Cellular culture of a bovine apatite as a bone substitute: preliminar tests

Cultivo celular de una apatita bovina como sustituto óseo: pruebas preliminares

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Abstract

The bone loss limits the possibility of dental rehabilitation, being necessary for many occasions to carry out bone reconstruction process for the placement of intraosseous implants and to improve the prosthetics profiles. The autologous bone is the ideal substitute, but there are other alternatives such as tissue of donor origin (homologous), animal origin (xenologous) and synthetic origin (alloplastic). At the engineering school of materials of the University of Valle, a bone substitute is being developed from bovine hydroxyapatite, which was obtained from veal bones previously washed (to eliminate fat and soft tissues) provided by the beef industry. These bones were fractionated, ground, and submitted to a thermal treatment up to 800 Celsius degrees. The samples were characterized by X-ray diffraction and Fourier transformed infrared spectroscopy (FTIR). In this paper, the osteoconduction of the material was evaluated; for this purpose, 15 samples were submitted to preliminary tests and then cultured with osteoblasts for 15 days. The surface characteristics of these samples were determined by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), these characteristics determined cellular functions such as adhesion, maturation and extracellular matrix formation. The results revealed adhesion and cellular growth, as well as the presence of deposits that are compatible with extracellular osseous matrix and that disclose organic and inorganic contents, which is an indicator for maturing.

Keywords: hydroxyapatite; bone grafts; xenograft; extracellular matrix; osteoblasts.

Resumen

Las pérdidas óseas limitan la posibilidad de rehabilitaciones odontológicas, siendo necesario en muchas ocasiones realizar procedimientos de reconstrucción ósea para la coloSCCión de implantes intraóseos y mejorar los perfiles protésicos, para lo cual se indican los sustitutos óseos. El sustituto ideal es el hueso autólogo, pero existen alternativas como el tejido proveniente de donantes (homólogo), el de origen animal (xenólogo) y el sintético (aloplástico). En la Escuela de Ingeniería de Materiales de la Universidad del Valle se está desarrollando un sustituto óseo a partir de hidroxiapatita bovina, la cual fue obtenida de huesos de ternera procedentes de la industria cárnica, los cuales fueron lavados para eliminar grasa y tejidos blandos, fraccionados, molidos y sometidos a tratamiento térmico a 800 °C. Las muestras fueron caracterizadas mediante difracción de Rayos X (DRX) y espectroscopía de infrarrojos por transformada de Fourier (FTIR); en este trabajo se evaluó la capacidad osteoconductora del material realizando pruebas preliminares a 15 muestras que fueron cultivadas con osteoblastos por 15 días. Se determinaron las características de superficie de dichas muestras con Microscopía Electrónica de Barrido (SEM) y Espectroscopia por Energía Dispersiva (EDS), con lo que se determinaron las funciones celulares como adhesión, maduración y formación de matriz extracelular. Los resultados mostraron adhesión y crecimiento celular, además de presencia de depósitos compatibles con matriz ósea extracelular con contenido orgánico e inorgánico, lo cual es un indicador de maduración.

Palabras clave: hidroxiapatita; injertos óseos; xenoinjerto; matriz extracelular; osteoblastos.

Introductión

Bones are vascularized and innervated organs that are composed of bone tissue, bone marrow and a surrounding connective tissue called periosteum. The bones perform a series of functions such as support to the muscles, protection of internal organs, blood production, calcium homeostasis, acid/base buffer, among others (Ratner, Hoffman, Schoen, and Lemons, 1996).

There are several types of cells involved in the formation and remodeling of bone tissue; among these are the osteoblasts, which are responsible for synthesizing the bone matrix; the osteocytes, which are terminally differentiated cells from mature osteoblasts that have remained inside a calcified matrix; the bone coating cells, also differentiated from osteoblasts; and finally, osteoclasts, whose role in bone metabolism is bone resorption (Rucci and Teti, 2016).

There are numerous studies on bone due (among other reasons) to that it is the second most grafted tissue; It is considered a complex tissue with a structure and a unique composition that allow it to adapt to the biological and biomechanical requirements of the system, but also has the capacity to self-regenerate; however, the ability to self-repair is limited by the loss of substance; in situations of large losses (critical size lesions), appropriate substitutes should be used, whose search has impulsed important developments in the field of tissue engineering (Igwe, Amini, Mikael, Laurencin, and Nukavarapu, 2011).

In recent years, the development of regenerative techniques has given impetus to the search for new materials for bone regeneration that would have the function of acting as temporary matrices stimulating the cells of the bone tissue to form and maintain the extracellular matrix, recovering part of the lost tissue. To this end, bone substitutes have been created that supply and regenerate the defects of the stomatognathic system, which originated for the most part by dental losses, facial traumas, and systemic diseases.

Substitutes must fulfill certain main functions to achieve their performance as a bone graft. Within these functions are:

- *Osteogenesis:* “It is the bone formation that occurs from the cells that are present in the graft, surviving the transplant, proliferating and differentiating into osteoblasts” (Kao and Scott, 2007).

- *Osteoinduction:* bone formation in places where the bone is not normally formed; "... it's the stimulation and recruitment of undifferentiated mesenchymal cells at the graft site. Once in the graft site, the stem cells are activated to differentiate into chondrocytes and osteoblasts "(Kao and Scott, 2007), can be active or passive (Daculsi, Fellah, Miramond, and Durand, 2013).

- *Osteoconduction:* it is the growth of tissue and mesenchymal stem cells in the structure of the graft material, this matrix supports and facilitates the growth of the host, the migration of osteoblastic cells and the eventual manufacture of new bone (Daculsi *et al*., 2013).

- *Osseointegration:* is the ability of the graft to integrate and join the receptor bone generating a structural resistance of the grafted material to compression, torsion, and shear. For the graft to be functional, there must be a quantity of new bone in the graft and join with the host bone (Kao and Scott, 2007).

Bony substitutes are classified depending on the material from which they are obtained:

- *Autografts:* tissue obtained from another site of the same organism that receives it (autologous graft). They are the best option for large defects, although they have disadvantages such as increased surgical trauma, increased pain for the patient and higher morbidity (Tortolini and Rubio, 2012).

- *Allografts (homografts):* tissue obtained from donor individuals of the same species (Kao and Scott, 2007).

- *Xenografts:* tissue obtained from a genetically different species to the host. Bovine bone is one of the most used xenografts (Kao and Scott, 2007).

- *Alloplastics:* materials synthesized in the laboratory, the most commonly used are ceramics and polymers (Tortolini and Rubio, 2012).

Autologous bone has been considered the *gold standard* for its osteogenic, osteoinductive and osteoconductive qualities (Wu, Li, and Lin, 2016), however, it has some limitations, such as the increase in surgical time for bone graft extraction, Donor site morbidity, graft resorption and limited availability.

In the case of regeneration of small-sized defects in the oral cavity, it is possible to use as donor sites the mental symphysis, the tuberosity of the maxilla and the mandibular branch (Tortolini and Rubio, 2012) and the feasibility of using the maxillary palatine process has been studied. As a possible donor site for the reconstruction of defects (Bernades, Guijarro, and Hernández, 2016). The maxillary palatine process shows a series of advantages, such as the location, size, and type of graft; but there are possible intra and postoperative complications (Wu *et al*., 2016).

Complications related to autografts and the limited supply due to the absence of an adequate number of donors, in the case of homografts, has made it necessary to use other sources such as xenografts and alloplastics.

In the last decades, hydroxyapatite has been considered as the most outstanding bioceramics with wide use in orthopedics and in dentistry, its wide use is explained by the similarity it presents in its chemical composition and its crystallographic similarity with the inorganic component of the extracellular bone matrix (Ayatollahi, Yahya, Asgharzadeh Shirazi, and Has San, 2015). Vallet (2010) had mentioned the arrival of third-generation bioceramics, which are considered bioactive materials.

The demand for bioactive ceramics has increased due to the biological properties that allow it to integrate easily with the recipient bone (Niakan *et al*., 2015). Bovine bone is considered a potential source for obtaining hydroxyapatite because it is an economical material, readily available and with characteristics very similar to human bone (Ayatollahi *et al*., 2015). Therefore, in this article, we present the preliminary characterizations to a ceramic material of bovine origin with potential use as a bone substitute.

Materials and méthods

The apatite particles used were obtained at the School of Materials Engineering of the Universidad del Valle (UV) from veal bones from the meat industry, which was initially washed to remove soft tissues, fractionated and subjected to a process drying at 130 °C for 24 hours, under air atmosphere; Later the fragments were ground with hammer mill and ball mill, to then perform the final heat treatment at a heating rate of 5 °C/min and 2 hours of holding at the temperature of 800 °C.

Initially, heat-treated bone particles (at 800 °C), uncultivated, were subjected to a series of characterizations, where it was expected to observe the influence of heat treatment and type of bone (tibia) on the results obtained, including porosity (microporosity and macroporosity), the presence or absence of organic compounds (such as fats and proteins), the microstructure and the degree of crystallinity of calcium phosphate were taken into account after cultivating large particles of approximately 500-600 μm with osteoblasts for 15 days.

An FTIR test was carried out on samples of commercial hydroxyapatite, on bone samples (cortical portion) and on the experimental samples in order to compare them and determine the presence of the organic component of the extracellular bone matrix. The spectra were obtained in an infrared spectrophotometer by Fourier Transform brand NICOLET 6700 FT-IR. The calcium phosphate samples were mixed with KBr and pressed to form pellets, which were analyzed in transmission mode in the range of 4000-450 cm^{-1}.

The samples were characterized by X-ray diffraction (XRD) to study the increase in the degree of crystallinity of the mineral by increasing the temperature of the thermal treatment at 800 °C; The X-ray diffraction patterns of the analyzed particles were obtained in a PANalytical X'Pert Powder ray equipment with Brentano Bragg geometry, using Kα Cu radiation (λ = 1,54056 Å). The sample was placed in a rotary sample holder and the diffraction was recorded in an angular range of 10 ° <2θ <70 °.

For cell culture assays, particles from the samples were selected with heat treatment at 800 °C; Fifteen particles of an average size of 500 μm x 600 μm were selected by screening; cultured with murine osteoblasts from a previously characterized primary culture; maintained in the middle of osteogenic growth (OGM ™) for 15 days, in incubation cabin with 5 % CO_2 at 37 °C and change of medium every 3 days; after completing the culture time, they were removed and fixed in ascending alcohols (50 %, 60 %, 70 %, 80 %, 95 %, and 100 %) and dried in biological safety cabinets at room temperature; Once dehydrated, they were analyzed by SEM and EDS to study cell adhesion and extracellular bone matrix formation.

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Results and discussión

The Fourier transform infrared (FTIR) test is usually used to detect the presence of organic and inorganic components. In this work, the samples of the apatite obtained were compared with samples of cortical bone and commercial hydroxyapatite samples. Apatite obtained the spectra were different from those of the other two studied, evidencing the absence of organic components of the extracellular bone matrix.

The infrared spectrum of the bone has been reported by some researchers, showing the presence of the main inorganic species, phosphate, and carbonate groups, and also organic components such as amide functional groups of the constituents of bone proteins, ie collagen (Boskey and Camacho, 2007). The bands associated with the amide groups of bone proteins at wavenumbers 1250, 1560 and 1650 cm^{-1}, actually appeared in the FTIR of the dehydrated bone in the present investigation, at wavenumbers 1250, 1549.4 and 1652.6 cm^{-1}; On the other hand, there are also presence of absorption bands related to the C-H bonds at wavenumbers 2852.6 and 2925.9 cm^{-1}, as shown in Figure 1. To analyze the bands corresponding to the inorganic components that appear in the spectrum.

To analyze the bands corresponding to the inorganic components that appear in the femoral bone spectrum of Figure 1, they can be divided into three main categories, phosphate bands, carbonate bands, and hydroxyl group bands. A strong and relatively broadband at 1043.6 cm^{-1}, relatively strong and narrow bands at 472.5, 565.9 and 600 cm^{-1}, and others at 961.5 and 1089.0 cm^{-1} appearing in the FTIR spectrum are related to phosphate groups; the bands that appear at 874.7, 1417.5 and 1456.2 cm-1 are associated with the carbonate groups with type B substitution. Continuing with the spectrum analysis, we find that the bands at 1250, 1549.4, 1652, 6, 2852.6 and 2925.9 cm^{-1} have disappeared after heat treatment of the bone (800 °C). It can be inferred that the above may be due to the removal of carbon and the amide groups associated with the proteins present in the bone in the form of carbon dioxide gas in the presence of oxygen in the air. The bands originated by the phosphate groups (1041.4, 464.8, 565.0, 601.7, 960.4 and 1089.0 cm^{-1}) and carbonate (874.7, 1419.8 and 1454.9 cm^{-1}) continue appearing at approximately the same wavelengths as for untreated bone, confirming that all the bands observed in the FTIR of natural calcium phosphate are related to the inorganic components of the bone that were heat-treated (Younesi, Javadpour, and Bahrololoom, 2011).

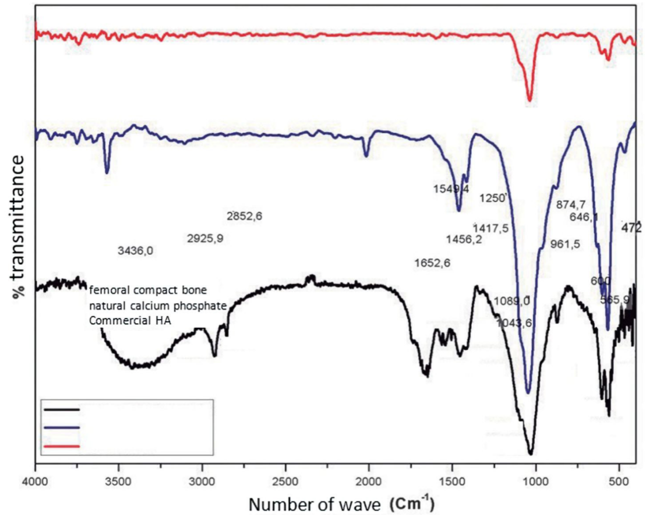


Figure 1. Spectral analysis of the three samples analyzed. Black: Compact bone, Blue: Apatite, Red: Commercial hydroxyapatite, FTIR technique
Source: the authors.

Figure 2 presents the XRD patterns of femoral compact bone (dehydrated at 130 °C), Natural Apatite (bone particles that were heat-treated at 800 °C) and commercial HA used as a reference in this investigation. It can be observed that when carrying out the heat treatment (800 °C) to particles of compact bone, the diffraction patterns show a significant increase of the peaks, however, the diffraction peaks of the dehydrated bone (130 °C) are very wide and little elongated. This allows us to demonstrate the increase in the degree of crystallinity of the mineral by increasing the temperature of the thermal treatment.

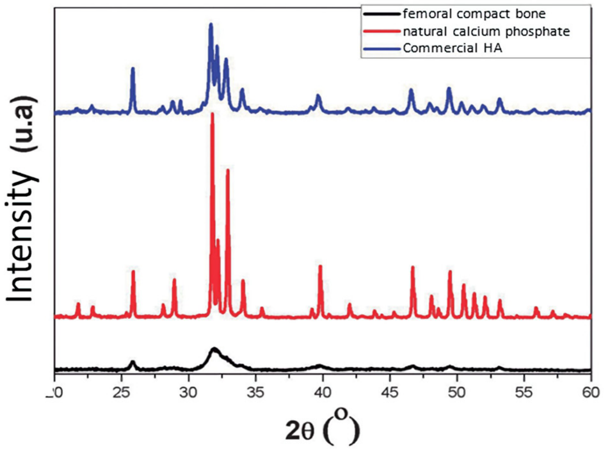


Figure 2. DRX patterns made to the three samples. Black: Compact bone, Blue: Apatite, Red: Commercial hydroxyapatite, FTIR technique
Source: the authors.

Similar observations have been reported by Shipman, Foster, and Schoeninger (1984), they analyzed the results of the XRD patterns in terms of alteration in the size of the crystal, finding that there was a gradual increase in the size of the associated HA crystal. with the increase of the temperature of the heat treatment. On the other hand, it must be noted that some chemical factors can affect the crystallite size, among which is the denaturation of the bone matrix during calcination through the release of water since the mineral crystals recrystallize and the elimination of the Networks of collagen fibrils influences the size of the crystallites of bone ash (Rootare and Craig, 1977).

In the SEM images of Figure 3, corresponding to natural calcium phosphate, it can be seen that the thermal treatment of compact bone particles of the ground tibia at (800 °C) results in the formation of large grains. The formation of large particles in the thermal process may be due to the tendency of calcium phosphate grains to agglomerate and grow at high temperatures (Meejoo, Maneeprakorn, and Winotai, 2006). In Figure 3, the micrograph of natural calcium phosphate at low magnification (150X) shows the presence of grains with irregular geometry, of different sizes and very angular. When making a greater increase (2000X) to the particles, it is observed that they are completely populated with irregularly shaped pores, which had sizes from 4 to 14 μm and some cavernous regions of up to 50 μm. This result is very important since it has been found that when calcium phosphates are porous they offer surface chemistry that leads to the new bone formation (Hendriks, Riesle, and Blitterswijk, 2010; López, 2003).

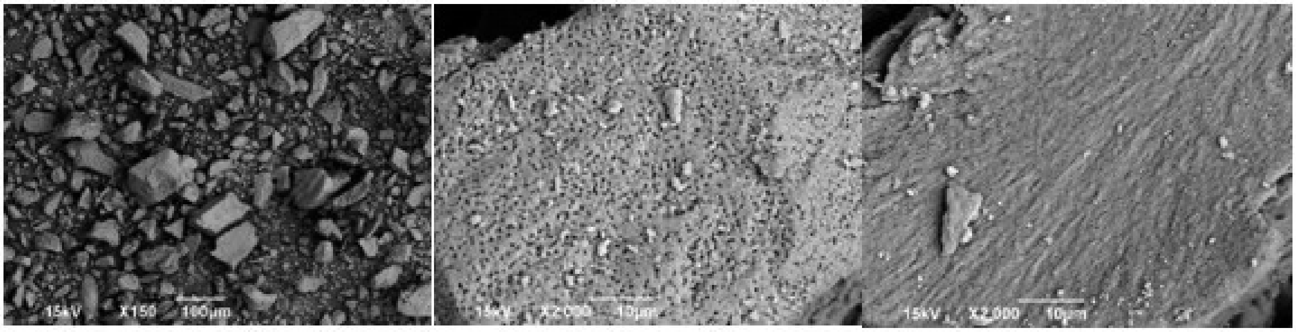
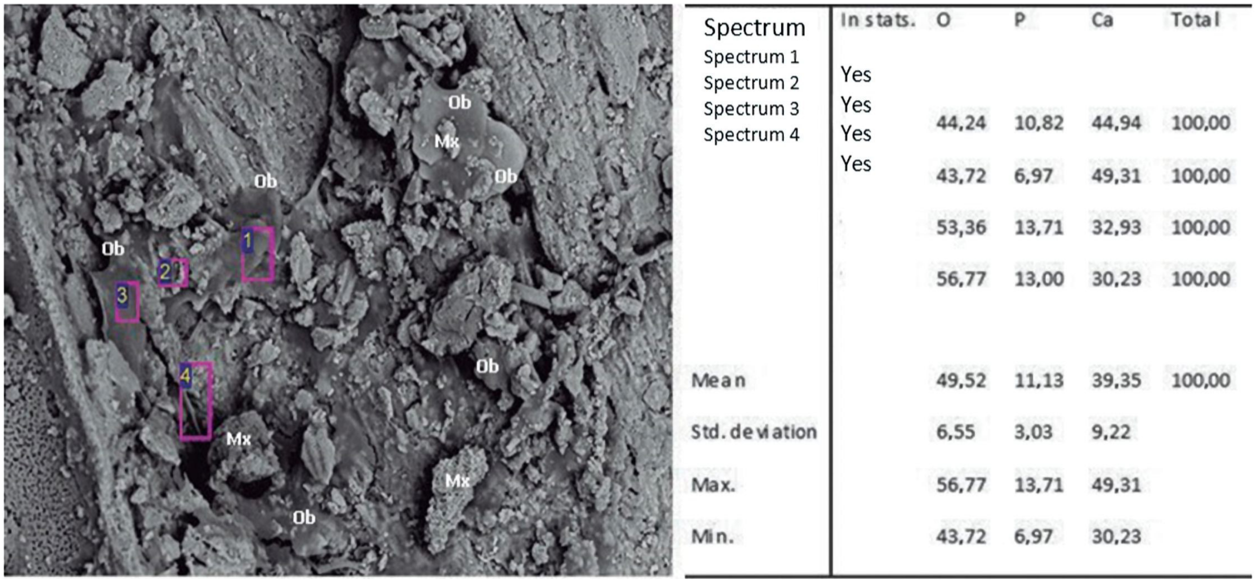


Figure 3. Sample of natural apatite, treated at 800 ° C. SEM technique, SEM image at 150X (right) and 2000X (center and left)
Source: the authors.

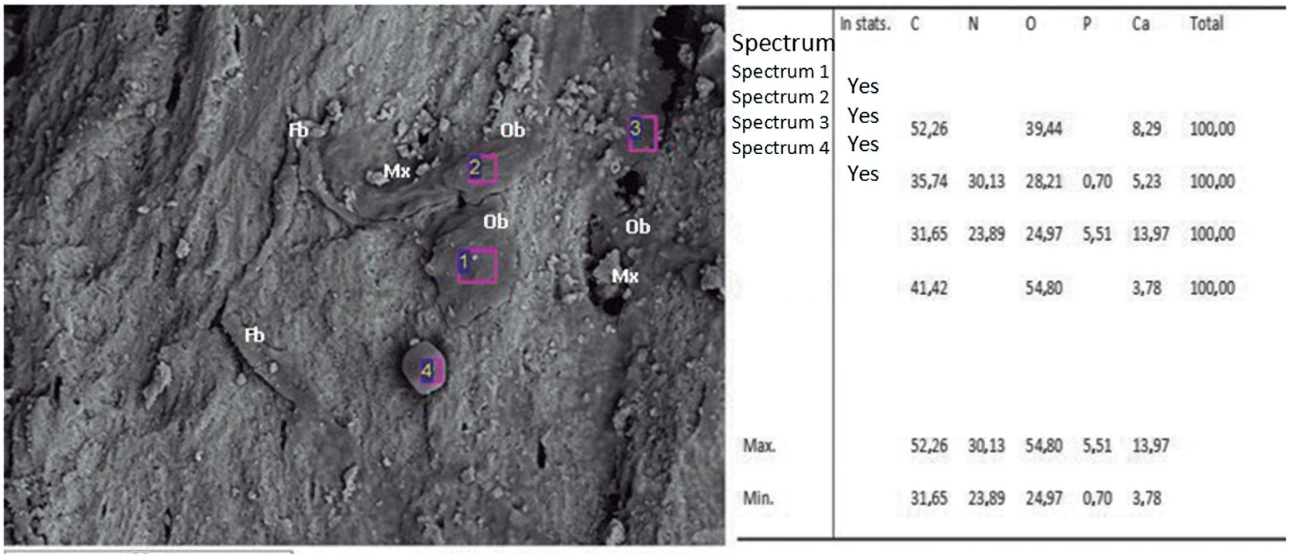
The SEM analyses performed on all the cultured samples reveal how the cells (osteoblasts) took advantage of the irregular surfaces of the grains to anchor themselves and produce extracellular matrix (Figure 4).



Spectrum	In stats.	O	P	Ca	Total
Spectrum 1	Yes	44,24	10,82	44,94	100,00
Spectrum 2	Yes	43,72	6,97	49,31	100,00
Spectrum 3	Yes	53,36	13,71	32,93	100,00
Spectrum 4	Yes	56,77	13,00	30,23	100,00
Mean		49,52	11,13	39,35	100,00
Std. deviation		6,55	3,03	9,22	
Max.		56,77	13,71	49,31	
Min.		43,72	6,97	30,23	

Figure 4. Apatite sample after 15 days of culture; the presence of inorganic components of the extracellular bone matrix (P and Ca) is observed. Ob: Osteoblast; Mx: Deposit of extracellular bone matrix. SEM Technique - EDS at 150 X
Source: the authors.

The deposits of material observed in the micrographs show fibrillar structures compatible with collagen and calcium phosphate deposits, evidenced by dispersive energy analysis (DES); additionally, 2 of the 15 samples showed the presence of nitrogen (Figure 5), indicating the presence of proteins in the extracellular matrix formed.



Spectrum	In stats.	C	N	O	P	Ca	Total
Spectrum 1	Yes	52,26		39,44		8,29	100,00
Spectrum 2	Yes	35,74	30,13	28,21	0,70	5,23	100,00
Spectrum 3	Yes	31,65	23,89	24,97	5,51	13,97	100,00
Spectrum 4	Yes	41,42		54,80		3,78	100,00
Max.		52,26	30,13	54,80	5,51	13,97	
Min.		31,65	23,89	24,97	0,70	3,78	

Figure 5. Apatite sample after 15 days of culture; In addition to evidencing the presence of inorganic components of the extracellular bone matrix (P and Ca), nitrogen is observed, indicative of protein formation. Ob: Osteoblast; Mx: Deposit of extracellular bone matrix SEM Technique - EDS at 150 X
Source: the authors.

Finding calcium phosphate and nitrogen in the analyzes performed are important insofar as they show how the extracellular matrix is being formed and mineralized; according to the characterization made by Siggelkow et al., (1999) in a culture of murine osteoblasts. The proliferative stage occurs between days 0 and 12, the maturation between days 12 and 20 and the mineralization from day 20, in that investigation the crops were suspended at day 15, for this time the expected was the finding of collagen and presence of matrix proteins, as well as calcium and phosphorus deposits from the osteoblasts.

Nudelman, Lausch, Sommerdijk, and Sone (2013) show how calcium phosphate that initially precipitates in extracellular matrix formation under in vitro conditions does not crystallize as a stable product but subsequently transforms into a more stable phase. Regarding protein contents, these are represented in the collagen proteins whose function is to assemble the extracellular matrix and non-collagenous proteins that guide the assembly of the matrix and its mineralization.

Conclusions

The results of the present investigation showed that particles of natural apatite of bovine origin allowed the adhesion and proliferation of osteoblasts on its surface, with the formation of deposits of material compatible with organic and inorganic components of the extracellular bone matrix.

References

Ayatollahi, M. R., Yahya, M. Y., Asgharzadeh Shirazi, H., y Hassan, S. A. (2015). Mechanical and tribological properties of hydroxyapatite nanoparticles extracted from natural bovine bone and the bone cement developed by nano-sized bovine hydroxyapatite filler. *Ceramics International, 41*(9), 10818–10827. doi: https://doi.org/10.1016/j.ceramint.2015.05.021

Bernades Mayordomo, R., Guijarro Martínez, R., y Hernández Alfaro, F. (2016). The anterior maxilla as a potential source of bone grafts: a morphometric cone beam computed tomography analysis of different anatomical areas. *International Journal of Oral and Maxillofacial Surgery, 45*(8), 1049–1056. doi: https://doi.org/10.1016/j.ijom.2016.03.001

Boskey, A., y Camacho, N. P. (2007). FT-IR imaging of native and tissue-engineered bone and cartilage. *Biomaterials, 28*(15), 2465–2478. doi: https://doi.org/10.1016/j.biomaterials.2006.11.043

Daculsi, G., Fellah, B. H., Miramond, T., y Durand, M. (2013). Osteoconduction, Osteogenicity, Osteoinduction, what are the fundamental properties for a smart bone substitutes. *Irbm, 34*(4–5), 346–348. doi: https://doi.org/10.1016/j.irbm.2013.07.001

Hendriks, J., Riesle, J., y Blitterswijk, C. A. van. (2007). Co-culture in cartilage tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine, 1*(3), 170–178.

Igwe, J., Amini, A., Mikael, P., Laurencin, C., y Nukavarapu, S. (2011). Nanostructured scaffolds for bone tissue engineering. *In Active implants and scaffolds for tissue regeneration* (pp. 169–192). Berlin, Heidelberg: Springer. doi: https://doi.org/10.1007/8415_2010_60

Kao, S. T., y Scott, D. D. (2007). A Review of Bone Substitutes. *Oral Maxillofacial Surgery Clinics, 19*(4), 513–521. doi: https://doi.org/10.1016/j.coms.2007.06.002

Khairallah, M., y Almeshaly, H. (2016). Present Strategies for Critical Bone Defects Regeneration.*Oral health case Rep 2016, 2:3,* 2:3.

López, M. E. (2003). Hidroxiapatita macroporosa obtenida en la Universidad de Antioquia: síntesis, caracterización y comparación con el hueso esponjoso y calcinado de bovino. *Revista Facultad de Ingeniería, 30*(30), 109–124.

Meejoo, S., Maneeprakorn, W., y Winotai, P. (2006). Phase and thermal stability of nanocrystalline hydroxyapatite prepared via microwave heating. *Thermochimica Acta, 447*(1), 115–120. doi: https://doi.org/10.1016/j.tca.2006.04.013

Niakan, A., Ramesh, S., Ganesan, P., Tan, C. Y., Purbolaksono, J., Chandran, H., ... Teng, W. D. (2015). Sintering behaviour of natural porous hydroxyapatite derived from bovine bone. *Ceramics International, 41*(2), 3024–3029. doi: https://doi.org/10.1016/j.ceramint.2014.10.138

Nudelman, F., Lausch, A. J., Sommerdijk, N. A. J. M., y Sone, E. D. (2013). In vitro models of collagen biomineralization. *Journal of Structural Biology. 183*(2), 258-269. doi: https://doi.org/10.1016/j.jsb.2013.04.003

Ratner, B. D., Hoffman, A. S., Schoen, F. J., y Lemons, J. E. (1996). *Biomaterials Science: An Introduction to Materials in Medicine*. San Diego, CA, USA: Academic. doi: https://doi.org/10.1016/B978-0-08-087780-8.00148-0

Rootare, H., y Craig, R. (1977). Vapor Phase Adsorption of Water on Hydroxyapatite. *Journal of dental research, 56*(12), 1437–1488.

Rucci, N., y Teti, A. (2016). The "love-hate" relationship between osteoclasts and bone matrix. *Matrix Biology, 52*, 176–190. doi: https://doi.org/10.1016/j.matbio.2016.02.009

Shipman, P., Foster, G., y Schoeninger, M. (1984). Burnt Bones and Teeth: An Experimental Study of Color, Morphology, Crystal Structure and Shrinkage. *Journal of archaeological science, 11*(4), 307–325.

Siggelkow, H., Rebenstorff, K., Kurre, W., Niedhart, C., Engel, I., Schulz, H., ... Hüfner, M. (1999). Development of the osteoblast phenotype in primary human osteoblasts in culture: Comparison with rat calvarial cells in osteoblast differentiation. *Journal of Cellular Biochemistry, 75*(1), 22–35.

Tortolini, P., y Rubio, S. (2012). Diferentes alternativas de rellenos óseos. *Avances En Periodoncia E Implantología Oral, 24*(3), 133–138.

Vallet, M. (2010). Tendencias en Biomateriales. *Revista de La Fundación de Ciencias de La Salud, Eidon, 33*, 6–10.

Wu, J., Li, B., y Lin, X. (2016). Histological outcomes of sinus augmentation for dental implants with calcium phosphate or deproteinized bovine bone: a systematic review and meta-analysis. *International Journal of Oral and Maxillofacial Surgery, 45*(11), 1471–1477. doi: https://doi.org/10.1016/j.ijom.2016.04.020

Younesi, M., Javadpour, S., y Bahrololoom, M. E. (2011). Effect of heat treatment temperature on chemical compositions of extracted hydroxyapatite from bovine bone ash. *Journal of Materials Engineering and Performance, 20*(8), 1484–1490. doi: https://doi.org/10.1007/s11665-010-9785-z