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Physico-chemical characterization and biocompatibility of hydroxyapatite derived from fish waste

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ABSTRACT

The aim of this study was to synthesize hydroxyapatite (HAP) powder from fish waste. The powder was characterized through X-ray diffraction, Fourier transform infrared spectroscopy, ion exchange chromatography, scanning electron microscopy and plasma emission spectrometry. The cyto- and genotoxicity was carried out to demonstrate biocompatibility in vivo by means of rat subcutaneous tissue test. The results showed that the visible crystalline nature of typical apatite crystal structure when they were calcined at 800 °C. Infrared spectroscopy analysis showed similar composition to HAP standard with the presence of carbonate ion demonstrated by wave number values of 871 cm^{-1} and 1420 cm^{-1} for calcinations at 800 °C. The scanning electronmicrographies depicted the crystal morphology and porous nature with average pore size of ~10 μm. Plasma emission spectrometry and ion exchange chromatography confirmed the presence of Ca and P in the samples. The mean of calcium content was 36.8; Mg was 0.8, Na was 0.7 and K was 0.5. Rat subcutaneous tissue test revealed that HAP presented biocompatibility. Furthermore, the lack of cyto- and genotoxicity in blood, liver, kidney and lung were noticed after 30 days of HAP implantation. Taken together, our results demonstrated that HAP from fish waste exhibits a great potential for using as biomaterial since is represents a simple, effective, low-cost process and satisfactory degree of biocompatibility.

1. Introduction

Bone fractures represent important pathological events in medicine, with more than 500,000 bone graft procedures performed per year only in the USA (Axelrad et al., 2007). Herein, many researchers have struggled to treat non-union fractures as a result of searching many bone replacement graft materials, such as autografts, allografts and synthetic bone substitutes (Drosse et al., 2008). The use of autologous bone grafts as bone substitutes is considered the gold standard so far. Nevertheless, they contain some problems, as follows: donor site morbidity, the need of additional surgeries, and the relative small amounts of available bone from the donor (Giannoudis et al., 2005). To overcome the drawbacks of currently available bone grafts, some alternatives have been purposed. Among them, the use of allogenic bone grafts has been considered for this purpose, but their utilization is limited due to potential risks of rejection and/or infection (Gabbai-Armelin et al., 2017). Herein, it has been developed synthetic bone substitutes, such as hydroxyapatite (HAP), calcium phosphate (CaP) ceramics and polymer-based materials (Dorozhkin and Ajaal, 2009; Hutmacher et al., 2007). HAP is highlighted because it is the major bioactive component of bone, having high mechanical strength that is desirable for regenerating bone tissue (Cheng, 1987; Koutsopoulos, 2002).

Fish is an important source of food around the world because it contains several nutritional compounds beneficial for human health. The main constituents of fish are protein, fat, carbohydrates, sodium, potassium, calcium, magnesium, vitamin B6 and vitamin B12, which are necessary to promote general health (Kawarazuka, 2010). Recently, much emphasis has been given to produce HAP from biowastes such as marine coral, starfish, and fish bones (Bardhan et al., 2011). It has been reported that type, availability as well as other processing parameters are important considerations for large scale production of HAP from biowastes (Venkatesan et al., 2011). There are a few recent reports describing the potential of fish bones for synthesis of HAP (Liao et al., 1999; Mondal et al., 2010; Boutinguiza et al., 2012; Piccirillo et al., 2013). The conversion of the biowaste has some advantages, such as, extraction of HAP and other useful products and solid waste management of fishery industry (Mondal et al., 2014). It is important to stress

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that considerable quantities of fish scales are generated by fish processing units in coastal cities around the world (Bentes et al., 2012). The degradation of these wastes is detrimental to the environment creating foul smell and pollution for general population (Mondal et al., 2014). In fact, these fish processing units represent a critical environmental problem so far.

The aim of this study is to extract HAP from unexplored biowastes of whitemouth croaker. The powder was characterized through X-ray diffraction, Fourier transform infrared spectroscopy, ion exchange chromatography, scanning electron microscopy and plasma emission spectrometry. The cyto- and genotoxicity was carried out to demonstrate biocompatibility in vivo.

2. Materials and methods

2.1. Synthesis of hydroxyapatite powder from whitemouth croaker fish

All experimental procedures were approved by the Ethics Committee of Federal University of Sao Paulo, UNIFESP, Brazil (Protocol. number 231533).

Whitemouth croaker (*Micropogonias furnieri*) was collected from biowaste generated by the Seafood Market at the Santos city, Sao Paulo state, Brazil. After processing for food, the fish waste (~1 kg) was washed thoroughly with tap water for 24 h followed by external washing with 1 N sodium hydroxide (NaOH) solution for 24 h for de-proteinization. Alkali-treated fish was again thoroughly washed in distilled water overnight. After that, the dried samples were treated with hydrogen peroxide (H_2O_2) at 30% for 24 h for whitening. The samples were washed in distilled water three times for removal H_2O_2. Finally, treated fish bones were subjected to calcinations at 800 °C for 5 h. The obtained HAP was milled to form powder ceramics.

2.2. Physico-chemical characterization of HAP powder

The crystalline phase of the synthesized HAP powder was identified by X-ray diffraction analysis (XRD) (D8 Advance, Bruker-AXS). Identification of the phases was realized by comparing the experimental XRD pattern to standards complied by the International Centre for Diffraction Data (ICDD) using the cards 00–009–0432 for hexagonal HAP structure. Fourier-transform infrared spectroscopy (FTIR) (Agilent Technologies, model Cary 630 FTIR Spectrometer) analysis of HAP powder was carried out in the range of 4000–400 cm^{-1} to detect the presence of various functional groups.

Morphologic analysis was conducted by scanning electron microcopy (TEM, Jeol Gem Microscope). The sample was coated with 100% gold by a sputter-coater (BAL-TEC MED 010, Balzers, Germany) and analyzed by scanning electron microscope. Image J software was used to evaluate the images of HAP particles. Ion exchange chromatography (Metrohn, USA) and plasma emission spectrometry (ICP-OES) (Perkin Elmer precisely, model Opticol Emission Espectrometer, Optima 2100 DV) were also used in this setting.

2.3. Biocompatibility tests in vivo

2.3.1. Animals and experimental design

The study was conducted using 20 male rats (*Rattus, norvegicus, albinus, Wistar*) weighing 180–200 g. The animals were kept in cages identified according to the groups. All animals received standard rat chow (Nuvital, PR, Brazil), and filtered water ad libitum. For surgical interventions, animals were anesthetized with Tanohalo® (Halotano, SP, Brazil). Anesthesia was made by individual inhalation using a hermetic box.

Prior to the surgical insertion of the HAP into the animals' subcutaneous tissues, their dorsal regions were submitted to trichotomy and antisepsis with alcohol at 70%. A surgical incision in the median region was then performed with a #15 blade (Embramac, SP, Brazil).

Table 1
Classification scores used to distinguish the intensity of the inflammation in the subcutaneous tissue of animal (Stanford, 1980).

Scores	Level of inflammation
0	None
1	Slight
2	Moderate
3	Severe

Laterally to the incisions, the subcutaneous tissue was raised and a gentle dissection was made. Thus, fragments from HAP with 0.5 cm^2 were inserted in the subcutaneous tissue of animals. After implantation, the cutaneous flap was replaced and sutured with resorbable polyglactin, and the skin was disinfected with povidone iodin. The health status of the rats was monitored daily. After 7, 15, and 30 days, five animals were killed with an overdose of anesthetics. A total of five animals were used as negative controls.

2.3.2. Microscopic evaluation

The tissues containing the HAP and controls were detached and fixed in 10% formalin for 48 h. The specimens were processed and embedded in paraffin for histological processing. Longitudinal sections of 4 μm were taken and stained with hematoxylin-eosin and analyzed under light optical microscopy.

The sections were microscopically evaluated regarding the presence or absence of inflammation. When present, the type of inflammatory cells, intensity of inflammation surrounding the biomaterial was evaluated. The scores were made in accordance with recommended standard practices for biological evaluation of dental materials (Table 1) (Stanford, 1980). The occurrence of destructive processes, such as necrosis and neoplastic transformation were also considered.

2.3.3. Cytotoxicity and genotoxicity evaluation in multiple organs

Ten male Wistar rats (aged 12 weeks and weighing 280–320 g) were used in this study. Rats were randomly distributed into two groups (containing five animals each): Control group and HAP group. In this group, fragments of HAP with 0.5 cm^2 were inserted in the subcutaneous tissue of animals as described above. All animals were euthanized 30 days after implantation.

For evaluating the cytotoxicity, liver, kidney and lung from all animals were detached and fixed in 10% formalin for 48 h. The specimens were processed and embedded in paraffin for histological processing. Longitudinal sections of 4 μm were taken and stained with hematoxylin-eosin and analyzed by light optical microscopy. The sections were microscopically evaluated regarding the presence of biomaterial, inflammatory process, necrosis area and/or neoplastic transformation.

The single cell gel (comet) assay was made for blood, liver, kidney and lung cells according to Tice et al. (2000) with some modifications. Central fragments from these organs were collected and minced in 0.9% NaCl using wood spatula. The resulting suspensions were centrifuged at 800 rpm for 5 min and were added to 120 μL 0.5% low-melting-point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100% and 10% DMSO), for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer (pH > 13) for 20 min and electrophoresed for another 20 min, at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized onto 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol and stored until analysis. DNA was stained by adding 100 μL ethidium bromide (50 μg/mL) onto each slide. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination.

A total of 50 randomly captured comets per animal (25 cells from each slide) were examined blindly by one expertise observer at 400× magnification using a fluorescence microscope (Olympus) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Sufolk, Haverhill, UK) calibrated previously with according to manufacturer's instructions. To measure DNA damage, tail moment was considered (the product of the tail length and the fraction of DNA in the comet tail).

2.4. Statistical analysis

The results obtained to inflammatory host response and the single cell gel (comet) assays were analyzed by the Kruskal-Wallis test and Newman Keuls test for individual comparisons. SigmaStat software, version 1.0 (Jadel Scientific, Chicago, USA), was used. The significance level adopted in all tests was 5%.

3. Results

3.1. Physico-chemical characterization of HAP from fish waste

The crystalline phase analysis of the HAP powder from fish was carried out by XRD analysis. The analysis indicates the visible crystalline nature of typical apatite crystal structure. It is also noticed that crystallographic behavior of HAP resembles biological apatite. The peak of highest intensity was 31.77° as obtained at 2Θ value for samples calcined at 800 °C (Fig. 1). Also, it was verified the positive correlation to the pure HAP ICDD i.d. number 89–6438.

Infrared spectroscopy analysis was used to characterize the different functional groups of HAP ($Ca_{10}(PO_4)_6(OH)_2$. Fig. 2 shows infrared spectroscopy from HAP power calcined at 800 °C. Such findings show similar composition to HAP standard with the presence of carbonate groups demonstrated by wave number values of 871 cm^{-1} and 1426 cm^{-1}.

The scanning electronmicrography (Fig. 3) of HAP powder displayed the typical crystal morphology of HAP. HAP was agglomerated to HAP particles. The micrograph depicts the porous nature with average pore size of ~8 μm.

The mean of calcium content was 36.8; Mg was 0.8, Na was 0.7 and K was 0.5. Such findings were made in triplicate and the results shown in Table 2. ICP-OES and ion exchange chromatography confirmed the presence of Ca and P in the samples (Tables 2, 3). The Ca/P ratio of HAP powder calcined was 1.4.

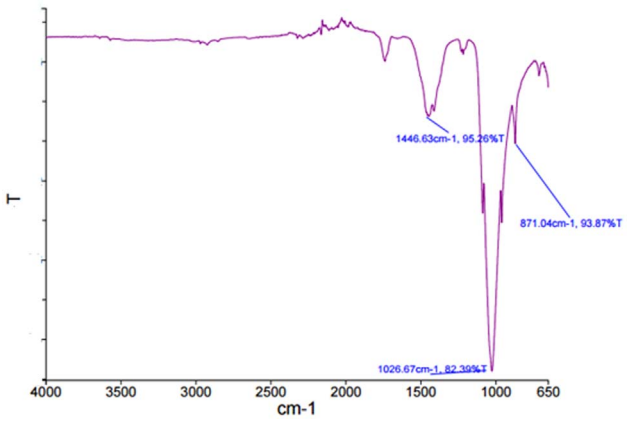

Fig. 2. Infrared spectroscopy from HAP samples after chemical treatment and calcination at 800 °C.

3.2. Biocompatibility tests

3.2.1. Rat's subcutaneous tissue analysis

Histopathological analysis from rat's subcutaneous test is shown in Fig. 4. All animals that received HAP in the subcutaneous tissue, it was possible to detect tissue reactions closely related to cytotoxicity in a time-exposure manner. At day 7, moderate to intense inflammatory process as a result of interstitial edema, a good deal of mononuclear inflammatory cells (lymphocytes), congested vessels and the presence of biomaterial were detected. Furthermore, the histological sections were characterized for the presence of smooth collagen fibers and few fibroblasts. At day 14, a significant regression of the inflammation was observed, in most sections. This was characterized as moderate to mild inflammatory process by the presence of non-immune granulomatous process. Some giant multinucleated cells were observed in this group. Moreover, there were connective tissue with collagen fibers, fibroblasts and blood vessels. At 30 days, few fragments of HAP surrounded by giant multinucleated cells were observed in this period. A well-organized connective tissue was detected, with tissue proliferation into the biomaterial in some of the cases. Inflammation was absent or slight with few lymphocytes.

In the negative control group (non-implanted), all animals presented ordinary appearance of epithelium, and connective tissue composed by collagen fibers and fibroblasts. Such findings are presented in Table 4.

The intensity of inflammatory process after HAP implantation

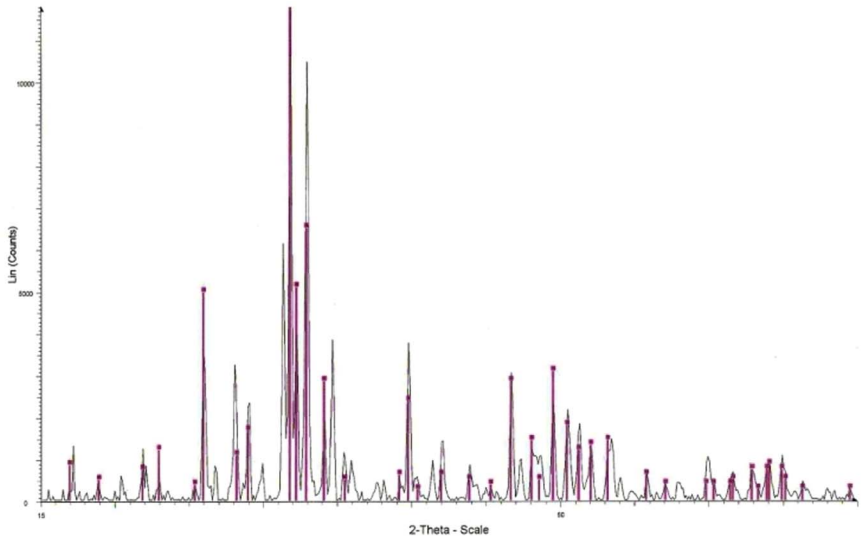

Fig. 1. X-ray diffraction analysis from samples calcined at 800 °C.

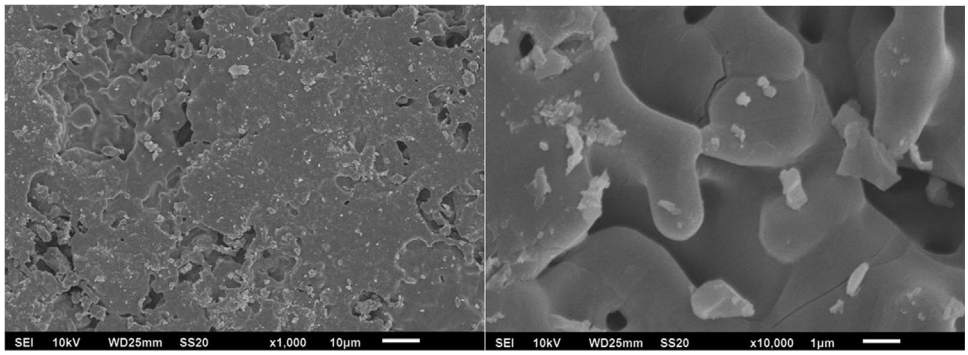

Fig. 3. Scanning electronmicrographies images of porous HAP particles from whitemouth croaker.

Table 2
ICP-OES from HAP samples calcined at 800 °C.

Cátion	Sample 1% (m/m)	Sample 2% (m/m)	Sample 3% (m/m)	Mean % (m/m)	S.D. % (m/m)
Ca	36.8	36.6	36.9	36.8	0.2
Mg	0.8	0.8	0.7	0.8	0.1
Na	0.7	0.8	0.6	0.7	0.1
K	0.4	0.6	0.5	0.5	0.1

Table 3
Ion-exchange chromatography analysis from HAP samples calcinated at 800 oC.

Anion	Sample 1% (m/m)	Sample 2% (m/m)	Sample 3% (m/m)	Méan % (m/m)	S.D. % (m/m)
PO_4^{3-} (like P)	19.5	19.7	19.7	19.6	0.1
Cl^-	1.3	0.5	0.5	0.8	0.5
SO_4^{2-}	0.4	0.4	0.4	0.4	0.0

revealed that animals in the 7 days group showed a high intensity of inflammatory process. After 14 days, a decrease of intensity of inflammatory process was detected. Significant statistically differences ($p < 0.05$) were found in this group when compared to 7 days group. The same picture occurred to 30 days group i.e. a significant decrease of inflammatory process was detected. Significant statistically differences

Table 4
Histopathological analysis from rat's subcutaneous tissue implanted with fish HAP.

Groups	Biological event[a]				
	Inflammatory process	Multinucleated giant cells	Necrosis	Fibrosis	Metaplasia
Control	0	0	0	0	0
7 days	4	2	0	0	0
14 days	4	4	1	3	0
30 days	2	2	0	4	0

[a] Total number of animals.

Table 5
Intensity of inflammatory process (expressed as mean ± S.D.) in animals after HAP implantation in the subcutaneous tissue.

Groups	Days of implantation		
	7	14	30
Control	1 ± 0	1 ± 0	1 ± 0
HAP	3.5 ± 0.5	2.5 ± 0.5*	2.2 ± 0.4*

* $p < 0.05$ when compared to 7 days group.

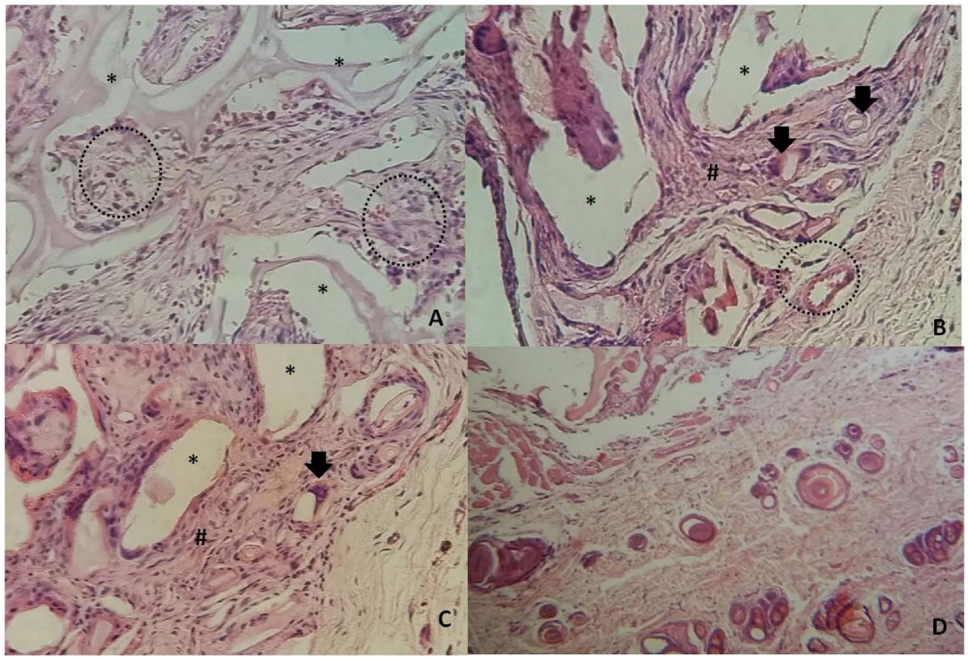

Fig. 4. Photomicrographies of rat subcutaneous tissue after HAP implantation A – 7 days after implantation. Note the presence of intense inflammatory process (circle), congested vessels, tissue injury and the presence of biomaterial (*). B – 14 days after implantation. In this group, it was noted the presence of biomaterial (*), inflammatory granulomatous process with giant multinucleated cells (arrow) and fibrous area (#). C- 30 days after implantation. In this period, few fragments from biomaterial was detected with fibrous area (*) and giant multinucleated cells (arrow). D- negative control. H.E. stain, x40 magnification.

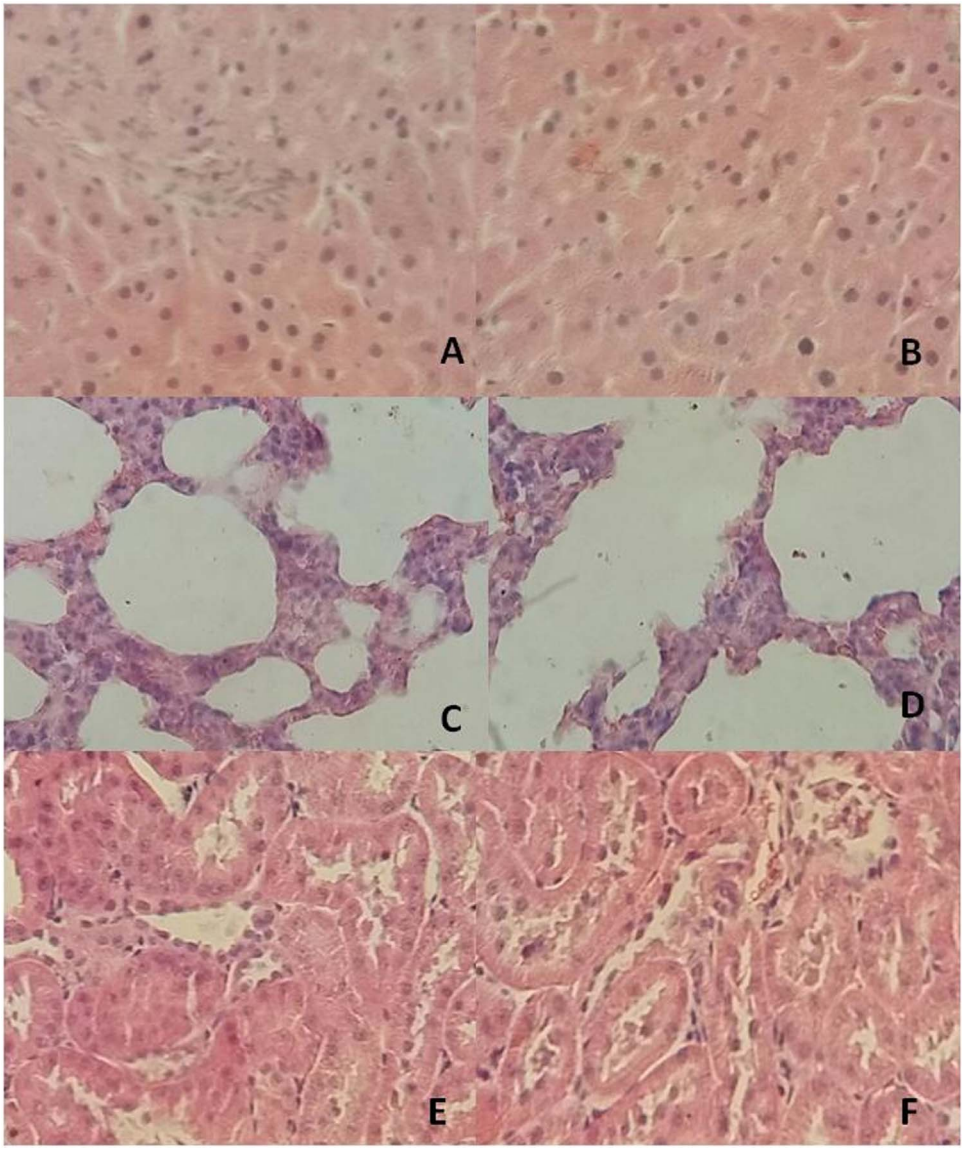

Fig. 5. Photomicrographies of rat multiple organs after HAP implantation for 30 days. Liver: A – control group; B – HAP group. Lung: C- control group; D – HAP group. Kidney: E – control group; F- HAP group. H.E. stain, x40 magnification.

($p < 0.05$) were noticed when compared to 7 days group as well.

In the negative control group, no inflammatory process was noticed for all animals evaluated. Such findings are summarized in Table 5.

Fig. 5 shows the results of cytotoxicity in multiple organs following subcutaneous implantation of HAP. After 30 days, no remarkable differences were detected in liver, kidney and lung for all animals evaluated in this study. Furthermore, the lack of genotoxicity was observed in peripheral blood, liver, kidney and lung cells after HAP implantation. Such findings are demonstrated in Table 6.

Table 6
DNA damage (tail moment expressed as Mean + S.D.) in multiple organs of rats after 30 days of HAP implantation.

Organs	Control	HAP
Blood	0.7 ± 0.3	0.9 ± 0.3
Liver	0.8 ± 0.4	0.8 ± 0.2
Kidney	1.0 ± 0.4	1.3 ± 0.4
Lung	1.0 ± 0.3	0.9 ± 0.4

$p > 0.05$.

4. Discussion

The results of this study demonstrated that HAP from whitemouth croaker preserved the chemical characteristics when calcined at 800 °C. Tang et al. (2010) have demonstrated good results for HAP calcined at 1200 °C. The same results were obtained by Juraida et al. (2011) and Ozawa and Suzuki (Ozawa and Suzuki, 2002). Regarding infrared spectrometry, our results revealed a decrease of wave number in the carbonate group at 871 cm^{-1} e 1412–1547 cm^{-1}. This finding is fully in line with others (Piccirillo et al., 2013). Taken as whole, we assume that HAP was successfully obtained from fish waste using simple and low cost techniques at lab scale. The whitemouth croaker was chosen because it is abundant in South America, without seasonal influences. To the best of our knowledge, the use of biowaste from whitemouth crocker for obtaining HAP has not been addressed so far.

The biomaterial can be considered biocompatible when it present low toxicity, and none inflammatory response (Hauman and Love, 2003). If there is the presence of inflammation, it should be mild (Hauman and Love, 2003). For biocompatibility tests in vivo, the biomaterial should be nontoxic to biological tissues and/or cells (Lawrence et al., 1963). According to the results of the present study, the HAP showed satisfactory biocompatibility in the subcutaneous assay. As expected, inflammatory process occurred after 7 days of implantation

since it is practically impossible some biomaterial does not induce any inflammatory response after close contact with living tissues and/or organs. The main important finding, however, was that inflammatory process decreased after 14 days of implantation, being nearly absent after 30 days of exposure. Therefore, HAP extracted from fish waste showed good biocompatibility in the rat subcutaneous test. By comparison, Mondal et al. (2014) investigated the biological response of Labeo – rohita derived from HAP scaffold. The results showed that the biomaterial presented potential osteoinduction, as a result of the presence of pre-osteoblasts in vivo.

Despite local biocompatibility found in this setting, there is a need for further investigation in order to prove your viability with regard to biological acceptance in host organism. For this purpose, we purpose studies able to analyze the biocompatibility of the material in different target-tissues as a result of host systemic response. In view biocompatibility tests available in the scientific literature so far, genotoxicity assays are of great interest since genotoxicity is largely accepted as an important and useful indicator of carcinogenicity (Sarasin, 2003). Taking into consideration that HAP could be used for a long time in medical and/or dental applications, there are reports that particles of this material are genotoxic to human fibroblast cells in vitro (Hulbert et al., 1970; Tsaousi et al., 2010). Therefore, the condition must be evaluated, even if the biomaterial displays a weakly genotoxic response. Moreover, biomaterials should be evaluated for cytotoxicity, since cellular death plays a crucial role during non-genotoxic mechanisms of carcinogenicity (Auletta and Ashby, 1988). Thus, the study aimed to evaluate the cyto- and genotoxicity in multiple organs after HAP implantation in rat subcutaneous tissue for 30 days. These analyzes are mandatory to prove the viability of the biomaterial for putative clinical application.

In this study, HAP did not induce a significant increase in the level of DNA strand breaks in peripheral blood cells after 30 days of implantation. The same results were obtained to liver, kidney and lung cells. Taken together, our results support the hypothesis that HAP does not induce genotoxic effect (DNA strand breaks) as a result of systemic host response in these target-cells. By comparison, a previous study conducted by our research groups have demonstrated no significant statistically differences ($p > 0.05$) for DNA breakage in blood and liver cells after implantation of internal fixture materials in rat tibia following 30 days. 90 days (Piozzi et al., 2009). The same finding was detected to alumina coated with HAP (Kido et al., 2014). As above mentioned, it is generally accepted correlation between genotoxic and carcinogenic effects of a variety of chemicals (Auletta and Ashby, 1988). Both genetic damage or repair is relevant when evaluating chemical carcinogenesis. This is because tumorigenesis is dependent of genotoxicity, and cell proliferative status (Sasaki et al., 2002). Cytotoxicity was not present for all tissues evaluated after 30 days of HAP implantation in this setting. Some studies have postulated no immunogenic effect of HAP particles in vitro (Mondal et al., 2014). It is important to stress that no single test is capable of detecting all genotoxic and/or cytotoxic agents. For further elucidation on local or systemic effects induced by HAP, further tests are welcomed.

In conclusion, our results demonstrated that HAP obtained from fish waste of whitemouth croacker exhibits a great potential for using as biomaterial since is represents a simple, effective, low-cost process and satisfactory degree of biocompatibility.

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Conflict of interest

None declared.

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