González, Ramón; Merino, Nelson; Rodríguez, Pedro; Rodríguez, Víctor M.
In vivo transformation of a calcium carbonate (aragonite) based implant's biomaterial to bone. A histological, chemical and FT-IR study
Centro Nacional de Investigaciones Científicas
Ciudad de La Habana, Cuba

Available in: http://www.redalyc.org/articulo.oa?id=181226086002
**In vivo** transformation of a calcium carbonate (aragonite) based implant’s biomaterial to bone. A histological, chemical and FT-IR study

**Ramón González, Nelson Merino, Pedro Rodríguez** and **Victor M. Rodríguez**

Biomaterial’s Laboratory, National Center for Scientific Research, P.O. Box 6414, Playa, Havana City, Cuba. **Department of Experimental Surgery, Higher Institute of Medical Sciences, Havana City, Cuba.**


**Palabras clave:** HAV, péptidos sintéticos, antigenicidad, especificidad.

**Key words:** biomaterials, bone implants, hydroxyapatite, aragonite.

**RESUMEN.** Se estudió la respuesta del tejido óseo a implantes de carbonato de calcio aragonito (CCA). Se utilizaron implantes de Hidroxiapatita Coralina (HA). en la misma forma y proceder quirúrgico como biomaterial de control comparativo. Se investigó el efecto de la composición química sobre la capacidad de curación y la velocidad de reabsorción de ambos biomateriales implantados en el fémur de ratas. Los animales fueron sacrificados a los 3, 7, 15, 21 y 60 d y los implantes fueron recuperados para los análisis posteriores. La evaluación se realizó mediante técnicas de microscopía óptica, estudios histológicos y radiográficos, análisis químico cuantitativo de calcio y fósforo y espectroscopia IR de Transformada de Fourier (FT-IR). Se encontró que ambos biomateriales fueron bien asimilados y se integraron al tejido óseo circundante. En el caso de la Hidroxiapatita se observó que a los 60 d el defecto óseo fue reparado, pero el biomaterial mantuvo la misma apariencia inicial. Por el contrario, en ese mismo período, el implante de coral (CCA) disminuyó su tamaño y su apariencia resultó más parecida a la del tejido circundante. Los estudios histológicos mostraron la misma capacidad osteogénica en ambos biomateriales en los primeros 7 d de implantados, pero a los 15 d se aprecia una mayor velocidad de sustitución del tejido fibrovascular por hueso en el caso del CCA. La cinética de biodegradación calculada a partir de los análisis químicos (relación Ca/P) y de los espectros IR coincide con las observaciones histológicas y muestra una mayor velocidad de reabsorción de los implantes de carbonato de calcio (CCA). Se encontró que la transformación del CCA produce una fase similar a la del hueso debido a la pérdida paulatina del anión carbonato y la incorporación del fosfato correspondiente. El CCA también presentó una mayor capacidad osteogénica en comparación con la HA. A los 60 d el (42.5 ± 3.2) % del CCA originalmente implantado se transformó en fosfato de calcio y la velocidad de este proceso se incrementó de (0.22 ± 0.08)%/d a los 7 d hasta (0.7 ± 0.1)%/d a los 60 d de implantado. Los implantes de HA no experimentaron cambios en el periodo analizado (60 d). En conclusión, igual que la hidroxiapatita porosa, el biomaterial de aragonito estimula la proliferación de tejido vivo hacia el interior de los poros, pero con una mayor velocidad de reabsorción y de generación de nuevo hueso. Estos resultados sugieren que la velocidad de reabsorción de este tipo de biomaterial de implante puede ser controlada en dependencia de la relación fosfato/carbonato en su composición química inicial.

**ABSTRACT.** The response of bone tissue to the implantation of calcium carbonate aragonite (CCA) has been studied. Implants of coralline hydroxyapatite (HA) in the same forms and surgical procedure were used as a control biomaterial for comparison. The effect of chemical composition on healing and resorption rate after implantation of both biomaterials into the femur of rats was investigated. The animals were sacrificed at 3, 7, 15, 21 and 60 d after surgery and the implants were recovered for analysis. Light microscopy, histological and radiographic techniques, quantitative chemical analysis of Ca and P and FT-IR spectroscopy were used for evaluation. It was found that both implanted biomaterials showed a good assimilation and integration to bone tissue. In the case of hydroxyapatite the defect was repaired but the biomaterial remained the same until 60 d. In contrast, the coral implant decreased in size and appeared like the surrounding bone tissue. Histological study showed that both biomaterials had a similar behavior in their osteogenic capacity until 7 d postimplant but not at 15 d when CCA induced in a faster rate the complete substitution of fibrovascular tissue for bone tissue. The kinetics of biodegradation calculated from chemical (Ca/P ratio) and FT-IR analysis support the histological observation and pointed out the high reabsorption rate of the calcium carbonate implants. It was found that the transformation of CCA produce an inorganic phase similar to the bone due to the loss of carbonate anion with the incorporation of phosphate. It had also a faster rate of osteogenic capacity in comparison with hydroxyapatite. At 60 d the (42.5 ± 3.2) % (means ± S.D.) of the implanted original CCA was transformed in to calcium phosphate phase. The rate of reabsorption was increased from (0.22 ± 0.08)%/d at 7 d to (0.7 ± 0.1)%/d at 60 d post-implantation. In the HA implants no changes of the chemical composition were observed. In conclusion, just like porous hydroxyapatite, aragonite based biomaterial stimulates the proliferation of the living tissue inside the pores, but with a faster rate of reabsorption and new bone generation. These results lead to suppose that the rate of reabsorption of such type of implant’s biomaterial may be controlled depending on the relation phosphate/carbonate in its initial chemical composition.
INTRODUCTION

Natural coral and its structurally similar derivate in the form of porous hydroxyapatite (HA) have been used for many years as bone substitute in reconstructive surgery. Both biomaterials showed a good biocompatibility and osteointegration but their reabsorption rate are different. Whereas the coral is quickly reabsorbed, hydroxyapatite is considered as essentially non-reabsorbable.

When HA is placed in contact with the bone tissue, formation of osteoprogenitor cells and proliferation of bone on the surface and within the pores is observed. HA has been successfully applied for the restoration of damaged or lost bone in areas such as dentistry, maxillofacial and orthopedic surgery. But due to its non-reabsorbable nature, it has had a limited use in the treatment of large lesions in sites of low osteogenic potential or when the remodeling of the osseous structure is needed. On the other hand, corals undergo rapid degeneration and might result in loss of its mechanical properties and in incomplete repair of bone defect.

For these reasons, the rate of reabsorption of these biomaterials is a very important aspect to keep in mind in its clinical use, according to the site and type of lesion to be repaired.

The efforts carried out to try to explain the mechanisms that take place in the biodegradation as well as the determination of the reabsorption rate of biomaterials with base of calcium carbonate are even insufficient and in some cases they produce contradictory results. One of the main aims of this work was to make a comparative study of the behavior in vivo of the implants of calcium carbonate aragonite (CCA) from coral and coralline hydroxyapatite (HA) in bone (femur) of rats. To determine the resorption rate of CCA through the changes in chemical composition and phase transformation of the implants during 8 weeks of postoperative evolution.

MATERIALS AND METHODS.

The biomaterials used were porous calcium carbonate (CCA) from natural coral containing up to 1% of calcium phosphate (hydroxyapatite) in granules of 0.4-0.5 mm of diameter and porous hydroxyapatite CORALINA HAP-200 (HA) with the same shape and dimensions of the particles and macropore average size between 75 and 340 µm. Implants were supplied by the Biomaterial’s laboratory at National Center for Scientific Research and were sterilized in autoclave (120 ºC, 2 bar, 30 min).

The study was carried out in 25 rats Sprague Dawley, both sexes with an average weight of 250-300 g, which were anesthetized with sodium pentobarbital (4 mg/100 g weight) through intraperitoneal route. Previous cleaning and antisepsis of operatory region with a 3% iodine solution, a 2 cm long incision of the skin was made in the right posterior leg, the muscles were dissected carefully until the bone was exposed, and a cavity of 3 mm of diameter was made in the diaphysis, penetrating the medullar zone. To avoid heating an aerator was used at a low speed and constant irrigation with sterile saline solution.

The cavity was carefully cleaned, eliminating the remaining of bone fragments and filling it with the corresponding biomaterial; no isolated granules in the adjacent soft tissues were left.

Finally the muscular planes were confronted and sutured with catgut 3-0 using a curved atraumatic needle, and the skin was sutured with silk 3-0, in aseptic conditions.

With this procedure the CCA was implanted into the right femur and the HA in the left one.

The animals were divided into five groups and placed in cages identified according to the days of sacrifice; food and water was given ad libitum. The location and evolution of the implants were monitored by means of simple radiography.

The animals were sacrificed at 3, 7, 15, 21 and 60 d of evolution after surgery with an overdose of ethyl ether. Both legs were isolated and the femur exposed after the removal of the soft tissue. The macroscopic appearance of the implants was observed with a Carl Zeiss stereo microscope. Finally the specimens under study were fixed in 10% neutral formaline solution for further histological evaluation.

RESULTS

The clinical evolution of the animals was satisfactory, with proper healing of the wound in the implanted zone. Neither local nor systemic adverse responses to the materials implanted were observed.

Radiographic evolution

Both biomaterials HA and CCA were more radiopaque than the surrounding tissue during all the time (up to 60 d) due to its greater density than the bone and showed a good assimilation.

In the case of Hydroxyapatite, it could be observed to the naked eye and through the stereo microscope that the defect was well repaired and the biomaterial remained the same until 60 d. However, after this time, the coral implant was hardly appreciable decreased in size, and...
with a great resemblance of the surrounding bone tissue (Fig. 1).

**Histological studies**

In all implants a fibrovascular tissue invasion was observed inside the pores and in close contact with the biomaterial, with no inflammatory response to the implanted materials, although the histological evaluation of surrounding tissue was qualitatively different for each type of biomaterial.

The characteristics observed in the samples of both implants were as follows.

**Hydroxyapatite (Fig. 2)**

3 DAYS: A highly vascularized connective tissue invaded the pore area and a fibrinous clot was observed next to the fibrovascular tissue. No inflammatory response to the biomaterial was observed.

7 DAYS: The fibrovascular tissue was partially invaded by bone tissue, distributed in the pore cavities and covered with osteoblasts. The red bone marrow was compressed against the bone wall (Fig. 2a).

15 DAYS: A very similar aspect to 7 d, the new bone tissue being more prominent (Fig. 2b).

21 DAYS: Complete substitution of the fibrovascular tissue by bone tissue. The bone marrow started to

---

**Fig. 1.** Photography at first sight through the stereoscopic microscope of the implants of CCA at 3 d (upper left) and 60 d (upper right) and HA (lower left and right) at the same time respectively after surgery. (H&E 15x).
Fig. 2. Histological appearance of HA implants at 7 (a), 15 (b) and 21 d (c) after surgery. (H&E 250x).
take up the empty areas of the implant (Fig. 2c).

60 DAYS: Similar to 21 d. The bone marrow has taken up the implant area.

**Calcium carbonate** (Fig. 3)

3 DAYS: Presence of new connective tissue, very vascularized. An adjacent fibrin clot was observed. No giant cells (Fig. 3a).

7 DAYS: The fibrovascular tissue was partially invaded by bone trabecules in the pore region. Numerous osteoblasts surrounded the new bone tissue (Fig. 3b).

15 DAYS: The fibrovascular tissue has disappeared and only the proliferated bone trabecules were observed (Fig. 3c).

21 DAYS: Similar to 15 d.

60 DAYS: Similar aspect to 21 d. The osseous trabeculae net was prominent, and the cavities were occupied by the remaining implant material.

As can be seen, both biomaterials have a similar behavior in their osteogenic capacity until the 7 d post-implant, but not at 15 d when calcium carbonate has induced the complete substitution of fibrovascular tissue for bone tissue, while in the case of HA this process was finally completed at 21 d (Fig. 2c).

**Chemical and FT-IR studies**

The chemicals and FT-IR analysis support the histological and microscopic observations. In the case of the calcium carbonate implants, the transformation of the biomaterial produce an inorganic phase very similar to the bone due to the loss of carbonate anion and at the same time with the incorporation of phosphate. The Infrared Spectroscopy is a very useful analytical tool for monitoring the evolution of the bone implants. The figure 4 shows FT-IR spectra of carbonate type implant at different evolution time (a-e) and the spectrum of the bone (f).

In the spectra corresponding to earlier states it is possible to observe that the \( \text{CO}_3^2- \) bands (1788, 1476, 1083, 858, 713 and 700 cm\(^{-1}\)) are the most representative and the intensity of these bands decreases with the time, while the intensity of the \( \text{PO}_4^3- \) bands (1037, 871, 604 and 564 cm\(^{-1}\)) and water bending mode (1655 cm\(^{-1}\)) increases. There is a clear evidence of the stepped transformation of CCA implants to the calcium phosphate phase present in bone. The spectrum of the 60 d old implant (Fig. 4e) is very similar to the bone spectrum (Fig. 4f).

Chemical analysis of calcium and phosphorous showed that the molar ratio Ca/P in CCA implants decrease toward the half values of the bone composition (Fig. 5). At 60 d the (42.5 ± 3.2) % (means ± S.D.; n = 3) of the originally implanted CCA was transformed in to calcium phosphate phase. The rate of resorption was increased from (0.22 ± 0.08) %/d at 7 d to (0.7 ± 0.1) %/d at 60 d post-implantation. In the HA implants no changes on chemical composition were observed.

**DISCUSSION**

The results demonstrate clearly that Calcium Carbonate is a biocompatible material with the bone, it is osteoconductor and compared to hydroxyapatite it has a greater osteogenic capacity as it stimulates the formation of bone tissue at a faster rate. However in the hydroxyapatite implants the whole substitution of fibrovascular tissue within the pores and around the implant is completed only some time later.

The quantitative determination of the chemical and structural changes of the inorganic phases of both biomaterials support the histological studies. Furthermore, these results prove that the process of in vivo absorption of Aragonite based biomaterial is in connection with the formation of a new hydroxyapatite phase, resembling the composition and structure of the natural bone tissue.

However, we observed that the CCA shows a very high reabsorption rate. From a critical point of view this fact can be undesirable in restorative surgery of bone with low osteogenic potential such as cranial or other cortical bones, particularly in great defects. The risk of the reabsorption of the biomaterial before the complete healing effect of the newly formed bone must be observed. For this reason it is necessary to be careful in the indication and application of the surgery treatment with such material.

The CCA from coral reef constitutes a unique and particular product due to its morphology and chemical composition. Its biocompatibility and capacity for healing the damaged bone tissue, demonstrated in the present study, was qualitatively similar to the one obtained by other authors with such type of biomaterials.

There are remarkable differences in the literature about the in vivo behavior of the magnitude of the biodegradation of the biomaterials of calcium carbonate as well as the interpretation of the processes or mechanisms that take place. Some authors have reported that the coral is reabsorbed completely in 12 weeks while others have not found total resorption in 6 years. However, in different clinical applications for 4 years, not enough results had been obtained to make definitive conclusion. In opinion of the authors, these mechanisms are very complex and they depend among other factors of the physical, chemical, morphological, mechanical and other properties of the own biomaterial, as well as of the characteristics of the biological environment and host reaction in the site where they are implanted.

The increment of the resorption rate of 0.2 %/d at the 7 d up to 0.7 %/d at the 60 d post-implantation of CCA found in this study denotes an acceleration of the process of degradation under the influence of the biological activity. This increased osteogenic property can be related to partial implant resorption in conjunction with the biological action. CCA is more soluble than HA and contributes to the surrounding tissue with the initial calcium and phosphate ions necessary for the osteogenesis and at the same time stimulate the formation of the new bone. This process rises to a progressive substitution (by steps) of the implant by the new formation of bone.

At the same time these results lead to suppose that the rate of reabsorption of such type of implant’s biomaterial may be controlled depending on the relation phosphate/calcium in its initial chemical composition.

In conclusion, aragonite based biomaterial can constitute an excellent implant’s biomaterial for the bone tissue reconstruction. Just like porous hydroxyapatite, it stimulates the proliferation of the living tissue inside the pores, but with a faster rate of bone generation, which is very useful in some clinical applications because the lesion restoration times is decreased. Its property to be reabsorbed, stimulate the formation of the new bone in its place and at the same time contribute to remodel the treated bone structures. But it is necessary to be careful in order to avoid failure of the implant by its complete resorption before the formation of the new bone.
Fig. 3. Histological appearance of CCA implants at 3 (a), 7 (b) and 15 d (c) after surgery (H&E 250x).
ACKNOWLEDGMENTS

The authors thank Elena González, Amelia Capote and Humberto García for technical assistance. To professor Jesús A. Núñez for the English revision of this manuscript and express their gratitude by the collaboration received from project VIII.6 of the Program CYTED (Ibero American Program for the Development of Science and Technology).

BIBLIOGRAPHY