown in Figure 5. A and the XRD pattern of the prepared HAp of calcined bone powder at 800℃ as shown in Figure 5. B. The result express strong similarities in all peaks as compared to the standard hydroxyapatite which is considered a great assurance of the high purity and HAp nanoparticles crystallinity of the prepared HAp in this study to induced a pure hydroxyapatite powder. Besides, the result of XRD appealed to declare that the heat treatment for 800℃ in the calcination process have not affected the chemical structure of HAp extracted from bovine bone.

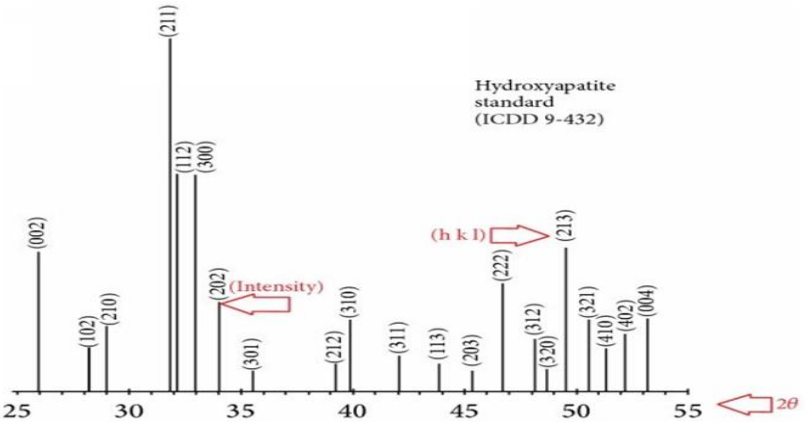

Figure 5-A: XRD spectra of ICDD standard of pure hydroxyapatite

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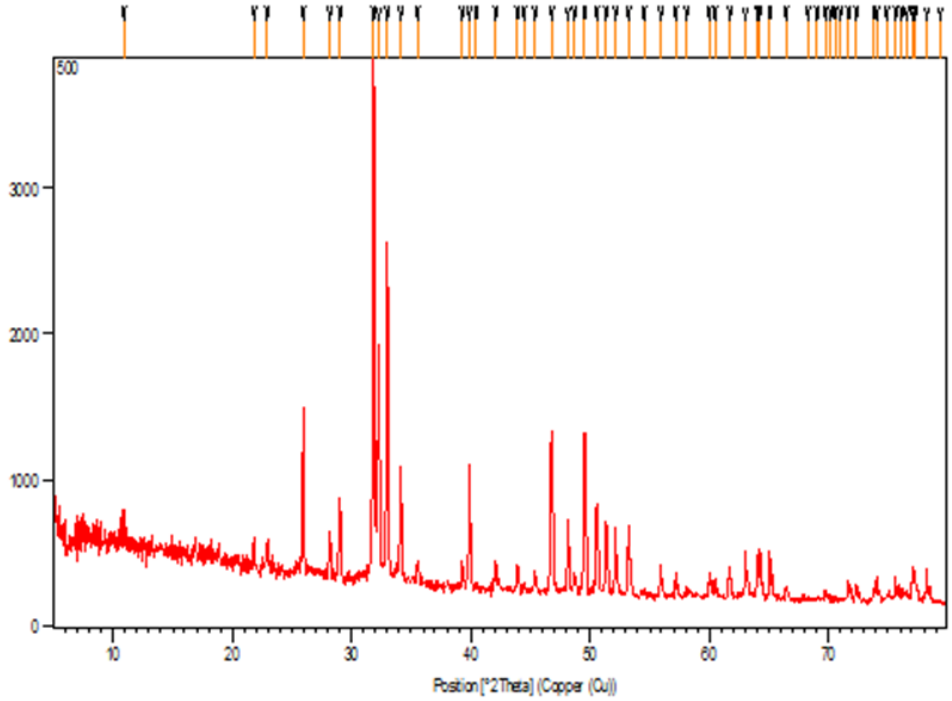

Figure 4-B: XRD spectra of calcined bone powder at 800℃

Hydroxyapatite as Anti-inflammatory in cell and serum

That interaction between serum proteins and cell membrane receptors and nanoparticle shape can change cytotoxicity [18]. It has been extensively researched how nanoparticle size, chemistry, and charge affect inflammation [19], however, there is no information about the manner in which the geometry of nanoparticles influence the inflammatory response. The rate that cells able to absorb nanoparticles is significant influenced by the geometry of nanoparticles. Internalization most quickly for particles that have a rod shape. Next followed the forms, spheres and cylinders, although cubes are difficult on an interior level, a wide range of micro- and nanoparticles with different forms [20]. the generation of pro-inflammatory cytokines and reactive oxygen species (ROS) is also influenced. The needle form was associated with a greater degree of inflammation as compared to rods or spheres [21]. As a result of its chemical and physical similarities to bone, the impact of HA nanoparticles has been extensively utilized as a replacement to bone material in orthopedic applications. This is due to fact that their properties are comparable to those of bone [5]. On the other hand, HA has the potential to cause inflammation when its dispersed as nanoparticles. This is accomplished by stimulating monocytes and neutrophils [13,14] which results in an increase in the production of cytokines and chemokines, such as IL-1 and IL-18 and by causing neutrophils to degranulate and draw in leukocytes [17].

Conclusion

In summary, the aim of this work is to prepare a pure hydroxyapatite nanoparticle and applied as anti-inflammatory properties in macrophage cell in lab and serum of mice .However, the result were indicated to reduce the concentration of TNFα in macrophage cell and serum of mice by spectrophotometer at 480 nm.

Acknowledgment and Greeting

For college of pharmacy staff - University of Al-Qadisiyah - Iraq.

Conflict of interesting

There is no Conflict of interesting

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