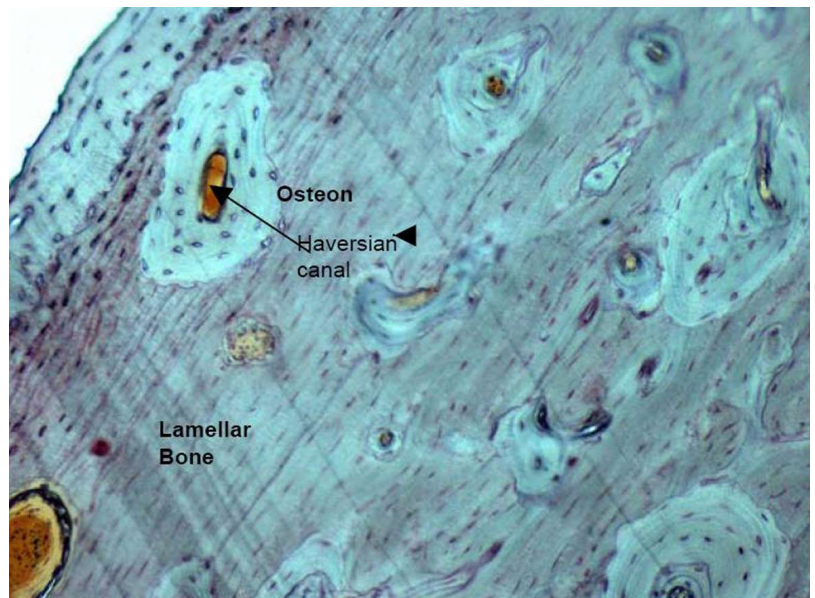
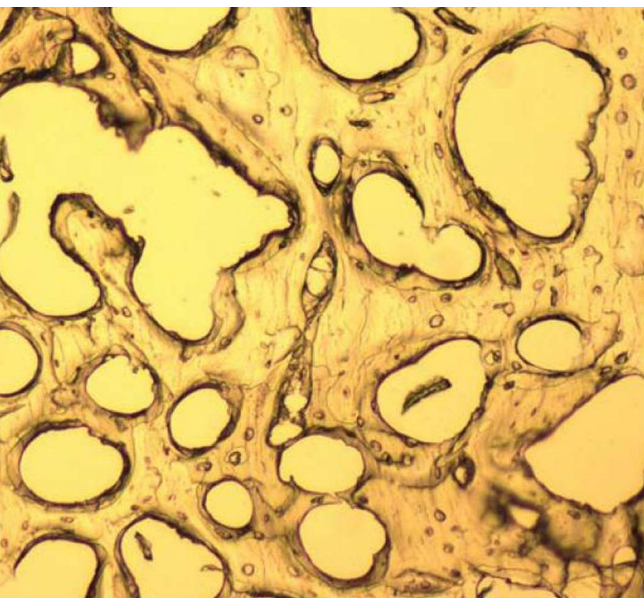


***IN VIVO* STUDIES OF BONE GRAFTS FOR MAXILLOFACIAL SURGERY**

JOSÉ VENTURA MACIEIRA DE SOUSA LOBATO



Dissertação de Doutoramento em Ciências Médicas
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Universidade do Porto
2007

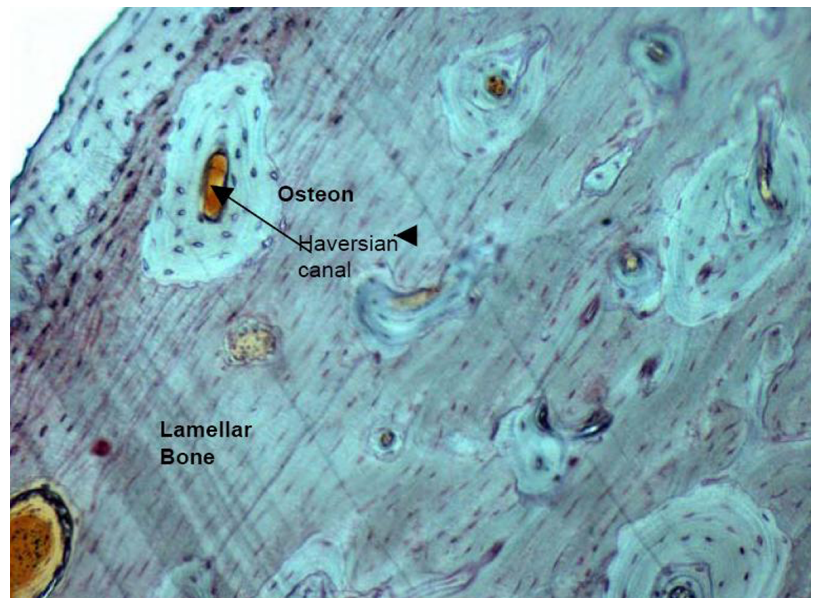
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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

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Dissertação de Candidatura ao grau de
Doutor em Ciências Médicas submetida ao
Instituto de Ciências Biomédicas de Abel
Salazar da Universidade do Porto

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Resumo

O estudo e o desenvolvimento de novos substitutos ósseos aumentaram de forma acentuada nas últimas décadas, principalmente devido às desvantagens e aos perigos dos auto-enxertos e dos alo-enxertos. Os auto-enxertos são considerados os enxertos ósseos ideais, no entanto a sua obtenção implica uma segunda intervenção cirúrgica, o que aumenta a morbidade do paciente. Alo-enxertos foram apresentados como uma alternativa aos auto-enxertos, provenientes essencialmente de osso de cadáveres. O enorme risco de transmissão de doenças priónicas e víricas (como o vírus do HIV, da hepatite B ou C) é enorme, para além do desenvolvimento frequente de reacções imunológicas de rejeição, que podem ser exuberantes. Hidroxiapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, é frequentemente utilizada como um biomaterial, devido à sua semelhança com a parte mineral do tecido ósseo humano. No entanto, foi demonstrado que a parte mineral do tecido ósseo humano é uma apatite de fosfato de cálcio multi-substituído. Deste modo, para que a HA tenha uma estrutura química muito próxima do tecido humano em questão, deve ter incorporado iões como o magnésio, flúor, sódio e silício. Em 1992, Santos e seus colaboradores demonstraram claramente que a bioactividade da HA poderia ser grandemente aumentada com a incorporação de um vidro baseado num sistema $\text{P}_2\text{O}_5\text{-CaO}$. Este biomaterial foi então patenteado com o nome de Bonelike[®]. A grande vantagem deste sistema reside na sua capacidade de incorporar diferentes iões na estrutura da HA, resultando num substituto ósseo sintético com uma composição química muito próxima da fase mineral do osso. Vários estudos *in vitro* e *in vivo* realizados com este substituto ósseo, o Bonelike[®], vieram a demonstrar a sua elevada bioactividade e a sua boa osteo-integração.

Esta tese pretendeu testar *in vivo*, no coelho, novas aplicações deste substituto ósseo e desenvolver uma versão de fácil aplicação, de grânulos de Bonelike[®] associados a um veículo biodegradável e biocompatível, que facilita a sua utilização em técnicas cirúrgicas de invasão mínima e adicionar moléculas terapêuticas. Pretendeu-se ainda testar em casos clínicos seleccionados, de quistos benignos ósseos da mandíbula ou da maxila, a aplicação destes grânulos de Bonelike[®]. Foram ainda realizados ensaios clínicos com implantes dentários de titânio, revestidos com Bonelike[®]. Nestes casos de implantologia, a osteo-integração do implante dentário foi grandemente aumentada. Finalmente, descreveu-se e discutiram-se os efeitos secundários dos bifosfonatos, como o zolendronato, em pacientes com mieloma múltiplo. Estes pacientes desenvolvem frequentemente osteonecrose da mandíbula ou da maxila, quando sujeitos a este tratamento prolongado.

Após resultados promissores obtidos *in vitro*, muitos testes animais foram desenvolvidos. Os grânulos de Bonelike® foram associados a duas matrizes reabsorvíveis disponíveis no mercado, FloSeal® e Normal Gel 0.9% NaCl® e a uma molécula terapêutica, o raloxifeno e foram testados em coelhos. Exame radiológico aos fêmures dos coelhos revelou uma elevada osteo-integração e uma regeneração dos defeitos ósseos induzidos e preenchidos por grânulos do substituto ósseo associados aos veículo e/ou ao raloxifeno. Durante o período de regeneração, os coelhos recuperaram rapidamente, não apresentando sintomas locais ou sistêmicos de rejeição. Após 12 semanas, foram sacrificados para análise histológica, que veio a confirmar a osteo-integração dos grânulos de Bonelike®, com a formação *de novo* osso. No caso de aplicação associada ao raloxifeno, no exame histológico não foi evidenciada actividade osteoclástica. Este resultado pode ser explicado devido á presença de raloxifeno, que bloqueia a actividade normal dos osteoclastos. Os grânulos de Bonelike® apresentavam-se perfeitamente envolvidos por novo osso, evidenciando-se ainda o desenvolvimento de uma rede de vascularização. Deste modo, a associação de grânulos de Bonelike® a matrizes reabsorvíveis parece ser um enxerto sintético de qualidade, para ser usado na regeneração de tecido ósseo. Adicionalmente, pode ainda funcionar como um sistema de libertação controlada de fármacos no local de regeneração.

Em cirurgia maxilo-facial e oral, Bonelike® foi usado para preencher importantes defeitos ósseos, após a remoção cirúrgica de quistos benignos, em 11 pacientes. Exame radiológico e os resultados histológicos claramente demonstraram a extensa formação *de novo* osso em torno dos grânulos, ao longo de uma interface parcial de biodegradação. Este efeito osteo-conductor bastante eficiente permitiu encurtar o tempo de regeneração destes defeitos ósseos na mandíbula e/ou na maxila destes doentes.

De modo a aumentar a osteo-integração de implantes dentários de titânio, estes foram revestidos por uma camada homogénea de Bonelike®. Os resultados histológicos de amostras biopsiadas demonstraram a formação *de novo* osso em torno dos implantes revestidos, com uma estrutura madura do tipo lamelar sem a presença de células inflamatórias nem de fibrose. Observações da microestrutura dos implantes revestidos com Bonelike® revelaram a presença de tecido ósseo aderente na sua superfície e uma estabilidade primária melhorada. Bonelike® provou ser um bom revestimento de implantes podendo ser utilizado no futuro, em implantologia.

Três pacientes com tecido ósseo exposto e com osteonecrose da mandíbula apresentavam um aspecto clínico em comum. Todos tinham mieloma múltiplo e estavam a ser tratados por administração endovenosa, com zolendronato, um bifosfonato, por longos períodos de tempo. Nestes 3 casos clínicos descritos, a suspensão do tratamento com zolendronato, uma antibioterapia intensa e a limpeza cirúrgica da osteonecrose da

mandíbula permitiram a recuperação evidente do quadro clínico. Deste modo, o tratamento com bifosfonatos em pacientes com mieloma múltiplo pode desencadear osteonecrose da mandíbula principalmente, após tratamentos de dentisteria ou aplicação de implantes dentários. A utilização de um substituto ósseo com as características descritas para o Bonelike[®] associado a uma matriz reabsorvível e ao raloxifeno, pode ser um tratamento bastante promissor para a osteonecrose da mandíbula desenvolvida por estes doentes.

Abstract

The research and development of new synthetic bone grafts increased over the past decades, mainly due to the disadvantages of autografts and allografts. Although autografts are considered the ideal bone graft, this type of bone graft requires an additional surgery, which increases morbidity to the patient and the volume obtained is relatively small. Allografts were presented as an alternative to autografts, but the risk of disease transmission, like HIV, hepatitis B and hepatitis C or any other disease still exists. In addition, there are reports of severe immunological reaction to the implant. Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is frequently used as a biomaterial, due to its similarity to the mineral phase of bone. However, it has been demonstrated that the mineral phase of bone is a multi-substituted calcium phosphate apatite, so in order to have a biomaterial with a closer chemical composition to the mineral phase of bone, different ions can be incorporated into the HA structure, like magnesium, fluoride, sodium and silicium. In 1992 Santos *et al* demonstrated that the bioactivity of HA could be enhanced by the incorporation of glass based on the P_2O_5 -CaO system. This new biomaterial was later patented as Bonelike[®]. The great advantage of this system is the ability to incorporate different ions into the HA structure, resulting in a biomaterial with a chemical composition closer to the mineral phase of bone. Several *in vitro* and *in vivo* tests with Bonelike[®] showed its high bioactivity and osteointegration.

This thesis was designed to address new applications of Bonelike[®], namely: to develop a user-friendly version of Bonelike[®] granules with the aim to obtain a system that would allow the association of therapeutic molecules to Bonelike[®] granules and whose application only require a minimal invasive surgery; to improve the osteointegration of titanium implants by coating them with Bonelike[®] and studying the biological response of different patients to the coated oral implants. Finally the secondary effects elicited by zolendronate, a bisphosphonate, characterised mainly by lytic bone lesions and osteonecrosis of the jaw in patients with multiple mieloma, were studied and reported.

After the promising results obtained *in vitro*, several animal tests were performed. Bonelike[®] granules were associated to two resorbable matrixes, FloSeal[®] and Normal Gel 0.9% NaCl[®] and to a therapeutic molecule, raloxifene hydrochloride. X-ray analysis of the rabbit femurs revealed high osteointegration and defect healing for all experimental conditions. During the healing period, rabbits easily recovered and no rejection symptoms were observed in the implantation site for all implanted samples. After 12 weeks histological analyses confirmed the osteointegration of Bonelike[®] granules and the new bone formation, with almost complete regeneration of the bone defects. Similar results were obtained after the histological analysis of Bonelike[®] granules associated with

FloSeal[®] and raloxifene hydrochloride. In this case no evidence of osteoclasts activity was observed which may be explained by the presence of raloxifene hydrochloride that is known to inhibit osteoclast activity. The Bonelike[®] granules were completely surrounded by new bone with vascular structures and cement lines indicating active bone regeneration, demonstrating the presence of an active angiogenesis, which is an extremely important process for bone regeneration. The Bonelike[®] associated to a resorbable matrix seemed to act as an excellent scaffold for bone regeneration. In addition, this system can act as a controlled release system for therapeutic molecules and therefore enhancing the osteointegration of Bonelike[®].

In oral and maxillofacial surgery, Bonelike[®] was used to regenerate bone defects after cyst removal in 11 patients. Radiographic examination and histological results clearly demonstrated an extensive new bone formation apposed on Bonelike[®] granules with a significant degree of maturation. These clinical applications in maxillary bone defects indicated perfect bonding between new formed bone and Bonelike[®] granules, along with partially surface biodegradation. This quick and effective osteoconductive response from Bonelike[®] reduced the time required to reconstruct the bone defected area of these patients.

In order to improve the osteointegration of titanium implants, they were coated with Bonelike[®]. The histological analysis of the biopsy samples showed new bone formation surrounding the Bonelike[®] coated implants with a mature lamellar-like structure without the presence of inflammatory cells or fibrous tissues. Microstructural observations of Bonelike[®] coated dental implants demonstrated that they had excellent bone remnants on their surface and an improved primary stability. Bonelike[®] proved to be an excellent coating for bone regeneration and therefore it maybe used in the future, in implantology.

Three patients with exposed bone and osteonecrosis of the mandible shared one common clinical feature: all of them were treated with bisphosphonate zoledronate, administered intravenously for long periods. In these 3 described clinical cases, surgical debridement without flap elevation, intensive antibiotherapy and the suspension of the zoledronate allowed a partial recovery of the patients. The purpose of this clinical report was to point out that patients suffering from multiple myeloma can develop bone osteonecrosis induced by the treatment with bisphosphonates. The use of bone substitutes like Bonelike[®] associated to a resorbable matrix and to therapeutic molecules like the raloxifene hydrochloride can be used to restore the bone tissue of patients suffering from ONJ, being this an attractive treatment for these typical clinical cases that develop ONJ.

Resumée

L'étude et le développement de nouveaux remplaçants osseux ont notoirement augmenté dans les dernières décades, principalement à cause des inconvénients et des dangers des autogreffes et des allogreffes. Quoique les autogreffes soient considérées des greffes osseuses idéales, elles exigent une seconde intervention chirurgicale, ce qui augmente la morbidité du patient. Les allogreffes, provenant essentiellement d'os de cadavres, ont été présentées comme une alternative aux autogreffes. Cependant, le risque de transmission de maladies prioniques et vireuses comme le virus HIV, de l'hépatite B ou C, est énorme, au-delà du développement fréquent de réactions immunologiques de rejection. L'hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, est fréquemment utilisée comme un biomatériel, grâce à sa ressemblance à la partie minérale du tissu osseux humain. Toutefois, des études ont montré que la partie minérale du tissu osseux humain est une apatite de phosphate de calcium multi-remplacé. De cette façon, l'hydroxyapatite doit posséder différents ions comme magnésium, fluor, sodium et silicium pour qu'elle ait une structure chimique semblable à celle du tissu humain. En 1992, Santos et al., ont prouvé que la bioactivité de l'HA pourrait être augmentée grâce à l'incorporation d'un verre fondé sur un système $\text{P}_2\text{O}_5\text{-CaO}$. Ce nouveau biomatériel a été alors patenté sous le nom de Bonelike®. Le principal avantage de ce système est sa capacité d'incorporer différents ions dans la structure de l'HA, tout en créant un remplaçant osseux synthétique dont la composition chimique est pareille à la phase minérale de l'os. Plusieurs études in vitro et in vivo réalisés avec ce remplaçant osseux, le Bonelike®, ont montré que sa bioactivité et son ostéointégration sont notables. Ce travail a voulu tester in vivo, sur des lapins, de nouvelles applications de ce remplaçant osseux: développer une version de Bonelike®, sous la forme de granules associés à un véhicule biodégradable et biocompatible qui puisse être utilisé à travers des techniques chirurgicales d'invasion minime et additionner des molécules thérapeutiques. On a encore voulu tester l'application de ces granules de Bonelike® dans des cas cliniques spécifiques de kystes osseux bénignes de la mandibule ou de la maxille. On a aussi réalisé des essais cliniques avec des implants dentaires de titanium, revêtus de Bonelike® et on a constaté une rapide ostéointégration de l'implant dentaire. Finalement, on a analysé et décrit les effets secondaires des biphosphates, comme le zolendronate, sur des patients avec myélome multiple. Ces patients, lorsqu'ils sont soumis à ce traitement pendant longtemps, souffrent souvent d'ostéonécrose de la mandibule ou de la maxille.

Après des résultats in vitro prometteurs, plusieurs tests ont été réalisés. Les granules de Bonelike® ont été associés à deux matrices resorbable disponibles dans le marché,

FloSeal® et Normal Gel 0.9% NaCl® et à une molécule thérapeutique, le raloxiphène, et ont été testés sur des lapins. L'analyse radiologique des fémurs des lapins a montré une bonne intégration et la régénération des défauts osseux grâce aux granules du remplaçant osseux associés au véhicule et/ou au raloxiphène. Les lapins ont rapidement récupéré pendant la période de régénération et n'ont présenté aucun symptôme de rejection. Après 12 semaines, l'analyse histologique a confirmé l'ostéo-intégration des granules de Bonelike® et la formation d'un nouveau os. Dans le cas de l'application associée au raloxiphène, l'analyse histologique n'a montré aucune activité ostéoclastique, ce qui peut être expliqué par la présence de raloxiphène qui empêche l'activité normale des ostéoclastes. Les granules de Bonelike® étaient complètement revêtus par le nouveau os et on a assisté au développement de la vascularisation. L'association de granules de Bonelike® à des matrices résorbables semble donc constituer une très bonne greffe pour la régénération du tissu osseux. Elle peut encore fonctionner comme un système de libération contrôlée de pharmacos dans la zone de régénération.

En ce qui concerne la chirurgie maxillo-faciale et orale, on a utilisé Bonelike® pour remplir des défauts osseux importants, après le remuement chirurgical de kystes osseux sur onze patients. L'analyse radiologique et les résultats histologiques ont clairement démontré la formation extensive d'un nouvel os autour des granules. Cet effet ostéoconducteur a permis de réduire la période de régénération des défauts osseux sur la mandibule et/ou sur la maxille des patients.

Ces implants dentaires de titanium ont été revêtus d'une couche homogène de Bonelike® pour augmenter l'ostéointégration. Les résultats histologiques d'échantillons soumis à une biopsie ont prouvé la formation d'un nouvel os autour des implants revêtus, avec une structure mature du type lamellaire sans la présence ni de cellules inflammatoires ni de fibrose. L'observation de la microstructure des implants revêtus de Bonelike® a montré la présence de tissu osseux adhérent sur la surface et une stabilité primaire augmentée. Bonelike® a prouvé être un bon revêtement d'implants et, dans l'avenir, il pourra être utilisé dans le domaine de l'implantologie.

Trois patients présentant tissu osseux exposé et ostéo-nécrose de la mandibule possédaient un aspect clinique commun. Ils avaient un myélome multiple et avaient pendant longtemps été soumis à un traitement intraveineux avec zolendronate, un bisphosphonate. Dans ces trois cas cliniques, la suspension du traitement avec zolendronate, une antibiothérapie intensive et l'antisepsie chirurgicale de l'ostéonécrose de la mandibule ont permis la récupération du cadre clinique. Le traitement en employant des bisphosphonates sur des patients avec myélome multiple peut donc provoquer l'ostéonécrose de la mandibule principalement après des traitements de dentisterie ou après l'application d'implants dentaires. L'utilisation d'un remplaçant osseux comme Bonelike® associé à une

matrice resorbable et au raloxiphène peut constituer un traitement prometteur de l'ostéonécrose de la mandibule de ces patients.

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Chapter 1

General Introduction

General Introduction

The research and development of new synthetic bone grafts increased over the past decades, mainly due to the disadvantages of autografts and allografts, widely reported in the literature¹⁻⁹.

Autograft is considered the ideal bone graft due to the lack of immunological response and its ability to provide osteoinductive growth factors, osteogenic cells and to act as structural scaffold¹⁰. Although, the two main disadvantages of this bone graft is the requirement for an additional surgery to harvest the tissue, which increases the blood loss, causing extra morbidity to the patient and also its limited supply^{1,2}.

The allografts were presented as an alternative to autografts, but the risk of disease transmission, like HIV, hepatitis B and hepatitis C or any other transmissible disease still exists, additional that are reports of severe immunological reaction to the implant³.

In 1987 a biomaterial was defined as *"a nonviable material used in a medical device, intended to interact with biological systems"*¹¹. An important characteristic of a biomaterial is its biocompatibility that can be described as *"the ability to perform with an appropriate host response in a specific application"*¹¹.

Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is frequently used as a biomaterial, due to its similarity with to the mineral phase of bone. Although, it has been demonstrated that the mineral phase of bone is a multi-substituted calcium phosphate apatite, so in order to have a biomaterial with a closer chemical composition to the mineral phase of bone, different ions can be incorporated into the HA structure^{12,13}, like magnesium¹⁴, fluoride¹⁵, sodium¹⁶ and silicium¹⁷⁻¹⁹.

In 1992 Santos *et al* demonstrated that the bioactivity of HA could be enhanced by the incorporation of glass based on the P_2O_5 -CaO system. This new biomaterial was later patented as Bonelike^{®20-22}. The great advantage of this system is the ability to incorporate different ions such as magnesium, sodium and fluoride, which resulted in a biomaterial with a chemical composition closer to the mineral phase of bone^{23,24}.

Several *in vitro* and *in vivo* tests with Bonelike[®] showed its high bioactivity and good osteointegration. Similar results were also obtained in preliminary clinical trials, where it was demonstrated that Bonelike[®] enhances bone regeneration.

So, this thesis was designed to address new applications of Bonelike[®], such as:

- Develop of a user-friendly version of Bonelike[®] with the aim to obtain a system that would allow the association of therapeutical molecules to Bonelike[®] granules and also a system that would require a minimal invasive surgery for its application, or would not require a second intervention.
- To improve the osteointegration and biocompatibility of titanium implants by coating them with Bonelike[®] and studying the biological response of different patients to the coated oral implants.
- Study the secondary effects of the use of an osteoclasts inhibitory molecule such as zolendronate, a bisphosphonate, in patients with multiple myeloma. The application of bone grafts like Bonelike[®] associated to raloxifene hydrochloride, a molecule that inhibits osteoclasts' activity, to restore the jaw osteonecrosis (ONJ) in patients suffering from multiple myeloma and treated with bisphosphonates should be considered.

Maxillofacial Anatomy

This thesis is particularly focused in the application of a bone graft, the patented Bonelike[®], in implantology and maxillofacial surgery. So, a much resumed description of the maxillofacial anatomy is firstly introduced in order to clarify any doubt concerning the anatomical localization and the diseases' pathophysiology reported and discussed though the text.

The Superior Maxillary is one of the most important bones of the face, from a surgical point of view, due to the number of diseases to which some of its parts are liable. Its detailed examination becomes, therefore, a matter of considerable importance. It is the largest bone of the face, excepting the lower jaw, and forms, by its union with its fellow of the opposite side, the whole of the upper jaw (Figure 1). Each bone assists in the formation of three cavities, the roof of the mouth, the floor and outer wall of the nose, and the floor of the orbit; enters into the formation of two fossae, the zygomatic and sphenomaxillary, and two fissures, the sphenomaxillary, and pterygo-maxillary, and serves for the reception of the superior teeth. Each bone presents for examination a body, and four processes, malar, nasal, alveolar, and palatine. It forms articulations with nine bones, two of the cranium – the frontal and ethmoid, and seven of the face, the nasal, malar, lachrymal, inferior turbinated, palate, vomer, and its fellow of the opposite side. Sometimes it articulates with the orbital plate of the sphenoid²⁵.

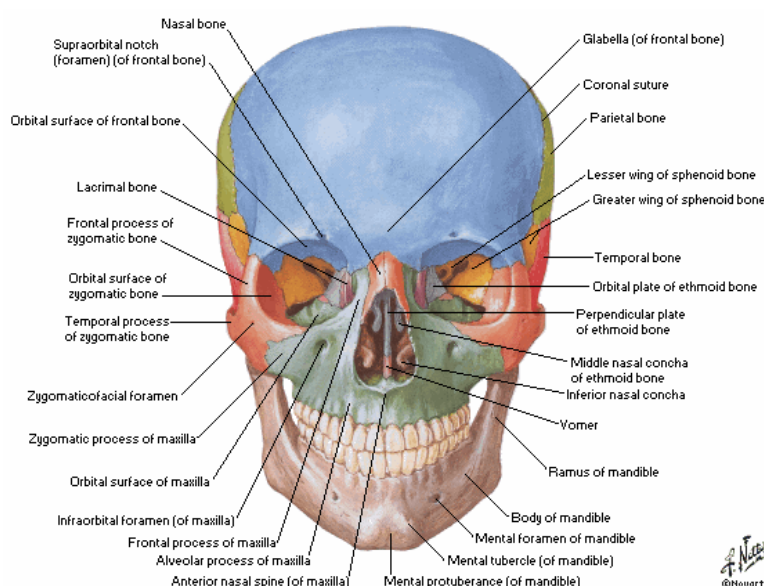


Figure 1 – Structure of the Skull (adapted from Netter²⁵)

The muscles that are attached to the superior maxillary are the *orbicularis palpebrarum*, *obliquus inferior oculi*, *levator labii superioris alaeque nasi*, *levator labii superioris proprius*, *levator angulioris*, *compressor naris*, *depressor alae nasi*, *masseter* and *buccinators*²⁵ (Figure 2).

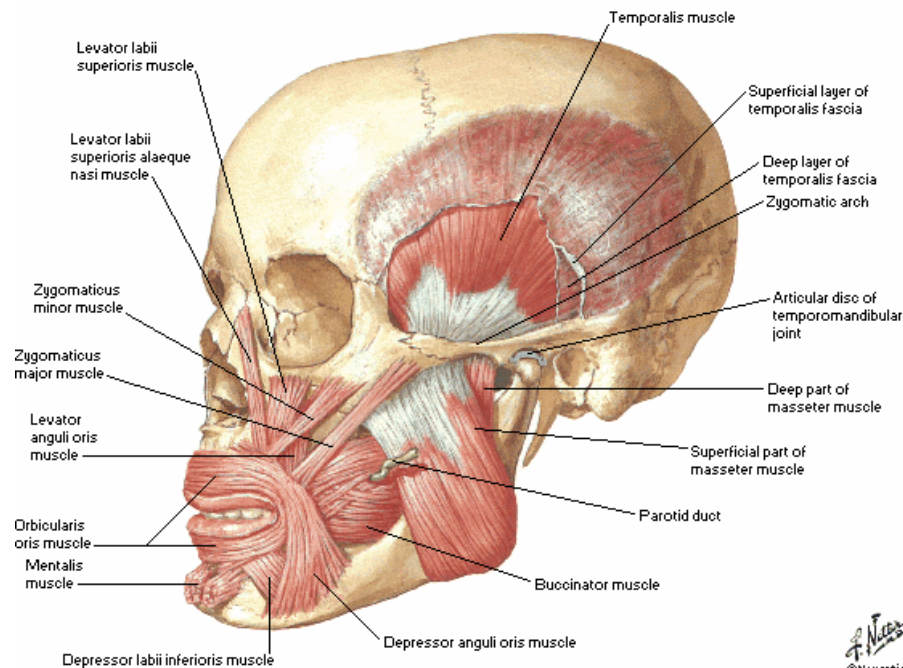


Figure 2 – Muscles involved in Mastication (adapted from Netter²⁵)

The Inferior Maxillary Bone (the jaw), the largest and stronger bone of the face, serves for the reception of the inferior teeth. It consists of a curved horizontal portion, the body, and of two perpendicular portions, the *rami*, which join the former nearly at right angles behind. It forms articulations with the *glenoid fossae* of the two temporal bones. Also several muscles, some of them very potent, are attached to this bone. Its external surface, commencing at the symphysis, and proceeding backwards, it is attached to the *levator menti*, *depressor labii inferioris*, *depressor anguli oris*, *platysma myodes*, *buccinator*, and *masseter*. Its internal surface, commencing at the same point, *geniohyoglossus*, *genio-hyoideus*, *mylo-hyoideus*, *digastric*, *superior constrictor*, *temporal*, *internal pterygoid*, and *external pterygoid* (Figure 3)²⁵.

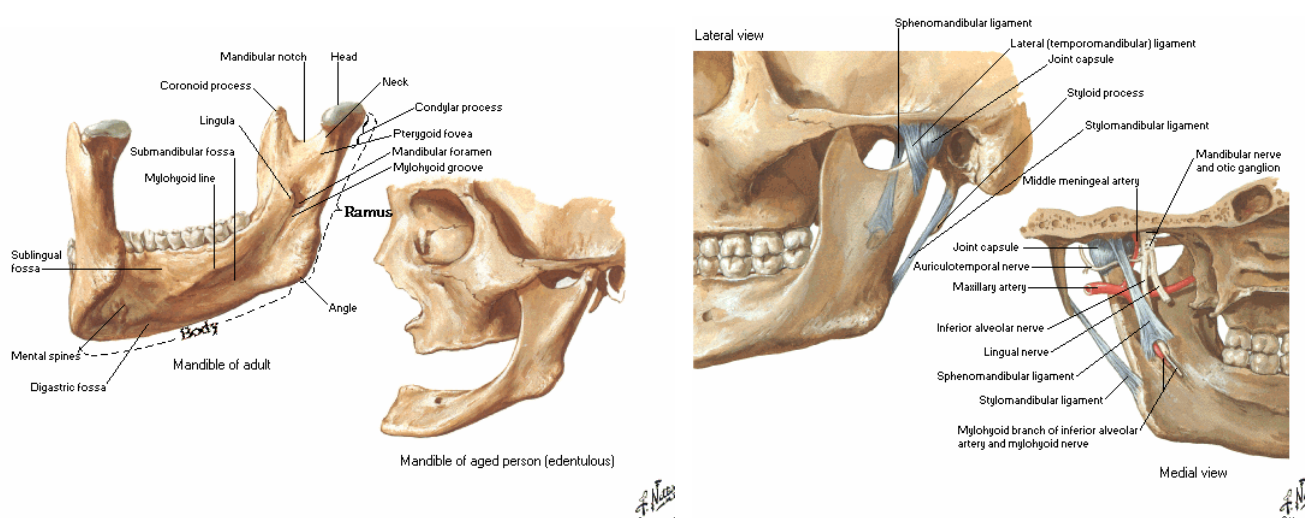


Figure 3 – Mandible (adapted from Netter²⁵)

As a result of several diseases, like mandibular neoplasia, alveolar cysts and jaw osteonecrosis in patients suffering from multiple myeloma and treated with bisphosphonates, a patient can lose part of the maxilla or of the mandible; therefore there is the need to restore the functionality and symmetry of these bones. If the resulting defect is very large, there is the need to fill the space with a bone graft.

Multiple Myeloma, an Example of a Bone Disease

Multiple Myeloma (MM) accounts for 10-15% of haematologic neoplasms and about 1% of all cancer deaths. MM presents two variants: non-secretory and secretory type. Within the secretory form there are several subtypes. Non-secretory MM accounts for 3% of myeloma patients²⁶. With more sensitive testing with the immunoglobulin free light chain assay²⁷, many of these 'non-secretory' patients are found to be oligosecretory. The presentation is similar to that of secretory myeloma with the exception that myeloma kidney does not occur²⁸. A reduction in background immunoglobulins is common and lytic bone disease is present in most patients. Median survival of these patients is at least as good as for those with secretory myeloma. Response is difficult to document, but quantification of serum free light chain is possible in about two thirds of these patients²⁷.

Immunoglobulin (Ig) D myeloma accounts for about 2% of all cases of myeloma²⁹. The presence of a monoclonal IgD in the serum almost always indicates MM or acute leukaemia (AL), but there have been some cases of IgD MGUS reported²⁹. Patients with IgD myeloma generally present with a small band or no evident M spike on serum protein

electrophoresis. Their clinical presentation is most similar to that of patients with Bence Jones myeloma (light chain myeloma) in that they have a higher incidence of both renal insufficiency and coincident amyloidosis as well as a higher level of proteinuria than in IgG or IgA myeloma. With an incidence of 19–27%, extramedullary involvement is more prevalent in patients with IgD myeloma. Though initial reports suggested that survival with IgD myeloma was inferior to MM, this was not the case in the Mayo Clinic series²⁶. IgE myeloma is a rare form of myeloma. A disproportionate number of cases have plasma-cell leukaemia. Only about 40 cases of IgE myeloma have been reported in the literature³⁰.

Waldenström macroglobulinemia (WM) should not be confused with IgM myeloma, which comprises less than 1% of myeloma cases²⁶. Patients with WM may have anaemia, hyperviscosity, B symptoms, bleeding, and neurologic symptoms. Significant lymphadenopathy or splenomegaly may also be present. Lytic bone disease is rare, but if present IgM myeloma should be considered. In WM, bone-marrow biopsy typically reveals infiltration with clonal lymphoplasmacytic cells, which are CD20-positive. The natural history and treatment options for WM differ from those of MM.

As mentioned previously, MM accounts for 10-15% of haematologic neoplasms and about 1% of all cancer deaths. The most common clinical presentation of MM is the recent onset of unexplained back pain or normochromic, normocytic anaemia in older patients. In recent times, however, up to 60% of new patients are first diagnosed when a serum or urine M-component is detected on routine laboratory testing²⁶.

The M protein (M component, monoclonal protein, myeloma protein, or M spike) is a hallmark of the disease. Ninety-seven per cent of myeloma patients have either an intact immunoglobulin or a free light chain that can be detected by protein electrophoresis, immunoelectrophoresis, or immunofixation of the serum or urine²⁶. M proteins are used to recognize the disease, to calculate myeloma tumour burden and kinetics, to stage myeloma patients, and to document their response to treatment. In a series of 1027 newly diagnosed cases of myeloma, the immunoglobulin type was IgG, IgA, IgD, and free light chain only (Bence Jones myeloma) in 52, 21, 2, and 16% of cases, respectively³¹. Less than 1% of myeloma cases are IgM. Ninety-three per cent of patients have a monoclonal protein detected in their serum. About 70% have a monoclonal protein detected in the urine. Of patients previously designated as non-secretory, approximately two thirds have a detectable immunoglobulin free light chain with the immunoglobulin free light chain assay²⁷. In general, there is a correlation for any given patient between M protein and the degree of bone-marrow plasmacytosis. Patients who have had, and been treated for, myeloma for a number of years may develop light chain escape or extramedullary disease that is relatively non-secretory. For these reasons, sole dependence on serum M proteins

is insufficient; periodic measurements of urinary protein and evaluations of skeletal radiographs are imperative^{26,27}.

Approximately 70% of MM patients are over 60 years of age and 90% are over 50 years. The diagnosis is frequently missed on the first evaluation of the patient. Up to 80% of MM patients present bone pain as the first clue of disease and more than 70% of MM patients develop one or more pathologic fractures during the course of their disease³²⁻³⁵. The cause of the bone disease is the local activation of osteoclasts by the clonal plasma cells. This involves the release of cytokines such as IL-1 β , tumour necrosis factor- α , IL-6, and most important, MIP-1 α and MIP-1 β . The latter is associated with an up-regulation of RANKL (receptor activation of NK- κ β ligand) and a down-regulation of OPG (osteoprotegerin, a natural antagonist of RANKL). Plasma cells may also be able to change the RANKL/OPG ratio through direct cell contact. In either case, the over expression of RANKL is associated with an increased generation of osteoclasts from monocyte precursors. Less commonly, MM may present as an isolated mass lesion, a solitary plasmacytoma. These lesions may be found in several areas, such as: skin, gastrointestinal (GI) tract and nasopharynx. They are not clinically distinctive and can only be defined as plasmacytomas by biopsy. The renal manifestations and the hypercalcemia are two important clinical features. Occasionally the MM patient presents acute renal failure or sudden symptomatic hypercalcemia. The cause of the hypercalcemia is primarily the rapid destruction of bone by osteoclast-activating factors secreted by plasma cells and/or bone marrow stromal cells. Amyloid nephropathy with irreversible renal damage is less common. MM patients have an increased susceptibility to infection, due to the decreased rate of production of normal immunoglobulins, possibly because dendritic cell dysfunction or a leukocyte abnormality induced by the M-component. The major haematological manifestation of MM is anaemia, due to the decreased of the erythropoiesis. The degree of anaemia may be disproportionate to the degree of marrow involvement by plasma cells. Patients who have begun chemotherapy for the disease may have severe myelo-suppression. Less commonly, the M-component may interfere with platelet function, leading to bleeding, or with leukocyte function, leading to recurrent infections³²⁻³⁵. Since the first symptoms, the average survival time of patients with myeloma left untreated is 6 to 12 months, and with treatment about 3 years. When myeloma is diagnosed before onset of symptoms, the life expectancy can be higher. Available therapeutic options result in considerable toxicity and offer only a low prolongation of life. For these reasons most physicians do not begin treatment before the onset of symptoms³²⁻³⁵.

When clear signs of progression occur, or when the patient becomes symptomatic, therapy should be started. Until the early 1950s, radiotherapy and surgery were the only

treatments available for the myeloma patient. Although, both treatments could effectively palliate the majority of patients, these interventions have little impact on the overall course of the disease. With the development of effective chemotherapy, the role of these other treatments became of secondary importance in the overall management of the myeloma patient. With the recent use of new treatments like, hemibody irradiation, total body irradiation, and bone seeking radionuclides, as part of high-dose therapy regimens, radiation treatment may become recognized as an important part of the systemic management of disease in these patients. The recent development of the minimally invasive surgical technique kyphoplasty for the treatment of patients with vertebral compression fractures (VCFs) has led to a major improvement in the quality of their lives. So, for many years, the recommended therapy has been melphalan 6-9 mg/m² daily together with prednisone 40-60 mg/day given for 4-7 days and repeated every 4-6 weeks. To maximize effectiveness, the dose should be adjusted to produce a mild neutropenia, a granulocyte count of 1000-1500 cells/μL, or a platelet count of around 10⁴ cells/μL. Multidrug regimens and newer therapies have been used, especially for younger patients who can tolerate more aggressive therapy, and those patients who present progress on standard therapy. These multidrug regimens include combination of vincristine, doxorubicin, and dexamethasone (VAD) or the M² and C-VAMP protocols (vincristine, adriamycin, BCNU, melphalan, cyclophosphamide, and prednisone). Several clinical trials have reported promising results using maintenance therapy with interferon-α to prolong remissions. Promising results with thalidomide, alone or in combination with chemotherapy, especially with dexamethasone, are being reported³²⁻³⁵. New agents under trial include CC-5013 (Revimid), a potent immunomodulatory derivative of thalidomide, and PS 341 (Velcade). The latter is a proteasome inhibitor with apparent activity in refractory myeloma patients. Other therapeutic agents under consideration in the treatment of refractory patients include arsenic trioxide, antibodies against IL-6 and CD20, and Gleevec. In addition, the bisphosphonates (pamidronate, zoledronic acid) originally used to control hypercalcemia and bone lesions in MM, are now showing an effect on survival. It would appear that the inhibition of osteoclast activity helps to reduce IL-6 levels and induce myeloma cell apoptosis. Whether bisphosphonate therapy will have a positive impact in patients who lack bone is still an open question. High-dose chemotherapy (melphalan 200 mg/m²) with or without total body radiation followed by peripheral blood stem cell rescue (autologous transplantation) can result in a complete remission together with a prolonged survival. Autologous transplantation is not, however, a pathway to cure. Peripheral blood collections of CD34+ progenitor cells are almost certainly contaminated with malignant plasma cell precursors, which will lead to a future relapse. In patients with an HLA-matched sibling, allogenic marrow transplantation has been performed with

promising results, although the early death rate from marrow failure, infection and acute GVHD is very high, near 50%. Obviously, this approach can only be considered in a relatively small number of patients who are under 50 years old and have a HLA-matched sibling donor. Also a nonmyeloblastic allogeneic transplantation using an HLA-matched sibling or non-matched donor has been shown in early trials to be effective, based on the anti-tumour effect of the resulting graft versus host disease (GVHD)³²⁻³⁵.

Among the haematological malignancies, MM stands out for its destructive action on bone resulting in severe pain and disability, as referred previously. During the course of their disease, most patients will have severe and sometimes intractable pain due to progressive osteolysis and pathological fractures. Even patients responding to chemotherapy may have progression of the skeletal disease^{36,37}, and recalcification of the osteolytic lesions is rare. Bone loss, either from direct tumoral involvement or from generalised osteoporosis can lead to pathologic fractures, spinal cord compression, hypercalcemia, and pain, being the major cause of morbidity and mortality in these patients³⁸. These patients frequently require radiation therapy, surgery, and use of analgesics. The prevention or, at very least, inhibition of lytic bone lesions are very important aspects in the clinical approach of these patients. The bisphosphonates are potent inhibitors of osteolysis. These agents have been evaluated alone and as adjunctive therapy to primary anti-cancer treatment in patients with cancers involving the bone, and are now widely used in the control of myeloma bone disease. Recent studies show the efficacy and increased convenience of the newer, more potent imidazole-containing bisphosphonate zoledronic acid in the management of the skeletal complications of myeloma. A number of other types of new anti-bone-resorptive agents are also in early clinical development. Recent new surgical techniques such as kyphoplasty offer the opportunity to greatly improve the quality of life of myeloma patients with vertebral compression fractures³⁸.

These complications, like hypercalcemia and osteolytic lesions, result from an asynchronous bone turnover wherein increased osteoclastic bone resorption is not accompanied by a comparable increase in bone formation. This increase in osteoclastic activity is mediated by the release of osteoclast-stimulating factors, which are produced locally in the bone-marrow microenvironment by cells of both tumour and non-tumour origin³⁹. The enhanced bone loss results from the interplay between the osteoclasts, tumour cells and other non-malignant cells in the bone marrow microenvironment⁴⁰. The bisphosphonates are non-metabolized analogues of endogenous pyrophosphates (PP_i) that can be localized in bone and inhibit osteoclastic function. Pyrophosphates are natural compounds which contain two phosphonate groups bound to a common oxygen atom. They are potent inhibitors of bone resorption *in vitro*; however, when used *in vivo* these

compounds are readily hydrolyzed and are ineffective in reducing bone resorption³⁴. By simply substituting the oxygen atom by a carbon atom, the molecule becomes resistant to hydrolysis and yet remains active as an inhibitor of bone resorption. With the carbon substitution, these synthetic compounds, known as bisphosphonates, contain two additional chains of variable structure (called R1 and R2) that have given rise to a large number of different drugs. Most bisphosphonates contain a hydroxyl group at R1 that confers high affinity for calcium crystals and bone mineral. Marked differences in anti-resorptive potency result from differences at the R2 site³⁴. These drugs are poorly absorbed orally (usually <1%) and are also poorly tolerated orally, with significant gastrointestinal toxicity, particularly oesophagitis and oesophageal ulcers. The bisphosphonates are almost exclusively eliminated through renal excretion, and significant nephrotoxicity can occur with these compounds. Because bisphosphonates have high affinity for bone mineral, the drug is highly concentrated in bone. These molecules bind avidly to exposed bone mineral around reabsorbing osteoclasts, resulting in very high levels of bisphosphonates in the resorption lacunae; therefore, high concentrations of bisphosphonates are maintained within bone for long periods of time. Bisphosphonates are then internalized by the osteoclast, causing disruption of osteoclast-mediated bone resorption^{41,42}. Their potential for strong inhibition of osteoclastic bone resorption and high affinity for hydroxyapatite crystals have progressively extended the field of their clinical indications³²⁻³⁵. Such compounds are able to chelate Ca^{2+} ions very effectively, and its high affinity for Ca^{2+} crystals permits its binding to hydroxyapatite crystals in the mineralised bone matrix¹³. The exact mechanism of the bisphosphonates-mediated osteoclast inhibition has not been completely elucidate, but it has been established that these compounds affect bone turnover at various levels^{41,42}. On a tissue level, bisphosphonates inhibit bone resorption and decrease bone turnover as assessed by biochemical markers^{41,42}. On a cellular level, the bisphosphonates clearly target the osteoclasts and may inhibit their function in three possible ways: (1) inhibition of osteoclast recruitment⁴³, (2) reduction of the osteoclast life span⁴⁴, and (3) inhibition of osteoclastic activity at the bone surface⁴⁵. On a molecular level, it has been postulated that bisphosphonates modulate osteoclast function by interacting with a cell surface receptor or an intracellular enzyme⁴⁶. Several structurally related bisphosphonates have been synthesized by changing the two lateral chains on the carbon or by sterifying the phosphate groups⁴⁷. The resulting analogues vary extensively in their anti-resorptive potency⁴⁸, with analogues such as etidronate being the weakest, alendronate being stronger, and the new analogue, zoledronate, being the most potent^{47,49}. Intravenous bisphosphonates are the current standard for the treatment of hypercalcemia of malignancy (HCM) and prevention of skeletal complications associated with bone

metastases^{48,50,51}. Currently, zoledronic acid (2-[imidazol-1-yl]-1-hydroxyethylidene-1, 1-phosphonic acid, Zometa[®], 4 mg via a 15-minute infusion) and pamidronate (Aredia[®], 90 mg via a 2-hour infusion) are the only agents recommended by the American Society of Clinical Oncology (ASCO) for the treatment of bone lesions derived from breast cancer and multiple myeloma^{52,53}. Furthermore, zoledronic acid is approved by both the U.S. Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products for the prevention of skeletal complications in patients with multiple myeloma, bone metastases secondary to a variety of solid tumours, (breast, prostate and lung cancer) and malignant hypercalcemia⁵⁴⁻⁵⁷. These intravenously administered bisphosphonates significantly reduced the development of skeletal complications and improved the survival of patients⁵⁴⁻⁵⁷. Recent studies have demonstrated the efficacy and increased convenience of the newer, more potent imidazole-containing bisphosphonate zoledronic acid in the management of the skeletal complications of myeloma^{40,58} and also provides long-term reduction of bone pain in patients with bone metastases secondary to prostate cancer^{40,58}. If tolerated, it is common for these patients to be maintained indefinitely on bisphosphonates therapy⁴¹. The oral bisphosphonate preparations (alendronate and risedronate) are also potent osteoclast inhibitors, but are not as effective in the treatment of malignant osteolytic disease, and therefore are only prescribed for the treatment of osteoporosis⁴¹. Bisphosphonates-associated osteonecrosis of the jaws (ONJ) is currently a very topical subject. Initially, it was thought to be an extremely rare condition but in a retrospective review of multiple myeloma and breast cancer, ONJ was reported in 10.5% of those who received intravenous bisphosphonates at the Memorial Sloan-Kettering Cancer Centre in 2003⁵⁹. Osteonecrosis has not been seen at any other skeletal site in these patients. Bisphosphonates-associated ONJ is characterized by dehiscence of the oral mucous membranes, with exposure of the underlying mandible or maxilla where it can be observed bone necrosis. More than 50% of the cases have been diagnosed after surgery procedures, like extractions, implants and periodontal procedures. In some clinical cases, ONJ does not respond to any form of treatment that has yet been attempted, like interruption of the chemotherapy and bisphosphonates administration. Hyperbaric oxygen reportedly had no effect⁶⁰. Antibiotics cannot penetrate the necrotic tissue, being only used to manage cellulites in adjacent tissues. By default, a conservative and symptomatic treatment is the current recommendation. Patients receiving bisphosphonates infusions are asked to avoid oral surgery^{61,62}. The mechanism underlying the reaction is unknown, but it has been postulated that bisphosphonates inhibit new vessel formation. In many cases, dental extractions and other oral surgeries have been identified as precipitants. Cancer diagnosis, concomitant therapies (chemotherapy, radiotherapy and

corticosteroids) and morbid conditions (anaemia, coagulopathies, infection, and pre-existing oral disease) are documented risks factors⁶³.

Among other anti-resorptive agents, an analogue of the natural inhibitor of receptor activator of nuclear factor κ B (RANK) known as osteoprotegerin (OPG) presents promising results in terms of suppression of bone resorption markers^{31,64-66}. Notably, OPG binds tumour necrosis factor-related apoptosis-inducing ligand/Apo2 ligand (TRAIL), and, as a result, OPG can inhibit the induction of apoptosis of myeloma cells generated by TRAIL^{31,64-66}. Moreover, it is possible that the development of antibodies to OPG may occur in patients treated with the analogue, resulting in the prevention of its normal anti-bone resorptive function. To avoid these potential problems with the use of OPG analogues, a recombinant form of RANK ligand (RANKL), RANK-Fc, that is an antagonist of RANKL–RANK signalling, has been recently developed, and inhibits both bone disease and myeloma growth in a murine SCID-hu model of human myeloma. This recombinant protein is now being evaluated in clinical trials among patients with metastatic bone disease. In addition, inhibitors of Src activity show marked anti-resorptive capability and may enter clinical trials soon. The statins have shown the potential to increase bone density by their stimulatory effects on specific bone morphogenetic proteins (BMPs) involved in stimulating bone formation as well as their inhibitory effects on mevalonic acid biosynthesis which results in the lack of prenylation of critical cellular proteins such as the GTPases which are known to play key roles in both bone pathophysiology and myeloma growth^{31,64-66}.

Summarizing, the major clinical problems that arise in myeloma patients relate to the enhanced bone loss that commonly occurs in these patients. Recent improvements in radiological techniques have enhanced the ability to detect bony involvement more accurately. With the development of minimally invasive surgical procedures such as kyphoplasty that effectively treat vertebral compression fractures, it becomes increasingly useful to find these fractures in myeloma patients. Recent advances in the use of bone-seeking radiopharmaceuticals make these attractive therapeutic candidates to combine with the new anti-myeloma drugs (thalidomide, bortezomib and arsenic trioxide) since these latter agents are also radio-sensitizing. The results of two large phase III clinical trials show the benefit of adjunctive use of intravenously administered monthly bisphosphonates (zoledronic acid or pamidronate) in addition to chemotherapy in safely reducing bone complications in myeloma patients. Bisphosphonate treatment should now be considered for all myeloma patients with evidence of bone loss. Although preclinical studies suggest the potential anti-myeloma effects of especially more potent nitrogen-containing bisphosphonates, clinical trials - probably at higher doses given more slowly - will be necessary to establish their anti-tumour effects. ONJ is currently a very topical

subject. Bisphosphonates-associated ONJ is characterized by dehiscence of the oral mucous membranes, with exposure of the underlying mandible or maxilla where it can be observed bone necrosis. Most cases occur after surgery procedures, like extractions, implants and periodontal procedures. The mechanism underlying the reaction is unknown, but it has been postulated that bisphosphonates inhibit local angiogenesis. Dental extractions and other oral surgeries have been identified as precipitants and cancer therapies like chemotherapy, radiotherapy and corticosteroids, and morbid conditions, like anaemia, coagulopathies, infection, and pre-existing oral disease are documented risks factors. A number of promising new agents, including RANK-Fc, are in early clinical development for the treatment of myeloma bone disease.

Bone Grafts

A bone graft has three main functions: to restore skeletal integrity, to give mechanical support and enhance bone healing. In terms of biological response a perfect bone graft should be able to carry living cells (osteogenic), it should stimulate precursor cells in the implant site or surrounding environment to undergo phenotypic conversion into bone cells (osteoinductive) and its surface should allow bone formation (osteoconductive)¹⁰. In some clinical situations the ability of a material to provide support or fill a void is more important than its biological performance, such as in the case of a large proximal femoral graft in a revision total hip arthroplasty or in other cases such as in lateral spinal fusion it is more important that the bone graft stimulates bone formation⁶⁷.

A bone graft can be classified according to its origin; autograft, allograft, xenograft and alloplastic or synthetic graft.

Autografts

An autograft is considered the ideal bone graft; it is osteogenic, osteoinductive and osteoconductive¹⁰. This type of graft involves harvesting bone tissue from one site within the patient, such as: iliac crest or tibiae, and implanted in the defect site of the patient itself¹⁰.

Another advantage of the use of an autograft is the lack of an immune response, rejection or disease transmission. The autograft contains cartilage matrix, proteins, minerals and osteogenic marrow cells⁶⁸. The major drawback of this type of bone graft is

related with its limited supply and the need to subject the patient to a second surgery, which results in pain and morbidity at the donor site. These symptoms can persist even after wound healing^{1,2}.

According to the literature, skull trepanation dates back to 12 000 BC, and this technique was developed due to the need of healing wounds^{69,70}. It has been reported that, in 1810, Merrem was able to heal bone plates of an animal skull after trephining. Later on in 1821, von Walther applied a similar technique in human, being this the first recorded bone autograft in humans^{70,71}. In 1889, Seydel reported a new technique, where he removed tissue from the tibiae of a patient and implanted in his skull⁷⁰.

Autogenous cancellous bone, nonvascular cortical autografts and vascular cortical autografts are the three main types of autografts used clinically.

Autogenous cancellous bone grafts

The main source of this type of bone graft is the iliac crest and the proximal tibiae from the patient itself. As mentioned previously this type of bone graft is osteogenic, easily vascularised and integrates into the defect site without any signs of rejection or adverse immune response. The patient response to a cancellous bone graft occurs in several steps, being the first step, the reaction to haemorrhage and inflammation that resulted from the surgical procedure and during transplantation part of the cells are damaged, especially osteocytes, although the remaining osteoblasts and osteoprogenitor cells survive and are able to produce new bone^{67,72}.

The vascularisation of this type of bone graft is facilitated due to its porosity that allows the formation and infiltration of blood vessels and also bone cells^{72,73}. Following this stage, the necrotic bone is resorbed by osteoclasts, triggering the remodelling mechanism of a healthy bone. It has been reported that after 12 months the autogenous cancellous bone graft is part of the support structure⁷².

Nonvascular cortical autografts

The nonvascular cortical autografts offer a stronger mechanical support than the autogenous cancellous bone graft, although they are less bioactive, due to its low porosity, which complicates the vascularization and infiltration of bone cells or osteoprogenitor cells⁴. This is only achieved by osteoclastic resorption and vascular invasion of Volkmann's and Haversian canals. According to Burwell *et al*⁴, this process weakens the bone structure due to an excessive resorption. This graft has less

osteoprogenitors cells and haematopoietic cells and a higher rate of resorption than the cancellous bone graft.

Vascular cortical autografts

A vascular cortical autograft is harvested with a pedicle that will be anastomosed to the new site of implantation. It has been reported that this type of graft provides a limited structural support and that the interface between the graft and the surrounding tissue forms very quickly⁷². The percentage of cells that survive this surgical procedure is much higher in comparison with nonvascular cortical autografts. Goldberg *et al*⁷⁴ and Doi *et al*⁷⁵ reported that the vascular cortical autograft is remodelled due to local mechanical and cellular stimulation.

Disadvantages of autograft

As mentioned previously the autograft can be considered the ideal bone graft, although there are a few disadvantages associated with this type of bone graft, being the most important the requirement for an additional surgery^{1,2}. The surgical procedure required to harvest the bone tissue into the donor site results in increased blood loss, pain and an additional scar. These symptoms can persist even after the wound healed^{1,2}. Another limitation of this type grafts is related with the prolonged surgery time that can lead to complications⁶⁸. As reported earlier, the main source of autografts is the iliac crest, Seiler and Johnson⁷⁶ reported that this can cause arterial injury, urethral injury, hernia, chronic pain, nerve injury, infection, fracture, pelvic instability, cosmetic defects and haematoma caused by excessive blood loss.

Allografts

The allografts appeared as an alternative to the autografts. The allograft procedure involves the harvest of bone tissue from a human donor, besides the patient, therefore eliminating of one the surgeries. The common source of allograft is bone from cadavers.

The use of allografts it has been described since the 19th century. It has been reported that the first successful human allografts was performed in 1881 by Macewen^{70,77}, according to the literature, Macewen removed tissue from a boy's tibiae

and implanted in the humerus of another boy, being this procedure considered “*the first paragraph of a new chapter in the history of surgery*”^{70,78}.

The structural and biological performance of an allograft is dependent on the preparation method¹⁰. The most common allografts are the morsellised cancellous and cortical allograft, the corticocancellous and cortical allografts and demineralised bone matrix.

Morsellised cancellous and cortical allograft

Morsellised cancellous and cortical allograft is characterised by a porous structure which allows the bone ingrowth and vascularisation of the new formed bone. Due to the preparation methods, this type of bone grafts does not have living cells, so they are not osteoinductive.

Bulk corticocancellous and cortical allograft

Friedlander *et al*⁷⁹ described the use of bulk corticocancellous and cortical allografts in the reconstruction of bone defects after resection. This type of bone graft is usually preserved at by freezing or freeze-drying. The different method of preservation requires different procedures for the implantation. If this graft is freeze it can be implanted straight after thawing, but in the case of freeze-drying, the allograft has to be re-hydrated before implantation.

Demineralised bone matrix (DMB)

Reports regarding the use of demineralised bone grafts date from the late 19th century and it has been reported that Senn^{70,80} (1889) treated patients with long bones and cranial defects using decalcified xenogeneic bone implants.

There are several stages on the preparation of a demineralised bone matrix graft, being the first one the removal of the bone marrow, followed by the elimination of fat and finally the mineral content is dissolved with hydrogen chloride, leaving the collagen matrix intact⁷⁰.

In 1965 Urist^{70,81} studied implanted demineralised bone matrix allografts using several animal models, like rabbits, mice, rats and guinea-pigs. He implanted DMB intramuscularly of the different animals and found new bone formation, so he concluded that this fact was related to the proliferation of pluripotent ingrowing cells of the host and

to an inductive agent. Since then it has been shown that the inductive agent is indeed a series of glycoproteins belonging to the transforming growth factor family, being of particular interest the bone morphogenetic proteins (BMPs), of which BMP-2 and BMP-7 are known to have inductive properties^{70,81}. One limitation of this type of bone graft is related to its poor mechanical properties.

Disadvantages of allograft

The use of sterilization by gamma radiation (or ethylene oxide) and removal of blood and cellular constituents diminish the risks of infection and disease transmission of the allografts, although the risk of transferring viral contaminants such as HIV, hepatitis B and hepatitis C or the transmission of potential unknown diseases is still high³. Additionally this type of bone graft can induce severe immunological reactions.

The method of preparation and preservation will influence the mechanical structure and biological properties of the graft. For example if the tissue was freeze-dried or sterilized by gamma radiation, the graft will lose its osteoinductive and the osteogenic properties, due to the death of most of the cells during the preparation process. So, most allografts do not have cells, which results in the loss of its osteogenic properties^{10,72}. Urist found that the decrease in the osteogenic properties of the demineralised allograft was related to the amount of hydrochloric acid used in the process⁷⁰. The fresh allografts are rarely used clinically due to the high risk of disease transmission, and due to severe immunological reactions, as mentioned previously.

The limitations of autografts and allografts led to a great advance in the development of synthetic alternatives.

Synthetic Bone grafts

The limitations of the autografts and allografts procedures lead to great advances in the development of synthetic alternatives. Several types of materials have been proposed as bone substitutes namely: titanium, aluminium, stainless steel, cobalt-chromium alloys and titanium alloys, ceramic, polymers and glasses⁸².

The major advantages of the synthetic biomaterials are: they avoid the need for a harvesting procedure, therefore eliminating chronic pain at the harvest site; the risk of disease transmission is quite low, the immune response is lower than the one observed by immune response to allografts and there is no limited supply.

The metallic materials used clinically, like titanium do not present any of the requirements for an ideal bone graft, osteoconductive, osteoinductive or osteogenic properties, therefore they have poor osteointegration. These materials can provide mechanical support in load-bearing applications, although due to the mismatch in the mechanical properties of the materials and bone, this can cause stress shielding, leading to resorption of the surrounding bone⁸³.

Another class of materials used as bone grafts are polymers, such as, silicone rubbers, polyurethanes, hydrogels and polymethyl methacrylate (PMMA). The physical properties of this type of material can be tailored to specific applications, depending on the manufacture procedure. They can be prepared in the form of fibres, films, rods and viscous liquids. The PMMA bone cement it has been used often clinically, although several problems have been reported such as: the formation of bubbles during mixing and polymerisation that can function as stress points leading to cracks, its polymerisation is exothermic, so large amounts of heat are produced which can cause thermal necrosis of the surrounding bone and also methylmethacrylate is toxic, if it does not react in the cement, the monomer can enter the blood stream of the patient causing a reduction in blood pressure⁸⁴.

For the past decades a special interest in ceramics as bone grafts was demonstrated by researchers worldwide. The bioceramics can be defined as “*ceramic materials used in medical devices, intended to interact with biological systems*”, they can be used to fill spaces, as coatings or as a second phase in a composite⁸².

The first report on the use of bioceramics dates from 1894^{85,86} and describes the use of calcium sulphate, known as Plaster of Paris, to fill bone defects. Due to its poor mechanical properties and rapid deterioration caused by resorption, its used was limited.

The *in vivo* response to bioceramics depends on several factors, such as: tissue type, health and age, implant composition and phase, blood circulation in tissue and interface, surface morphology and porosity motion at the interface, chemical reactions, closeness of fit and mechanical load⁸². So, the materials can be classified according to biological reaction they elicit *in vivo* (Table 1)⁸². Some materials can elicit a toxic response that damages and/or kill cells or even due to the release of chemical substances that can go into the blood stream causing systemic damage to the patient⁸⁷.

Table 1 – Reaction induced by biomaterials after implantation⁸².

Implant	Consequence	Materials
Biologically nearly inert	This material induces a very small response from the host tissue, leading to the formation of a non-adherent fibrous capsule around the implant.	Zirconia, Alumina, Titanium
Bioactive	This material elicits a specific biological response at the interface of the material resulting in the formation of a bond between the tissue and the material.	Bioactive glasses, Bioactive glass-ceramics, HA, Bonelike®
Resorbable	Implant dissolves and /or is degraded by cells and replaced by tissue.	Tricalcium Phosphate, Bioactive glasses

The physical-chemical properties of the material influence the intensity and time duration of the inflammatory and wound-healing processes⁸⁸ caused by the implantation of the bone graft.

The implantation of an inert biomaterial induces a sequence of events leading to the formation of fibrous capsule. During the inflammation phase, plasma proteins and leukocytes (mainly neutrophils) migrate to the implantation site⁸⁹⁻⁹¹. After the migration of the leukocytes to the implant site, phagocytosis and the release of enzymes start, leading to the activation of neutrophils and macrophages. When the cellular mechanism does not have the ability to phagocytate the implant, enzymes of the macrophages stimulate fibroblasts to produce collagen to form a fibrous capsule around the implant. The biological response to bio-inert materials is not only dependent on its chemistry, but most important is related to movement, if it is not properly fitted the movement the capsule will be thicker until equilibrium is reached⁸². On the other hand if the implant is properly fitted the phagocytic response is transient, the capsule will be very thin and inactive soon after the implantation. In the presence of alumina or zirconia a very thin layer will form, but if the material is more chemical reactive the layer will be thicker⁹². A bioactive material can form an interfacial bond, due to its controlled rate of chemical reactivity, which leads to the formation of dynamic equilibrium at the interface. The formation of a bioactive interface between the host-tissue and the implant occurs when the tissue apposes directly the implant surface, leading to a biological fixation, which prevents movement of the implant⁸². A common characteristic of the bioactive implants is the formation of a hydroxyl-carbonate

apatite layer; which is similar to the composition and structure to the mineral phase of bone⁸².

Synthetic hydroxyapatite (HA) is used as a bone graft substitute, due to its similarity in composition to the mineral phase of bone and to its bioactivity. According to the literature HA has the ability to form an interface with bone, without the formation of a fibrous capsule⁹³⁻⁹⁵. A resorbable material is degraded by body fluids or digested by macrophages and it is extremely important that the degradation products are not toxic to the cells and can be easily disposed by the cellular mechanisms⁸². The main goal of this type of materials is to degrade at the same rate that new bone is formed⁸², although a high degree of solubility can cause problems regarding its mechanical performance while the regeneration is taking place and another issue related to this type of material is the difficulty in matching the dissolution rate of the material with the repair rate of the tissue. The tricalcium phosphate ceramic is a resorbable material and it can be degraded to calcium and phosphate salts in the body and be used as bone filler.

Nowadays, several bioceramics are used in the clinical, such as: bioactive glasses, HA, tricalcium phosphate⁸². The characteristics of the material should be optimised, depending on the function that the material should play in the body, for example a single crystal such as sapphire can be used as a dental implant due to its high strength, A/W glass-ceramic can be used to replace vertebrae due to its high strength and its ability to bonds to bone. Bioactive glass has low strength, although they bond very rapidly to bone, therefore they should be use in repair of bone defects⁸². As mentioned previously, HA is the most common ceramic used as a bone graft due to its similar composition to the mineral phase of bone, although the natural apatite should be described as a multi-substituted calcium phosphate apatite^{12,13}. So, one way to approximate the chemical composition of HA to the natural apatite is by the incorporation of different ions into the HA. The most common substitution is by carbonate ions^{96,97}, there are also reports regarding the incorporation of other ions present in the mineral phase of bone such as magnesium¹⁴, fluoride¹⁵ and sodium¹⁶.

Santos *et al*⁹⁸ developed a new composite a glass-reinforced HA. This systems comprises the incorporation of a glass of the P_2O_5 -CaO system into the HA lattice, being one of the most important advantages of this system the incorporation of different ions such as magnesium, sodium and fluoride, resulting in a material with a chemical composition similar to the mineral phase of bone^{23,24}. This composite was later patented as Bonelike®. The incorporation of a glass not only increases the bioactivity by reproducing the inorganic phase bone but also significantly improves the mechanical properties of the material, because during the liquid phase sintering process the porosity and grain size are reduced⁹⁹.

Glass-Reinforced Hydroxyapatite (Bonelike®)

It is known that the physical-chemical properties influence the biological response to the implant¹⁰⁰⁻¹⁰³. Therefore, during the development of this material all its physical-chemical properties were studied and optimised.

Through x-ray diffraction it was possible to observe that the incorporation of a glass induced the formation of secondary phases, such as α and β - tricalcium phosphate and also that the amount of glass incorporated influenced the percentage of secondary phases¹⁰⁴. This fact is due to decomposition of HA on the presence of reactive glass, which causes the hydroxyl groups to be driven out and affects the calcium phosphorous ratio. At very high temperatures the β - tricalcium phosphate converts into α - tricalcium phosphate without further decomposition of the HA phase. It is known that the presence of magnesium induces the formation of β - tricalcium phosphate and retards the formation of β - tricalcium phosphate into α - tricalcium phosphate¹⁰⁵⁻¹⁰⁸.

According to the literature the presence of secondary phases, namely β - tricalcium phosphate, influences the surface charge and wettability properties of the material¹⁰⁹. Another advantage of the incorporation of a glass into the structure of HA is related to increased mechanical properties. Lopes *et al*¹⁰⁵ found that the flexural bending strength of the Bonelike is two to three times higher than that of HA and also that the Bonelike® presents higher values of fracture toughness, although this property is highly depend on the chemical composition, the percentage of glass and on the sintering temperature¹¹⁰.

In vitro tests using osteosarcoma cell line MG63 and osteogenic-induced bone marrow cells showed that Bonelike® allows the attachment, proliferation and differentiation of these cell types. The cells cultured on its surface were able to express collagen type I, fibronectin and osteocalcin, characteristic of a normal osteoblastic culture. It was also possible to see that the osteogenic-induced bone marrow cells can induce the formation of a mineralised matrix¹¹¹⁻¹¹³.

Preliminary *in vivo* tests with Bonelike® using a rabbit a model demonstrated that this material was fully osteointegrated after 12 weeks of implantation, without the presence of any inflammatory cells. Additionally, the push-out test showed a strong interface implant/new bone, because the failure occurred essentially through the implant body²⁴.

All these results showed that Bonelike® is bioactive and allows the formation of new bone. Although, this material has an excellent biological response, in some clinical applications there is a need to develop new approaches, namely the incorporation of therapeutical molecules and possibility to have an injectable system.

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Chapter 2

Granular Bonelike®

Several animal studies with Bonelike® showed its excellent osseointegration and high bioactivity. Similar results were also obtained in preliminary clinical cases, where it was demonstrated that Bonelike® enhances bone regeneration.

In this chapter it is described the development of a user-friendly version of Bonelike® that can open-up new areas of application in Medicine. The aim was to develop a system that would allow the association of therapeutical molecules to Bonelike® granules and also that would only require a minimal invasive surgery for its application. Therefore, the first step was to test *in vivo* Bonelike® granules with a precise granulometry, followed by the association of a resorbable matrix and a therapeutical molecule, the raloxifene hydrochloride. These tests were performed using an animal model, the rabbit.

The two granulometries tested (150-250µm and 250-500µm) showed excellent osteointegration. After 12 weeks of implantation all the Bonelike® granules were surrounded by *de novo* bone and the Bonelike® granules were partially resorbed.

The histological and scanning electron microscopy (SEM) analysis showed that new bone was rapidly apposed on implanted granules and also that the presence of a matrix (FloSeal® or Normal Gel 0.9% NaCl®) and a therapeutic molecule (raloxifene hydrochloride) did not alter the proven highly osteoconductivity properties of Bonelike®. Therefore, the association of a resorbable matrix and a therapeutical molecule to a precise size of Bonelike® granules is one step-forward for the clinical applications of Bonelike® since it is much easier-to-handle when compared to granular materials.

Assessment of the Potential of Bonelike® Graft for Bone Regeneration using an Animal Model

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Abstract

Bonelike® graft that mimics the inorganic composition of bone tissue has been developed and characterized over the last decade. To evaluate the osteoconductivity of Bonelike® two granule size ranges, one ranging from 150-250µm and the other from 250-500µm were implanted in the femurs of New Zealand White rabbits, aiming at being clinically used in different medical applications, such as dentistry and orthopaedics. In order to facilitate the medical application of the Bonelike® graft the use of a commercially available polymeric vehicle was also analyzed. Radiological examination, histological studies and scanning electron microscopy (SEM) analyses revealed that the surface of Bonelike® granules was almost completely surrounded by new bone formation after 12 weeks of implantation, which proves its highly osteoconductive behaviour.

Introduction

Nowadays, it is possible to prepare synthetic bone substitutes that have similar composition to the mineral osseous tissue. This aspect is important to increase the regeneration and neo-formation of bone, since it promotes an ideal micro-environment where cellular adhesion, differentiation and mitosis are possible to occur. Some of these bone grafts have the ability of being re-absorbable in a time controlled way, in order to allow the correct process of natural re-construction of the involved bone tissue. Over the last decade, Bonelike® graft, which accomplishes the above mentioned requirements, was developed and characterized^{1,2}. For several medical applications of bone regeneration, the use of a vehicle to carry the biomaterial graft is considered as being a very relevant issue. In fact, this association not only facilitates the medical application of the bone graft but also will open-up new areas of application in medicine, namely those related to: (i) minimal invasive surgery and (ii) the possibility of associating therapeutic molecules that have crucial function in bone regeneration. The aim of this work is to evaluate the osteoconductivity and biofunctionality of Bonelike® in an animal model using two particle size ranges, 150-250 µm and 250-500 µm, aiming at being clinically used in several medical areas of bone regeneration. The possibility of associating the Bonelike® graft with a polymeric vehicle has also been analyzed. Rabbits were sacrificed 12 weeks after implantation, and the retrieved samples studied by radiographic examination, scanning electron microscopy (SEM) and Solo Chrome-R staining for histological studies.

Materials and Methods

Bonelike® preparation

Two different granules size of Bonelike® graft, one ranging from 150-250 µm and other from 250-500 µm were produced as follows. Firstly a P_2O_5 -CaO based glass with the composition of 65 P_2O_5 -5 H_5CaO -10 CaF_2 -10 Na_2O in mol% was prepared from reagent grade chemicals by using a platinum crucible heated at 1400°C for 2hrs. After pouring, the produced glass was crushed in an agate mortar and sieved to a particle size less than 75 µm. The Bonelike® was obtained by mixing 2.5% of glass with the laboratory prepared hydroxyapatite (HA) in isopropanol. The mixed powders were dried for 24h at 60°C and sieved to less than 75 µm and then isostatically pressed at 200 MPa. Finally, the obtained Bonelike® was again sintered at 1300°C for 1hr. Using standard crushing and sieving techniques, two particle size ranges were obtained, 150-250µm and 250-500µm.

In vivo studies

Healthy skeletally mature male New Zealand White rabbits (Charles River Laboratories, Spain) with a weight between 2.5-3.5 kg were used as experimental animals. National guidelines for the care and use of laboratory animals were always observed and the surgeries were done after approval from the Animal Ethics Commission from Instituto de Ciências Biomédicas Abel Salazar (ICBAS), University of Porto. General anaesthesia was performed using isoflurane and intravenous injection of pentobarbital sodium solution. Operations were carried out using a standard procedure in aseptic conditions. Incision sites were shaved, cleaned, and disinfected. A longitudinal incision was made on the lateral surface, extending from about 20 mm below the coxo-femoral joint for a distance of 25 mm, exposing the femur. In each femur, 3 holes of 3.0 mm diameter were drilled through cortex and into medulla, using a micro-burr with a 3.0 mm tip continuously flushed with a saline solution (NaCl 0.9%, Braun) to minimize thermal damage (see Figure 1).

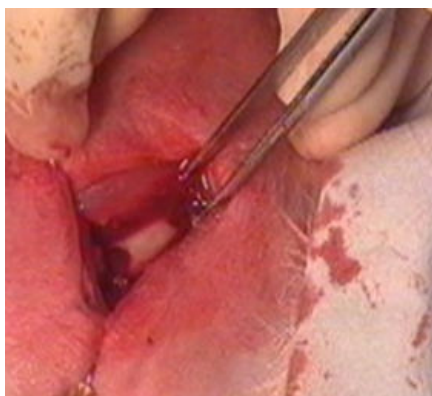


Fig.1. Rabbit left femur showing 2 of 3 holes with 3.0 mm diameter that were drilled through cortex and into medulla, using a micro-burr with a 3.0 mm tip.

To permit an accurate identification of the central portion of the defect, marker pins were placed at the proximal and distal margins of the femur. The defects were flushed with saline solution (NaCl 0.9%, Braun) to remove any residual bone. Bonelike® granules of both granulometries (150-250µm and 250-500µm) mixed and/not-mixed with autologous medullar blood were implanted in the 3.0mm diameter holes. A ready-to-use polymeric vehicle paste was also mixed with Bonelike® and its injectability assessed (Normal gel 0.9% NaCl, Monelycke, Portugal). For each experimental condition 6 distinct holes were prepared. Rabbits were sacrificed 12 weeks after implantation and the retrieved samples analyzed by radiographic examination, scanning electron microscopy (SEM) and Solo Chrome-R staining for histological studies.

Results and Discussion

Bonelike® is a synthetic bone graft composed of a mixture of hydroxyapatite, β - and α - tricalcium phosphate (TCP) phases^{3,4} as shown in Figure 2.

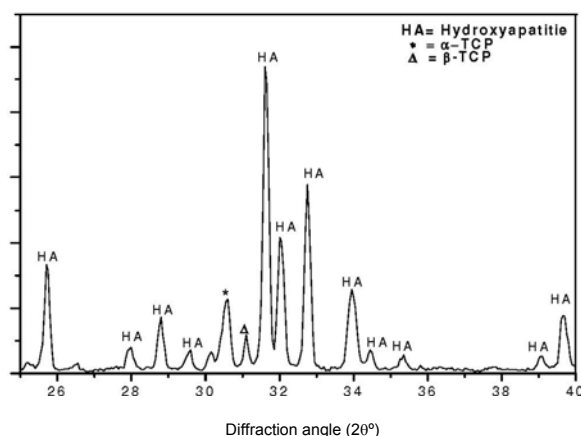


Fig.2. X-ray diffraction pattern of Bonelike®, which shows the presence of HA, α - and β -TCP phases

This phase composition is a result of the reaction of the hydroxyapatite matrix with the $\text{CaO-P}_2\text{O}_5$ based glass that occurs during the sintering process². Granules size can alter the particle packing characteristics and therefore it effect on bone defect regeneration. Hence, two sizes of Bonelike® granules were chosen and having in mind the two different potential fields of application, maxillofacial surgery and orthopaedics, where bone defects usually differs in size. Granules showed a randomised aspect ratio before implantation and in some of them an approximate acicular shape has been observed (Figure 3).

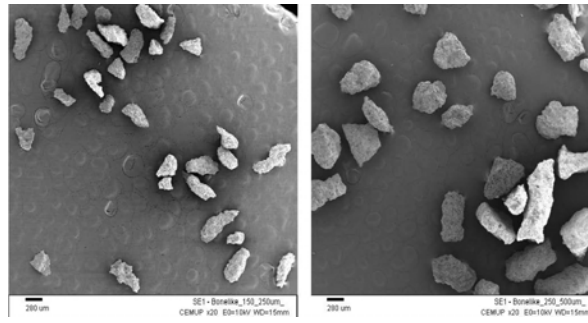


Fig.3. SEM images of Bonelike® granules with different sizes implanted in the femurs of rabbits.

Radiological examination revealed good osteointegration and defect healing for both particle size ranges of implanted Bonelike, as it may be observed in Figure 4 a-b.

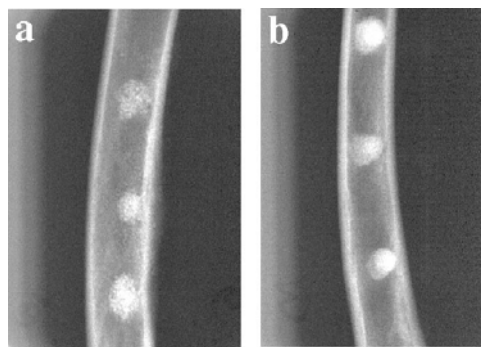


Fig.4. Radiological images of implanted Bonelike granules. For both granules size complete healing of the bone defect was observed, a) 150-250µm and b) 250-500µm

The osseointegration of Bonelike® granules and the new bone formation has been confirmed by both SEM and histological analyses, as depicted in Figure 5 a-b.

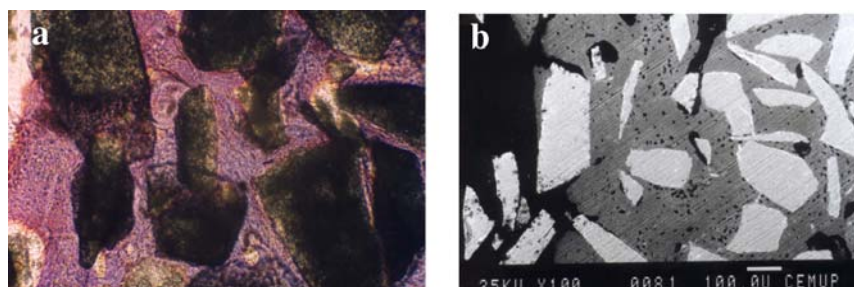


Fig.5. Solo Chrome-R staining and SEM images of Bonelike®/de novo bone formed after 12 weeks of implantation, granule size 250-500 µm.

After 12 weeks of implantation most of the Bonelike® granules are surrounded by *de novo* bone, which corroborates previous published results². The new bone formation occurred through an osteoconduction mechanism and resorption of Bonelike® granules was observed for both particle size ranges, which indicates that this bone graft may be

considered as partially resorbable for this implantation period. Radiological and histological analysis revealed that no complete resorption of Bonelike® granules occurred, which may be an important factor for medical applications where morphological contours of the anatomical site have to be preserved, as it occurs in several maxillofacial applications. Studies conducted with a polymeric vehicle demonstrated that the medical application of Bonelike® is facilitated. The influence of the polymeric paste on the osteoconductivity of Bonelike® is currently under study.

Conclusions

Bonelike® graft has proved to be highly osteoconductive for the two studied particle size ranges, 150-250µm and 250-500µm, and therefore it has a good potential material to be clinically used in several areas of reconstructive surgery, such as maxillofacial and orthopaedics.

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Assessment of Bonelike[®] Graft with a Resorbable Matrix using an Animal Model

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Abstract

Synthetic bone grafts have been developed to provide an alternative to autografts and allografts. Bonelike® is a patented synthetic osteoconductive bone graft that mimics the mineral composition of natural bone. In the present preliminary animals studies a user-friendly version of synthetic bone graft Bonelike® have been developed by using a resorbable matrix, FloSeal®, as a vehicle and raloxifene hydrochloride as a therapeutic molecule, that is known to decrease osteoclast activity and therefore enhanced bone formation. From histological and scanning electron microscopy evaluations, the use of Bonelike® associated with FloSeal® and raloxifene hydrochloride showed that new bone was rapidly apposed on implanted granules and also that the presence of the matrix and therapeutic molecule does not alter the proven highly osteoconductivity properties of Bonelike®. Therefore, this association may be one step-forward for the clinical applications of Bonelike® scaffolds since it is much more easy-to-handle when compared to granular materials.

Keywords: Bonelike®, FloSeal®, Raloxifene Hydrochloride, Animal Model, Histological Analysis.

Introduction

Nowadays, the life expectancy is two times higher than in the beginning of the 20th century (e.g. in the EUA in 1900 the life expectancy was approximately 48 years and now its around 75-80 years). So, the human body is subjected to higher cumulative stress that results in degradation of the tissues and hence new therapies are required to overcome these problems^{1,2}, such as bone grafts, that is the second most common transplantation tissue³. Recently, Murugan *et al*⁴ reported that, in Europe the number of bone grafting procedures was 287,300 in 2000 and it is expected to increase to approximately 479,079 in 2005. The worldwide use of bone grafts was estimated in 1 million, of which 15% were synthetic bone grafts and it was also suggested that future growth is mainly due the development of tissue-engineered composites, i.e. composites containing osteogenic cells and growth factors⁴.

A bone graft should have particular characteristics depending on its application, for example if a quick bond to bone is required then a highly bioactive implant material should be used. L. Hench defined a bioactive material as “a non-toxic, biologically active and that forms an interfacial bond with the host”¹.

The ultimate goal of a synthetic bone graft field is to mimic the biological properties of natural bone. Therefore, the morphology and properties of natural bone should be used as a standard that has to be met by the ideal bone substitute⁵. According to its origin bone grafts can be classified as autografts, allografts, and xenografts⁶. All of them present advantages and disadvantages⁷. Autograft, do not induce an immunological reaction and it has the ability to provide osteoinductive growth factors, osteogenic cells and structural scaffolds⁸, although they require an additional incision site and increased blood loss^{9,10}. With the use of allografts there is the risk of transferring viral contaminants such as HIV, hepatitis B, hepatitis C and the promotion of immunological reactions. Due to adverse antigenic responses xenografts are not considered suitable for bone grafting. Hence, synthetic bone graft substitutes have been developed and clinically used to provide an alternative to autografts and allografts.

The bone grafts can be classified into three types depending upon their biological properties namely osteoconductive, osteoinductive and osteogenic grafts³. In literature wide varieties of bone grafts materials have been reported for use on *de novo* bone formation *in vivo*^{3, 4, 7,11-13}.

Osteoconductive synthetic hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ has been marketed in a variety of forms and used as a graft material due to its chemical composition, which has similarities with the mineral phase of bone. The natural apatite can be described as a multi-substituted calcium phosphate apatite^{14,15}. Hence, one-way to improve HA bioactivity is by the incorporation of different ions into the HA lattice. Santos *et al* showed that the bioactivity of HA can also be enhanced by the incorporation of glass based on the $\text{P}_2\text{O}_5\text{-CaO}$ system, a material patented and recently marketed as Bonelike^{®16-20}. This system allows the incorporation of several ions, such as magnesium, sodium and fluoride resulting in a bone graft with a chemical composition similar to the mineral phase of bone. This novel biomaterial as a result of its controlled chemical phase composition of HA, α and β -tricalcium phosphate (TCP) and its microstructure showed to have better mechanical properties and enhanced bioactivity than the actual commercially HA²¹⁻²². The use of Bonelike[®] associated with a vehicle will facilitate the medical application of this bone graft and also allow the incorporation of biological molecules that can stimulate bone formation *in vivo*.

FloSeal[®] is a haemostatic sealant²³ composed of collagen-derived particles and topical bovine-derived thrombin²⁴⁻²⁶ and it has been proven to control bleeding in several medical applications, like vascular surgery, adenoidectomy, laparoscopy and partial nephrectomy^{24,27,28}. FloSeal[®] is easily used and it can be extruded from a syringe and applied topically to the bleeding area. This haemostatic agent has the ability to acquire

irregular shapes fitting the wounded site^{27,28}. When the FloSeal® is in contact with blood the collagen particles are hydrated and swell, restricting the blood flow. The thrombin present converts the patient fibrinogen into a fibrin polymer, originating a clot around the granules^{27,28}.

Estrogen is well known for its beneficial effect on osteoporosis^{29,30}. The raloxifene hydrochloride is a known selective estrogen receptor modulator (SERM). This molecule acts as an estrogen agonist on bone and liver, also increasing bone mineral density³¹, being therefore used for prevention of osteoporosis in postmenopausal women. It has also the advantage of being an antagonist on estrogen receptors in the breast and uterus decreasing the risk of cancer. This SERM can be described as [6-hydroxy-2-(4-hydroxyphenyl)benzo-[b]thien-3-yl][4-[2-(1-piperidinyloxy)phenyl]ethanone hydrochloride³¹. Raloxifene hydrochloride inhibits *in vitro* mammalian osteoclast differentiation and bone resorption in the presence of interleukin 6 (IL-6). Also produces a similar activity of TGF- β_3 (a cytokine associated with inhibition of osteoclast differentiation and activity) in ovariectomized rats³²⁻³⁵. Several studies shown that this molecule can prevent bone loss^{33,36,37}.

Recently, the authors have reported the potential of Bonelike® graft for bone regeneration for a period of 12 weeks by using an animal model³⁸ and in clinical applications^{39,40}.

The aim of this work was to assess *in vivo* the osteoconductivity and biofunctionality of Bonelike® granules associated to FloSeal® and raloxifene hydrochloride and also to verify the effect of both FloSeal® and raloxifene on the bioactivity of Bonelike®.

Materials and Methods

Material Preparation

In the present study, Bonelike® granules with a size ranging from 150-250 μ m were prepared as follows: a P₂O₅-CaO based glass with the composition of 65P₂O₅-15CaO-10CaF₂-10Na₂O (mol%) was prepared by mixing reagent grade chemicals using a platinum crucible at 1400°C for 2hrs. The prepared glass was crushed in an agate mortar and sieved to a granule size below than 75 μ m. The Bonelike® was obtained by mixing of 2.5 wt% of glass with laboratory prepared hydroxyapatite (HA) in isopropanol. The mixed powders were dried for 24h at 60°C and sieved to less than 75 μ m and then isostatically pressed at 200 MPa. The obtained Bonelike® was sintered at 1300°C for 1hr and finally using standard crushing and sieving techniques the desirable particle size range was obtained. Phase identification and quantification was assessed by X-ray diffraction and Rietveld analysis.

In vivo studies

Healthy skeletally mature male New Zealand white rabbits (Charles River Laboratories, Barcelona, Spain) with an average weight 2.5-3.5Kg were used as an experimental model. All animals were housed in a temperature and humidity controlled room with 12-12 hours light/dark cycles, one animal per cage, and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking in account human endpoints for animal suffering and distress. Animals were housed for 2 weeks before entering the experiment. All procedures were performed with the approval of the veterinary authorities of Portugal in accordance with the European Communities Council Directive 86/609/EEC, and from the Ethic Commission of ICBAS-Porto University. For surgery, rabbits were placed prone under sterile conditions and the skin from both legs scrubbed in a routine fashion with antiseptic solution. Under deep anaesthesia (ketamine 9 mg/100g; xylazine 1.25mg/100g, atropine 0.025mg/100 body weight, intramuscular), a longitudinal incision was made on the lateral surface extending from about 20 mm below the coxo-femoral joint for a distance of 25 mm, exposing the femur (Fig.1a). In each femur 3 holes of 3.0 mm diameter were drilled through the cortex and into medulla using a micro-burr with a 3.0mm tip as it is shown in (Fig.1b). The defects were rinsed with a saline solution (NaCl 0.9% Braun) to remove any residual bone.

Rabbit A was implanted with Bonelike® associated to FloSeal® and raloxifene hydrochloride (experimental samples), and Rabbit B and C were used as controls, being the rabbit B implanted with FloSeal® plus raloxifene hydrochloride and rabbit C with FloSeal® alone as shown in Table 1. Bonelike® granules (1.2 g) were mixed with matrix FloSeal® (5ml) and raloxifene hydrochloride (28 mg dissolved in 1 ml of dimethylsulfoxide – DMSO, in a final concentration of 1mM) as shown in (Figs. 1c,d) and then implanted into the bone defect (Fig.1e). The animals were sacrificed after 12 weeks of implantation and the retrieved samples analyzed by scanning electron microscopy (SEM) and histological analysis was performed on not decalcified slices.

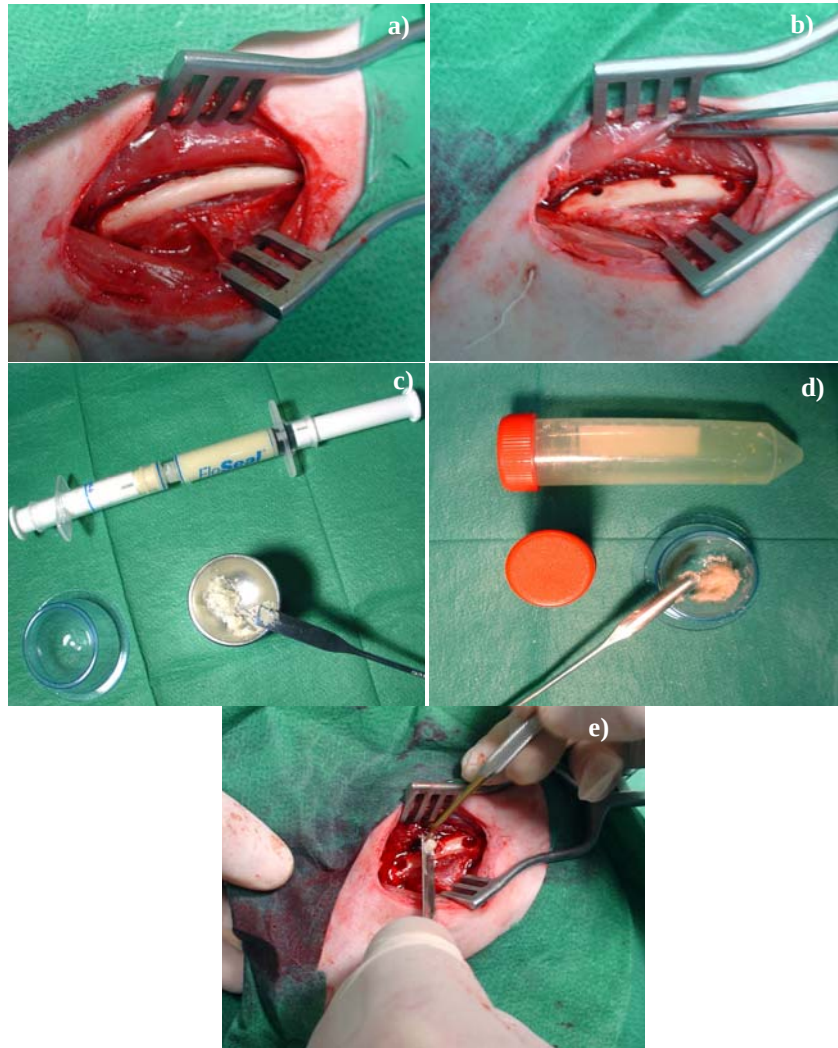


Fig.1. Surgical procedures: (a) exposed rabbit femur; (b) rabbit femur showing 3 holes of 3 mm diameter; (c) mixture of FloSeal® and Bonelike® granules; (d) mixture of Bonelike® granules with raloxifene hydrochloride and (e) implantation of FloSeal®, Bonelike® granules and raloxifene hydrochloride in the bone defects.

Histology Analysis

The retrieved experimental samples were immediately placed in a neutral formaldehyde fixative solution (6%) for seven days and then dehydrated in an increased percentage of alcohol solutions 70%, 80%, 90% and 100% and embedded in a methyl-methacrylate resin. After polymerisation, specimens were sectioned with a diamond saw and polished down to the thickness of $40 \pm 10 \mu\text{m}$ with a diamond disc to prepare the histological slices. These sections were stained with haematoxylin/eosin and solo-chrome R and finally examined using an Olympus BH-2 transmitted light microscope.

Results and Discussion

The incorporation of P₂O₅-CaO based glass into the HA, Ca₁₀(PO₄)₆(OH)₂, matrix, through a liquid sintering process leads to the formation of triple phase material, HA, β and α tricalcium phosphate (TCP). The percentage of β and α -TCP phases present on Bonelike[®] is dependent of the sintering cycle and the composition of the glass added¹⁷⁻²⁰. In the present study, the addition of 2.5 wt% P₂O₅-CaO glass resulted in the following composition: HA = 68.4%; β -TCP = 7.6% and α -TCP = 24% as shown in Fig.2, these results were obtained by X-ray diffraction and Rietveld analysis.

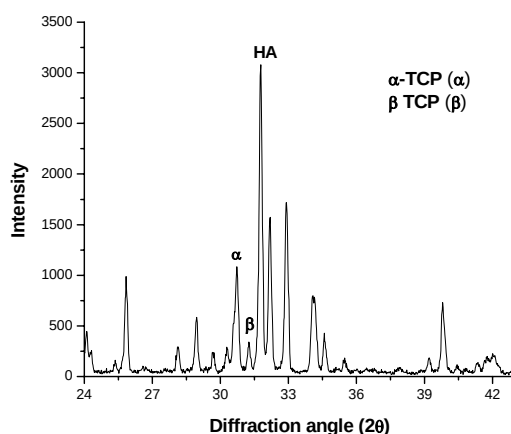


Fig.2. X-ray diffraction pattern of Bonelike[®] graft, which is composed of HA, β - and α -TCP phases.

Due to the presence of biodegradable β and α -TCP phases in the structure of Bonelike[®] a local enrichment in Ca and P in the physiological environment occurs, which stimulates new bone formation. The two phases β and α - TCP are known to exhibit *in vivo* bioresorbability while HA is bioactive. *In vitro* cellular evaluation of Bonelike[®] was exhaustively studied in the past^{21,41,42}.

Previous reported animal³⁸ and clinical applications of Bonelike[®] in maxillofacial surgery and implantology⁴³ and very recent reported study⁴⁰ on histomorphometric measurements, histological and SEM analyses of bone/implant interface in orthopedic applications proved the enhanced osteoconductive properties of Bonelike[®].

In the present *in vivo* study the authors used a FloSeal[®] as vehicle for the Bonelike[®] granules. In the presence of blood, the FloSeal[®] induces the formation of a blood clot that stabilizes the granules on the wounded site, diminishing the displacement and movement. It has been reported that the micromovements can induce the formation of a fibrous capsule leading to a poor osteointegration¹. Therefore, an easily use system was developed by the association of Bonelike[®] and a resorbable matrix like FloSeal[®].

Additionally, a therapeutically molecule was added to the system to further stimulate bone formation. In this study the molecule chosen was raloxifene hydrochloride that inhibits the osteoclast activity and therefore creates an unbalance on bone turnover towards bone formation.

To examine the healing of bone defects *in vivo*, histology is the most powerful method⁴⁴. The granules size (150-250µm) implanted with a randomized aspect ratio are considered as to be adequate for the regeneration of bone defects. The matrix used (FloSeal®) to prepare the Bonelike® graft paste seemed to have no effect on the osteoconductive properties of Bonelike® that has been clearly demonstrated in the past. During the 12 weeks healing period, the animals easily recovered and no rejection symptoms were observed in the implantation site for all implanted samples. Both SEM and histological analyses have confirmed the osteointegration of Bonelike® granules and the new bone formation, with almost complete regeneration of the bone defect.

The animal surgery details are described in Table 1. Rabbit A was implanted with 150-250µm size Bonelike® granules associated to the resorbable matrix FloSeal® and raloxifene hydrochloride as a therapeutic molecule. After 12 weeks of implantation, the Bonelike® granules were completely surrounded by *de novo* mature bone (Fig. 3). On the SEM image (Fig. 3a) it is possible to observe the completely osteointegration of the Bonelike® granules and bone growth formed among the granules and the presence of new osteon. Additionally an extensive surface dissolution of Bonelike® granules could be observed in (Fig. 3b,c). No evident of osteoclasts activity seems to have taken place which may be explained by the presence of raloxifene hydrochloride that is know to inhibit osteoclast activity. Similar results were observed on the histological slices in Fig. 3b where the granules were completely surrounded by new bone (fibroreticular) with vascular structures and cement lines indicating active bone regeneration. Once again an extensive dissolution of Bonelike® granules was observed on the histological analysis as shown in Fig.3c, which indicates that this bone graft undergoes some dissolution *in vivo* while new bone formation is occurring.

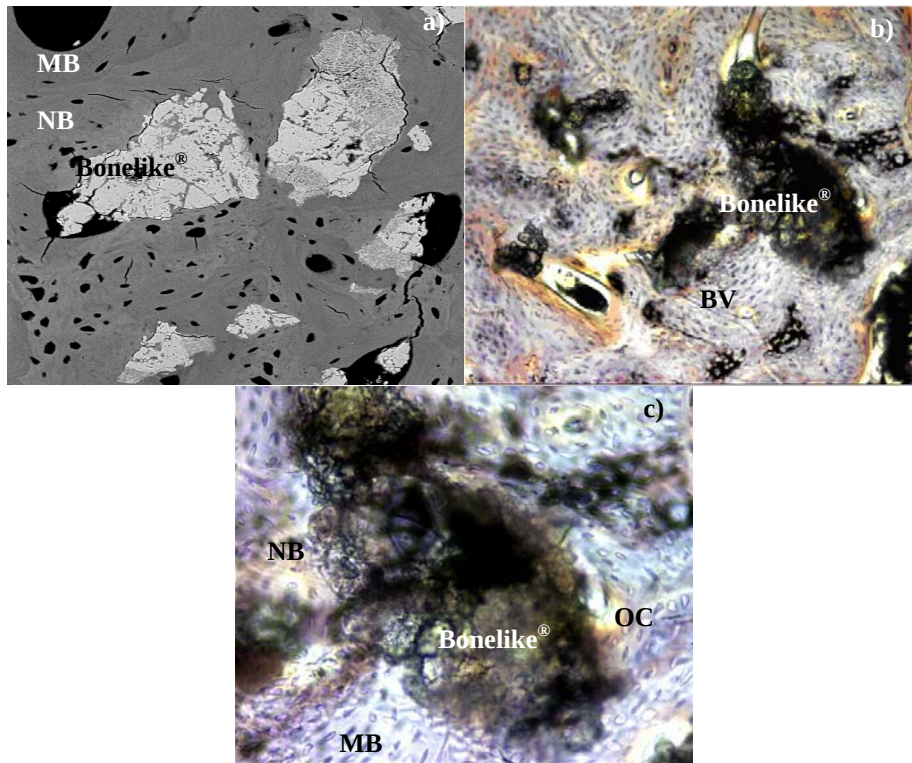


Fig.3. (a) Scanning electron microscopy image (200 x); (b) Haematoxylin-eosin staining images of the experimental samples (20x) showing Bonelike[®] granules involved in the *de novo* mature bone tissue and (c) degradation of Bonelike[®] granules observed by histological analysis (200x) in Rabbit A. (NB - New Bone, MB - Matured Bone, OC – Osteocytes and BV – Blood vessel).

The osteoblasts secrete bone matrix and after a certain period of time and some of the osteoblasts become entrapped in *lacunae* and then are called osteocytes. The number of osteoblasts that become osteocytes varies depending on the rapidity of bone formation. The more rapid the formation, the more osteocytes are present per unit of volume. As a general rule, embryonic (woven) bone and repair bone have more osteocytes than mature bone. After their formation, osteocytes gradually lose most of their matrix-synthesizing machinery and become smaller. The space in the matrix occupied by an osteocyte is called the osteocytic *lacuna*⁴⁴. Narrow extensions of these *lacunae* form enclosed channels, or *canaliculi*. This vascularization process which implied the formation of channels in the tissue is demonstrated in Fig. 3c. Several blood channels without signs of inflammation throughout the osteoid matrix have been observed and no inflammatory cells and fibrous tissue have been found. The presence of blood vessels is due to active angiogenesis process that is an extremely important process for bone regeneration. The development of a vascular network is essential to maintain the cellular viability through the supply of oxygen, nutrients and also to remove the metabolic products of the cellular activity. This network is also an important one on the transport of the surrounding progenitor

cells and metabolic active molecules that are involved on the bone regeneration process⁴⁵.

As expected bone regeneration was also observed on the controls (Fig. 4a,b) in Rabbits B and C. FloSeal® is resorbed in the body within 6-8 weeks due to the biodegradation behavior of this biological glue²³. Therefore, the histological evaluation revealed no remnants of FloSeal®, as may be observed in Fig.4. Additional analyses are being performed in order to assess the new bone formation rate of the three implanted systems and try to clarify the exact influence of the addition of FloSeal® and raloxifene hydrochloride on the osteoconductive properties of Bonelike® graft.

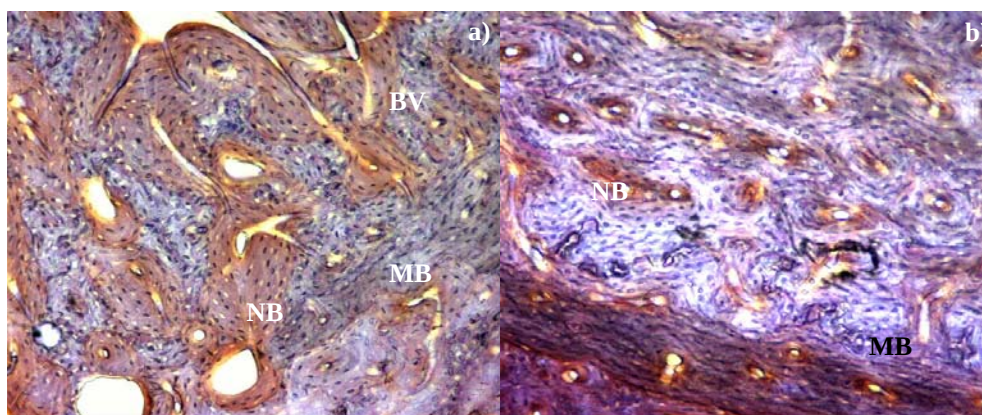


Fig.4. Haematoxylin-eosin staining images (20x) (a) FloSeal® plus raloxifene hydrochloride (b) raloxifene hydrochloride alone showing bone regeneration in rabbits B and C. New bone with different degree of maturity may be observed.

(NB - New Bone, MB - Matured Bone and OC - Osteocytes).

As a summary, the results obtained so far with Bonelike® associated to FloSeal® and raloxifene hydrochloride permit to conclude that the osteoconductive properties of the ceramic material were not significantly affected by the presence of this vehicle and by the therapeutic molecule. This system is easier to use than the standard granulated bone graft material. Furthermore, this system allows the incorporation of several therapeutic molecules, such as bone morphogenetic proteins, antibiotics and anti-inflammatory drugs.

Conclusions

The Bonelike® graft associated to FloSeal® seemed to serve as an excellent scaffold for bone regeneration. In addition the association of Bonelike® to a resorbable vehicle can act as a controlled release system to osteoinductive molecules and therefore has potential to increase the osteointegration of Bonelike® graft. Further studies are under process, to

assess the synergistic effect of the association of highly osteoconductive Bonelike® graft with several therapeutic molecules.

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Chapter 3

**Bonelike® Coatings
Clinical Applications**

Titanium is the most common material used in oral implantology due to its strength, comparatively low stiffness, light weight and bioinertness, although its osteointegration is poor. So, one way to improve the osteointegration and biocompatibility of the titanium implants is to coat them with a bioactive ceramic. It has been reported that a double-layer HA-P₂O₅/CaO glass (Bonelike®) coating has an enhanced bioactivity in comparison with HA and positive effect on bone cells proliferation and function.

The aim of this chapter is to describe the biological response of different patients to Bonelike® coated oral implants.

The first clinical case describes the case of a 40 year old male that was totally edentulous except for 1.8 include. The technique used for the implantation of eleven titanium coated dental implants with Bonelike®, (6 on the maxilla and 5 on the mandible), was the standard *ad modum Brånemark*. After 3 months, the central implant on the mandible was removed due to bad positioning and histological analyses were performed. The remaining implants were followed by clinical and radiographic examinations.

The histological analysis performed from the biopsy sample, showed a direct contact between the surface of the coating and the bone matrix. No inflammatory cells and fibrous tissues were observed. Due to the close bonding between new bone and Bonelike®, it was almost impossible to distinguish any discontinuity at the interface. The SEM analysis showed similar results, where it was possible to see excellent bone remnants on its surface and therefore improved primary stability. The radiological follow-up corroborated the previous results, an excellent osteointegration of the Bonelike® coatings.

Due to the excellent results obtained on the previous case, 27 titanium Bonelike® coated implants were placed (18 in the maxilla and 9 in the mandible) in 7 patients in order to have a more extensive clinical study. Similar results were obtained for each case. The radiological follow-up showed a good osteointegration of all the Bonelike® coated implants.

Titanium Dental Implants Coated with Bonelike[®]: Clinical Case Report

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Abstract

The aim of the study was to evaluate the direct bone bonding and osseointegration of the commercial pure (cp Ti) implants coated with Bonelike® synthetic bone graft by plasma spraying. The Bonelike® coated implant was placed in the mandible of a 40-year-old patient and it was removed after a healing period of 3 months with a trephine of 6 mm diameter. The structure of the coating and new bone/implant interface of retrieved samples were evaluated using scanning electron microscopy (SEM) and histological analysis using light microscopy. In vivo microstructure observations of Bonelike® coated retrieved implants showed excellent bone remnants on its surface without any tissue and inflammatory signs observed. The reported Bonelike® coated (cp Ti) implants improved primary stability, which may increase the lifetime of the implant. Bonelike coated dental implants proved to be highly bioactive with extensive new bone formation and strongly bonded to Bonelike® coating.

Keywords: Bonelike® coatings; Plasma spraying; Dental implants; Clinical applications; Bone regeneration.

Introduction

Bone has a unique capability of self-regeneration and remodelling to a certain extent throughout life without leaving a scar^{1,2}. Itself-remodelling fails due to certain conditions such as trauma, bone metabolic diseases, neoplasm and others, synthetic bone grafts and coated implant materials can be used for bone regeneration in orthopaedic procedures and dental applications³. The study of biomedical implant surface and the effects of surface modifications have become popular in recent years because surface characteristics directly influence the biomaterial-tissue interactions⁴.

Metals such as pure titanium, tantalum, niobium, zirconium, cobalt–chromium alloy, Ti–6Al–4V alloy, and ceramic materials such as aluminium oxide, hydroxyapatite (HA), or β -tricalcium phosphate have been used for oral implants⁵. The mostly used biomaterial in oral implantology is commercially pure titanium (cpTi) because of its strength, comparatively low stiffness, light weight and bioinertness⁶. When metals are used as an implant material, their biocompatibility and osseointegration is lower when compared to coated metal implants with bioceramic materials^{5,7}. Therefore, in order to improve the osseointegration between titanium implants and host bone, there are different coatings that have been applied by a variety of methods⁸. Among them, plasma spraying appears to be the most favourable one in terms of chemical control, bio-corrosion resistance, process efficiency and the degree to which the substrate fatigue resistance is reduced⁸. *In vitro* and *in vivo* biocompatibility testing of titanium alloy with and without plasma-sprayed hydroxyapatite coating have been studied⁹.

Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is commonly employed as a coating layer on metallic implants for fast fixation and firm implant-bone attachment [6]. Furthermore, the commercially available plasma-sprayed HA coatings relatively thick, with low crystallinity and homogeneity, porous, and presents low bonding strength¹⁰. Many clinical studies were reported for HA coated implants^{11–13}. The HA coated implants have higher integration rate, promote faster bone attachment, and achieve direct bone bonding, when compared to non-coated¹⁴. However, there are many controversies regarding the long-term prognosis of coated dental implants. For example, an 8-year¹⁵ clinical retrospective study of titanium plasma-sprayed with HA coated implants showed that the survival rate was initially higher for HA-coated implants, but decreased significantly after 4 years of implantation. Most of long-term failures were due to inflammatory reaction. Tsui *et al.*^{8,16} reports some metastable and amorphous phases that appear in the HA coating during the plasma spraying process, which results in the low crystallinity of HA coating and poor mechanical strength¹⁷. Long-term animal studies and clinical trials of load-bearing dental and orthopaedic prostheses showed that HA coatings degrades with time, depending

upon the degree of crystallinity of the HA.

Over the past decade, Santos *et al.* have reported and developed a glass-reinforced HA composite by incorporating CaO–P₂O₅ based glass into the microstructure of HA through a simple liquid phase sintering process^{18,19} and this material was patented and recently registered as Bonelike[®] [20–23]. This system allows the incorporation of several ions, such as magnesium, sodium and fluoride resulting in bone graft with a chemical composition similar to the bone mineral phase and its microstructure presented improved mechanical properties and enhanced bioactivity than the actual commercial HA^{24,25}.

Earlier, HA and double-layer HA-P₂O₅/CaO glass (i.e. Bonelike[®]) coatings showed to have a positive effect on human bone marrow cells increasing osteoblasts differentiation^{26,27}. The glass reinforced HA composites (Bonelike[®] coating) present better characteristics for bone cell growth and function when compared with HA ones. In another previous *in vitro* study²⁸, the bioactive testing using simulated body fluid (SBF) has shown that during the immersion of Bonelike[®] coatings, dissolution of the coating surface occurred and apatite layer formation on the surface took place faster than on pure HA coatings. Hence, these results are strong indication that Bonelike[®] coatings induce faster mineralization *in vitro* than HA coatings²⁹.

In granular form Bonelike[®] has proved its highly bioactive behaviour on orthopaedics and dental applications. For example, a very recent study³⁰ on histomorphometric measurements, histological and scanning electron microscopy (SEM) analyses of bone/implant interface of retrieved samples have proved the highly osteoconductive properties of Bonelike[®] (500–1000µm) in orthopaedic applications. In this study, the quantification was performed by measuring the percentage of bone contact, i.e. percentage of the surface of the granules covered by new bone, which is considered as being an excellent indicator of osseointegration. In another recent report³¹ the osteoconductivity and bioactivity of the Bonelike[®] graft (250–500µm) in repairing surgical cystic bone defects was confirmed by several successful clinical applications. These clinical applications in maxillary bone defects indicated perfect bone bonding between new formed bone and the Bonelike[®] granules.

An implant elicits a biological response in the surrounding tissue, which determines its acceptance and long-term function. Bone-anchored titanium implants *ad modum Bränemark* have been in clinical use for several years³². Adverse tissue reactions ranging from mild reactions to those leading to the removal of the implant are few³² and were, in a latter follow-up, reported in about 10% of the observations. Various factors, including an operation technique minimizing tissue injury and the use of implants of titanium, probably contribute to the good clinical performance. This clinical performance maybe even more

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improved, when the implants are coated with Bonelike[®] graft, which proved its capacity to bond new formed bone.

The aim of the study was to evaluate the direct bone bonding and osseointegration of the commercial pure (cpTi) implants coated with Bonelike[®] synthetic bone graft by a plasma-sprayed method for dental oral applications.

Materials and methods

Bonelike[®] coating

Bonelike[®] powder was prepared as follows: a P₂O₅–CaO based glass with the composition of 65P₂O₅–15CaO–10CaF₂–10Na₂O (mol %) was prepared by mixing reagent grade chemicals using a platinum crucible at 1450°C for 2h. The prepared glass was crushed in an agate mortar and sieved to a granule size below 75µm. The Bonelike[®] was obtained by mixing of 2.5wt% of glass with laboratory prepared HA) in isopropanol. The mixed powders were dried for 24h at 60°C and sieved to less than 75µm and then isostatically pressed at 200 MPa. The obtained Bonelike[®] was sintered at 1300 °C for 1 h.

A commercially available titanium grade 4 (cpTi) implant (Titantec SA Company, Argentina) of 3.75 mm diameter and 10 mm length was used as a substrate. Plasma spraying was performed using automated equipment from Plasma Technik under atmospheric conditions with optimised deposition parameters. After substrate preparation, a Bonelike[®] coating was sprayed in order to obtain a uniform coating thickness of 60µm in all directions.

Clinical case

The aim was to longitudinally follow-up the biological behaviour of Bonelike[®] coated dental implants in the selected patient by clinical and radiographic parameters, and finally, to observe osseointegration of these implants by SEM, using retrieved samples after 3 months of implantation. In the present study, a 40-year-old male was totally edentulous except 1.8 include. The technique used for the implantation was the standard *ad modum Bränemark*³². Eleven titanium coated dental implants with Bonelike[®] were implanted, 6 on the maxilla and 5 on the mandible. After 3 months, the central implant on the mandible was removed due to bad positioning. The maximum torque force applied was 40 Ncm.

Histological analysis and characterisation

For histological analysis, the retrieved Bonelike[®] coated dental implant samples were immediately placed in neutral formaldehyde fixing solution (6%) during 7 days and then dehydrated in an increased percentage of alcohol solutions of 70%, 80%, 90% and 100% and embedded in a methylmethacrylate resin. After polymerisation, specimens were sectioned with a diamond saw and polished down to the thickness of $40\pm 10\mu\text{m}$ with a diamond disc to prepare the histological slices. These sections were stained with haematoxylin/eosin and solo-chrome R and finally examined using an Olympus BH-2 transmitted light microscope. A scanning electron microscopy (JEOL JSM 6301F) was used to analyse the microstructure of these coated implants. A post-operative radiological examination was performed according to the standard follow-up protocol.

Results and discussion

Fig. 1 (a,b) shows the SEM morphological characterisation of the Bonelike[®] coated dental implant with different magnifications. The coating has a microstructure composed of partially melted particles, characteristics of plasma spraying process. For the structure and chemical mechanism of bone physiology, it is essential to provide substances that are endogenous to the body for successful bone regeneration. Hence, to create the natural bone structure, these substances must be present on the interface at the same rate at which bone formation occurs. Therefore, in Bonelike[®] coated dental implants, there is a local enrichment in Ca^{2+} and P^{5+} in the in the physiological environment, which stimulates new bone formation.

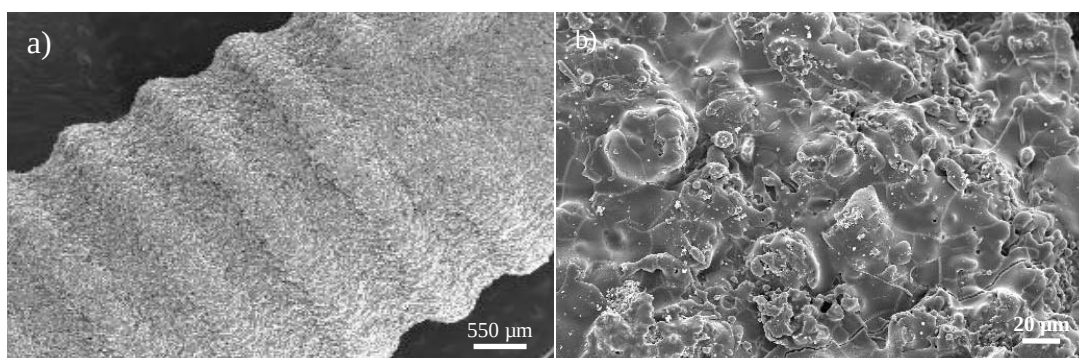


Fig.1. SEM image showing the surface morphology of the Bonelike[®] coated dental implant material at different magnifications (a)10 x and (b) 300 x.

Fig. 2 shows the clinical application of Bonelike® coated dental implants. In the present study, Bonelike® coated dental implants were implanted as shown in Fig.2 (a). Among 11 implants, 6 were placed on the maxilla and 5 on the mandible, as shown in the orthopantomogram in Fig.2 (b).

After 3 months of healing period, one mandibular implant was removed (Fig.2(c)) due to bad positioning, which allowed the study of the bone/implant interface and new bone regeneration.

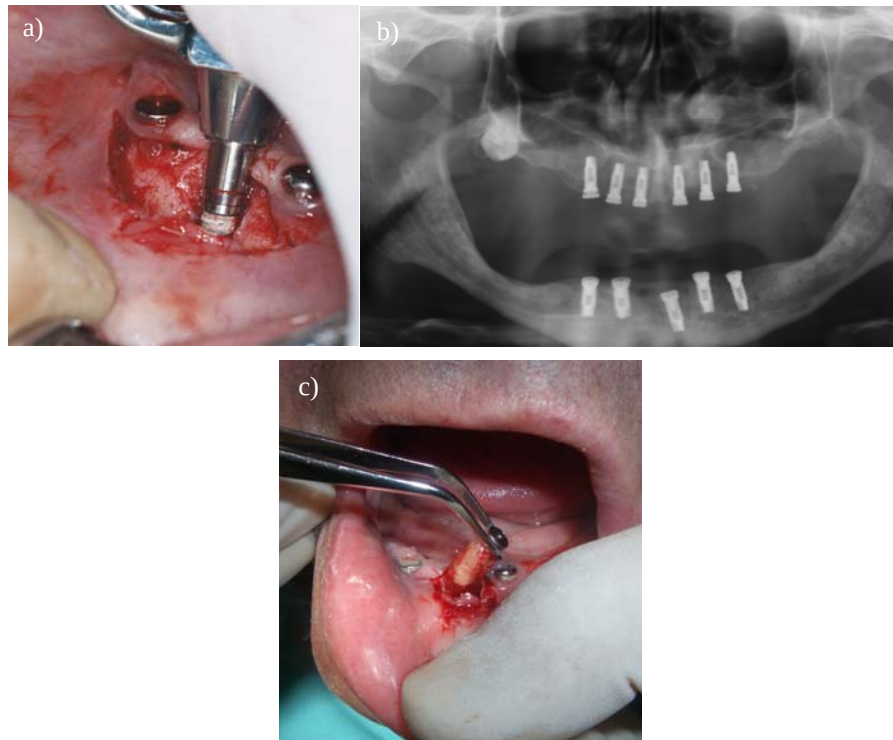


Fig.2. Clinical application: (a) implantation of Bonelike® coated dental implant in the mandible, (b) post-operative orthopantomogram image and (c) after 3months the central implant on the mandible was removed due to bad positioning and was used for histological analysis.

Literature reports that a removal of torque measurement is usually performed at the distal surfaces of the implants⁵. Implants could be successfully removed without failure of the coated implant due to the weak intrinsic mechanical properties of HA, which indicates the very strong interfacial bond between HA and bone. The average removal torque for different groups in one referenced study⁶ was ranging between 20.9 Ncm and 48.4 Ncm. Other group⁵ used 47.25 Ncm for the surfaces with HA deposition and in the present implant study, the surgeon decided to use 40Ncm.

Literature also reports that the plasma-sprayed hydroxyapatite coatings influence the osteoconductivity of commercially pure titanium implants [33]. A plasma-sprayed HA

coated implant exhibits greater tolerance than sandblasted cp titanium implant due to unfavourable conditions during healing, such as gaps in the interface or primary instability of the implant. Plasma-sprayed HA coated implants showed a high percentage of bone contacts. In the case of sandblasted cp titanium implants, filling of gaps with fibrous tissues was observed³³.

In order to assess the bone quality of the regenerated bone, histology is the most powerful method to examine the interface of the implant material and new bone³⁴. In this study it was shown that coated Bonelike® dental implant was actively involved in the bone regeneration process which maybe demonstrated by the strong bond between the new bone and the coating. Earlier studies^{30,31} with Bonelike® granules have also clearly demonstrated an extensive new bone formation with a significant degree of maturation in orthopaedics and maxillofacial applications.

Fig. 3 shows the histological analysis assessed from the biopsy sample. New bone ingrowth has been observed surrounding Bonelike® coated dental implants with a mature lamellar- like structure and a direct contact between the surfaces of the coating and the bone matrix established. No inflammatory cells and fibrous tissues have been found. Mature bone was clearly the major bone type observed around the retrieved sample. Solo-Chrome R staining histological image shows bone regeneration and a Ti-implant/bone interface with new bone at different magnifications of 100 x and 200 x (Fig. 3 a, b). New bone attached to Bonelike® is observed with a magnification of 200 x in Fig.3 (c,d). Due to intimate bonding between new bone and Bonelike® it was almost impossible to distinguish any discontinuity at the interface. Bio-affinity with highest osseointegration capacity and remodelling was observed in Fig. 3(e). Also radiological follow-up image (Fig.2b) showed osseointegration of the Bonelike® coatings.

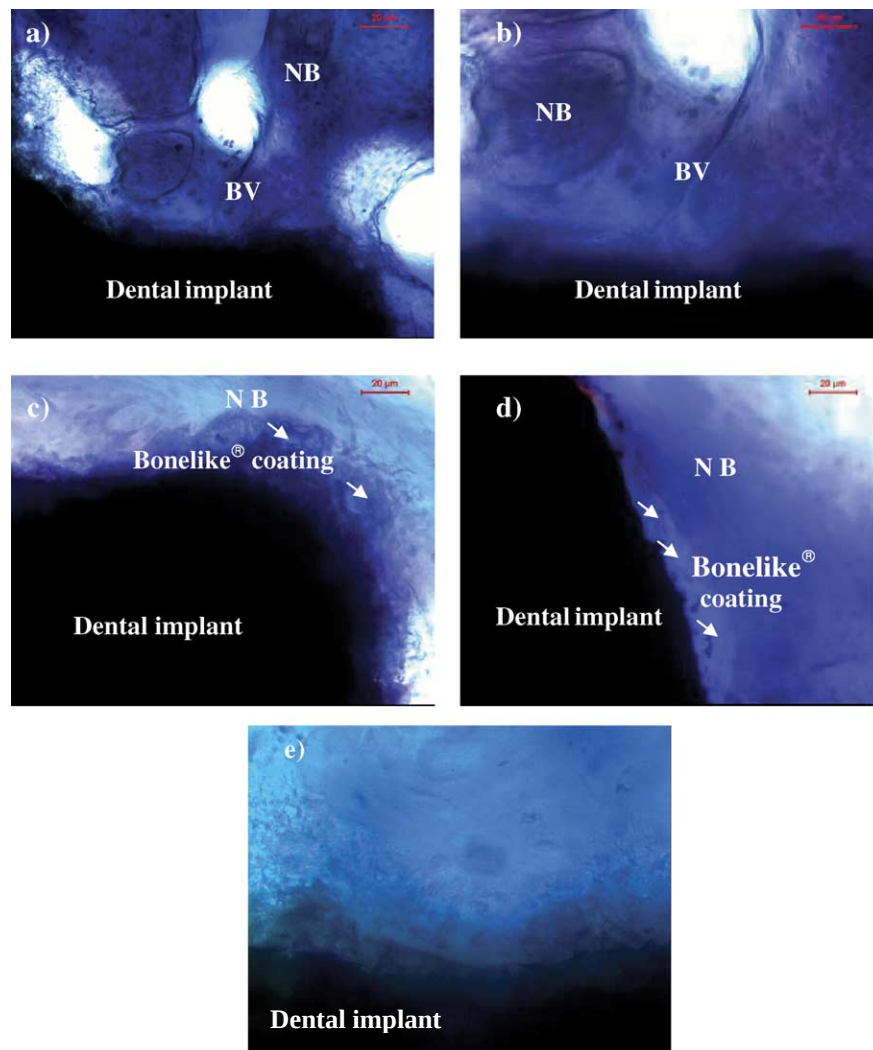


Fig.3. Histological images (Solo-Chrome R staining) show bone regeneration and Bonelike[®] implant/bone interface with new bone at different magnifications (a) 100 x, (b) 200 x. (c, d) new bone formed and Bonelike[®] osteointegrated may be observed at 200x and (e) due to intimate bonding between new bone and Bonelike[®] it is almost impossible to distinguish any discontinuity at the interface at 400 x (NB—new bone and BV—blood vessel).

The interfaces between dental implant / Bonelike[®] coating, and Bonelike[®] coating / new bone were also evaluated using scanning electron microscopy. Microstructure observations of Bonelike[®] coated dental implants demonstrated that they had excellent bone remnants on its surface and an improved primary stability of the coated implants was observed.

Fig. 4 is a cross-sectional view of the coated dental implant obtained by SEM. It is visible that the Bonelike® coating is well adherent to the dental implant substrate. In Fig. 4 (a, b) an extensive new bone formation and well adherent Bonelike® coating to the Ti-dental implant was observed. After 3 months of healing period the Bonelike® coating was well attached to the Ti-substrate and also the bone was apposed to the coating as seen in Fig.4(c). The new bone has grown through the micro and macroporosity of Bonelike® coating as shown in Fig. 4(d). Fig. 4(e) shows new bone formed apposed onto Bonelike® coating without the formation of gaps at the interface. In Fig.4 (f), it is possible to observe the new bone formed with a high degree of maturation after a 3 months implantation period.

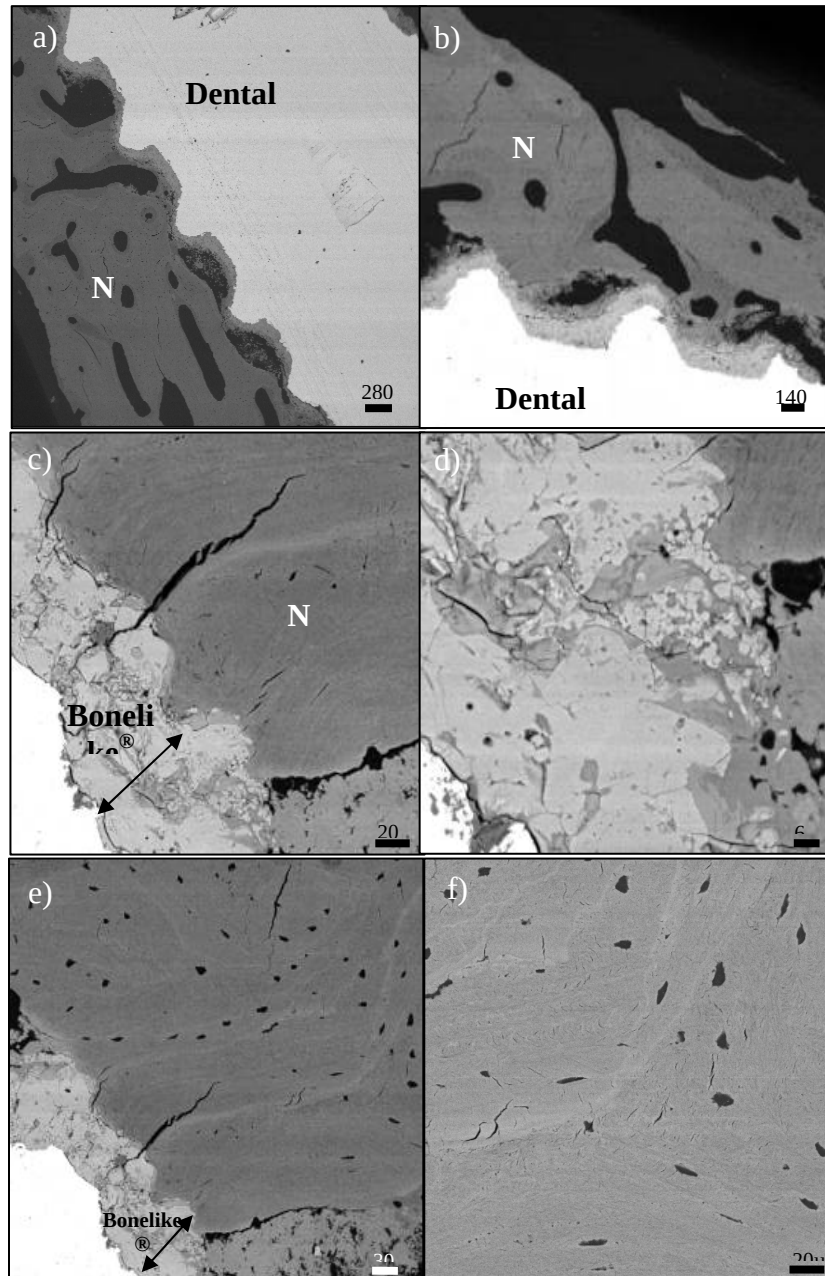


Fig.4. SEM image shows (a,b) an extensive new bone formation and well adherent Bonelike[®] coating to the Ti-implant. Bone was apposed on the coating; (c) a thick coating well attached to substrate. No significant Bonelike[®] coating dissolution after 6 months implantation; (d) new bone has grown through the micro and macroporosity of Bonelike[®] coating, which remained attached to the substrate; (e) an extensive new bone formation which was apposed onto Bonelike[®] coating without the formation of gaps at the interface at 200 x magnification and (f) new bone formed with a high degree of maturation after 3 months implantation as obtained at 400x magnification (NB - new bone).

The results of this study suggest that the Bonelike[®] played a significant role in the new bone formation process around the dental implants. Hence, Bonelike[®] proved to be an excellent coating for bone regeneration and therefore it maybe used in implantology.

Conclusion

On the reported clinical case, the direct bone bonding and a good osseointegration of the commercial pure (cp Ti) implants coated with Bonelike[®] synthetic bone graft was observed. According to these results, implants coated with Bonelike[®] showed a high osseointegration after 3 months of healing period and therefore these dental implants maybe clinical used when primary stability is needed.

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Clinical Applications of Titanium Dental Implants Coated with Glass Reinforced Hydroxyapatite Composite (Bonelike®)

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Abstract

Hydroxyapatite (HA) coated implants have a higher integration rate, promote faster bone attachment and achieve direct bone bonding, when compared to non-coated implants. There are many controversies regarding the long-term prognosis of coated dental implants namely in the interface between the metal and the ceramics. However dental implants coated with HA are known to improve primary stability. In this study commercially pure titanium dental implants were coated with glass-reinforced hydroxyapatite composites (Bonelike®) using plasma spraying technique. A total of 27 Bonelike® coated 3.75mm diameter and 10mm length implants were placed in the maxilla (18) and mandible (9) of 7 patients. Pre and post-operative radiological examination by orthopantomogram was performed according to the standard follow-up protocols. After a 3-month healing period, one patient's implant from the mandible was surgically removed due to bad positioning and assessed by light and scanning electron microscopy. The reported Bonelike® coated dental implants proved to be highly bioactive with extensive new bone formation and attachment.

Keywords: Bonelike® coatings; Plasma-spraying; Titanium dental implants; Clinical application; Bone regeneration.

Introduction

Good stability and biofunctionality of dental implants are the most important clinical goal in the oral implantology. It is desirable that this goal is achieved in the shortest possible healing time, with a very small failure rate and with minimal discomfort for the patient while bearing in mind the cost factor¹. The essential role of bone grafting cannot be fully appreciated without an understanding of the primary surgical, prosthetic and patient concerns in creating partially edentulous restorations. The most important surgical objectives for implant placement include: primary stability, atraumatic placement, avoidance of vital structures, surrounding bone formation and absence of load during integration². Therefore, to achieve the goals of surgical procedure, it is necessary to choose an adequate material of dental implants.

Dental implants are usually made from commercially pure titanium. Titanium is well established as a primary metallic biomaterial for implantology. Also, it shows a low toxicity, great stability with low corrosion rates and favourable mechanical properties compared to other metals³. There are certainly differences between coated and uncoated surfaces in terms of biological behaviour as there are depending upon surface topography. The survival rates reported for HA-coated implants were similar to that of uncoated titanium implants⁴. However, titanium alloys do not bond with the bone by a chemical and biological interaction, but simply by morphological connection to the bone. This insufficient adhesion to the bone, due to the lack of specific biological response from the living tissues, can cause the formation of a non-adherent fibrous tissue around the implant⁵ and commonly, a chemical degradation may also happen⁶. A faster osseointegration of dental implants can be achieved by modifying the surface properties of the original implants^{7,8}. Various coating methods⁹⁻¹³ have been developed for dental and orthopaedic implants. So far, the plasma spraying method appears to be the most favourable and also commonly used for clinical applications¹⁰. Control of processing of plasma-sprayed hydroxyapatite (HA) coatings on titanium prostheses is of vital importance in improving the quality of implants and their successful osteointegration due to its instability at high temperatures¹⁴⁻¹⁵.

The excellent biocompatibility of the plasma sprayed HA¹⁶⁻¹⁸ is exemplified by the rapid filling of cracks in the coating by tissues, as reported by *Wang et al*¹⁹. *In vivo* studies on histological findings in titanium implants coated with calcium phosphate ceramics implanted in rabbit tibia and dogs' studies show an extensive

osteointegration^{20,21}. However, there are many controversies regarding the long-term prognosis of coated dental implants²². Failure may be related to compositional and structural changes of the coating occurring during implantation [23].

*Pekka Laine et al*²⁴ reported clinical studies on failed dental implants with the main reason for implant failure being inappropriate prosthodontic reconstruction. Therefore, it is recommended that the prosthodontic work is carried out by a specialist, or at least by an experienced practitioner. The implant should always be inserted in a sufficient volume of bone. Inserting a thin implant is not a good solution since this leads to failure in osseointegration²⁴.

When compared with HA coated ones, earlier the authors reported that the glass reinforced HA composites (Bonelike®) coating present better characteristics for bone cell growth and function. HA and double-layer HA- Bonelike® glass coatings were showed to have a positive effect on human bone marrow cells increasing osteoblast differentiation^{25,26}. Bonelike® is a patented osteoconductive synthetic graft material and it is manufactured using a simple liquid phase sintering process, developed by *Santos et al*²⁷⁻³⁰. Its composition has the advantage of mimicking the mineral composition of natural bone.

The objective of this work is to study the osteointegration and functionality of 27 commercial pure (cp Ti) Bonelike® coated dental implants that were implanted in the 7 patients, to clinically assess the use of coated implants in the maxilla and mandible of patients by using histological, SEM and radiological analysis.

Materials and Methods

Bonelike® coating

Bonelike® powder was prepared as follows: a P₂O₅-CaO based glass with the composition of 65P₂O₅-15CaO-10CaF₂-10Na₂O (mol%) was prepared by mixing reagent grade chemicals using a platinum crucible at 1400°C for 2h. The prepared glass was crushed in an agate mortar and sieved to a granule size below 75µm. The Bonelike® was obtained by mixing of 2.5 wt% of glass with laboratory prepared hydroxyapatite (HA) in iso-propanol. The mixed powders were dried for 24h at 60°C and sieved to less than 75µm and then isostatically pressed at 200 MPa. The obtained Bonelike® was sintered at 1300°C for 1h.

Commercially available titanium grade-4 (cp Ti) dental implants (Titantec, Argentina) of 3.75 mm diameter and 10mm length were used as a substrate. Plasma spraying was performed using automated equipment from Plasma Technik under atmospheric conditions with optimised deposition parameters. After substrate preparation, a Bonelike® coating was sprayed in order to obtain a uniform coating thickness of 60µm.

Patients and Follow up

In the present dental implantological study, patients were selected strictly on the basis of their clinical needs, according to the radiological and physical examination performed by their medical doctor. Including criteria were: any age, any sex, patients with no infections, non-characterized alveolar maxillar or mandibular lesions. Exclusion criteria were: systemic unhealthy patients, infected cystic cavities, acute or chronic infection at local bone defect, bone inflammatory diseases, particularly osteomyelitis, malignant tumours, severe renal disfunctions, and patients with non- controlled bone metabolism. In this study, 7 healthy citizens of both sexes, 4 male and 3 female, ranging from 27 to 49 years with a mean age of 40 have been considered. Twenty seven Bonelike® coated dental implants were implanted in these 7 patients, 18 in the maxilla and 9 in the mandible, as shown in Table 1. A pre and post-operative radiological examination by orthopantomogram was performed according to the standard follow-up protocols. After 3 months of healing period, one implant from mandible due to bad positioning was surgically removed for SEM and histological analyses.

Table1: Clinical details of patients and total number of Bonelike[®] coated (cp Ti) dental implants were used in the surgery.

Nos.	Patients Details		No. of Implant		Total number of implants
	Age	Sex	Maxilla	Mandible	
1.	49	M	0	4	4
2.	48	F	1	0	1
3.	42	M	1	0	1
4.	27	F	1	0	1
5.	36	F	2	0	2
6.	40	M	6	5	11
7.	38	M	7	0	7

Material Characterisation

X-ray diffraction (XRD) was performed to identify the crystalline phases present in the microstructure of Bonelike[®] by using Siemens D 5000 diffractometer with Cu-K_α radiation ($\lambda = 1.5418\text{\AA}$). The scans were made in the range of 24-42° (2 θ) with a step size of 0.02° and a count time of 2sec/step. A scanning electron microscopy (JEOL JSM 6301F) was used to analyse the microstructure of these coated implants.

For histological analysis, the retrieved Bonelike[®]- coated dental implants were immediately placed in a neutral formaldehyde fixative solution (6%) for one-week period. Then the samples were dehydrated using graded series of ethanol solutions (70, 80, 90, 96 and 100%) and embedded in methyl-methacrylate resin. Non-decalcified sections of 40 $\pm$ 10mm were obtained from the resin blocks, after cutting these forms in the perpendicular direction of the bone length axis using a diamond blade microtome (Struers Accutom). The obtained slices were then stained with haematoxylin/eosin and examined using an Olympus BH-2 transmitted light microscope.

Results and Discussion

Literature reports that bone tissue neo-formation can occur directly on the titanium implant surface or indirectly with the interface of a fibrous tissue, which decreases the bone anchorage^{19,20,31-33}. Hence, the addition of osteoconductive biomaterials on the implant surface increases the osteointegration. Therefore, in the present study, Bonelike® coated cp Ti dental implants were used to accelerate local osteogenesis since its bioactivity has been proved both *in vitro* and *in vivo*.

X-ray diffraction indicated that the percentage of each phase in the microstructure of Bonelike® as determined by Rietveld analysis was as follows: HA = 67.2% β -TCP=8.2% and α -TCP=24.6%, as it may be seen in Fig. 1.

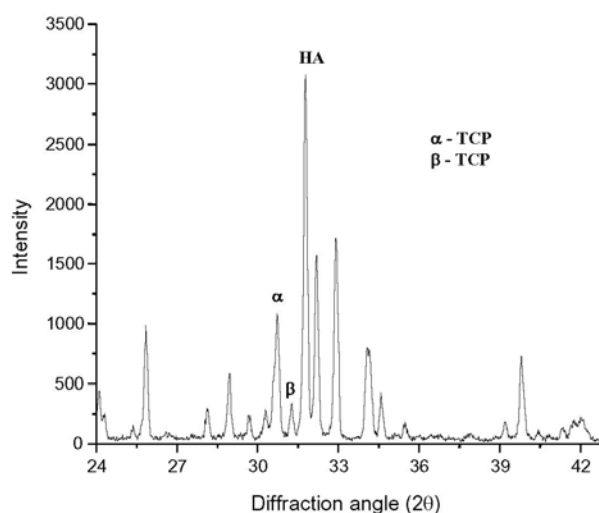


Fig.1. X-ray diffraction of Bonelike® graft, which is composed of HA, β - and α -TCP phases.

Because of the structure and the chemical mechanism of bone physiology, it is considered as essential for successful bone regeneration to provide substances that are endogenous to the body. To create the natural bone structure, these substances should ideally be re-absorbed at the same rate at which bone formation occurs. Bonelike® is composed of crystalline HA, β - and α -tricalcium phosphate phases and its biodegradation have proved to fulfil this requirement³⁴. Previous clinical applications of Bonelike® in maxillofacial surgery, implantology and orthopaedics³⁵⁻³⁷ have proved its

highly osteoconductive properties in the surgical field. Furthermore, earlier published data^{38,39} on histological studies of double layer HA/ Bonelike® plasma sprayed coatings, using the rabbit as experimental model, reported that the coatings did not show significant dissolution and ensured good contact between bone and implants. The high osteoconductivity demonstrated by the Bonelike® coatings led to the rapid establishment of bone/coating contact. Moreover, when these implants were placed in the bone marrow space, bone rapidly grew around the biomaterial surface, migrating from the cortex zone and results clearly indicated that Bonelike® coatings induced earlier new bone formation around the implant than HA coating ones³⁸.

To complement these previous studies, a total of 27 (# 18 maxilla and # 9 mandible) Bonelike® coated dental implants were analysed. Among them one implant, due to bad positioning, was removed for histological and SEM analyses in order to assess new bone formation.

The scanning electron microscopic image (SEM) of Fig. 2 (a) shows the morphology of the dental implants, supplied by Titantec SA (Argentina), after Al₂O₃ grit blasted, and the surface of the dental implant coated with Bonelike® graft in Fig. 2 (b). This coating has a microstructure composed of partially melted particles with macro and micro-porosity, which are characteristic of the plasma spraying process.

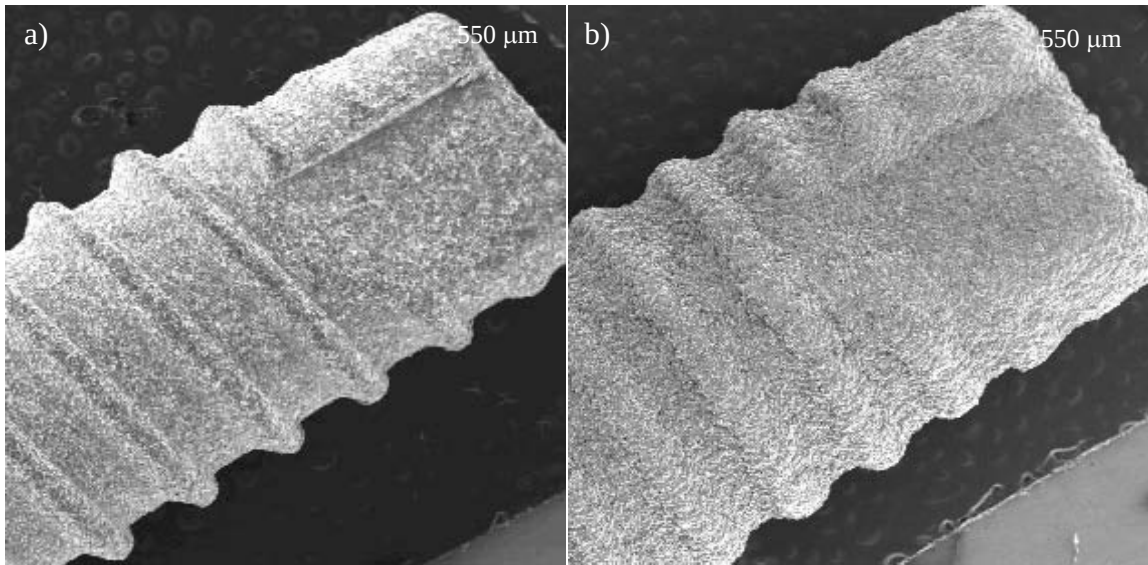


Fig.2. SEM images show the surface morphology of the dental implants (a) as supplied (cp Ti) dental implant of Titantec (SA company, Argentina) (b) Coated with Bonelike® graft on the surface of the dental implant (magnification 10x).

Fig. 3 represents an example of clinical application of Bonelike® coated dental implants in maxilla. In Fig. 3 (a) it is represented a pre-operative orthopantomogram image of the patient and Fig. 3 (b) an operative image of the maxilla where the coated implant was surgically inserted. In Fig. 3 (c) and Fig. 3 (d) is shown two implants inserted in the maxilla and the post-operative orthopantomogram obtained after a healing period of 6 months, respectively.

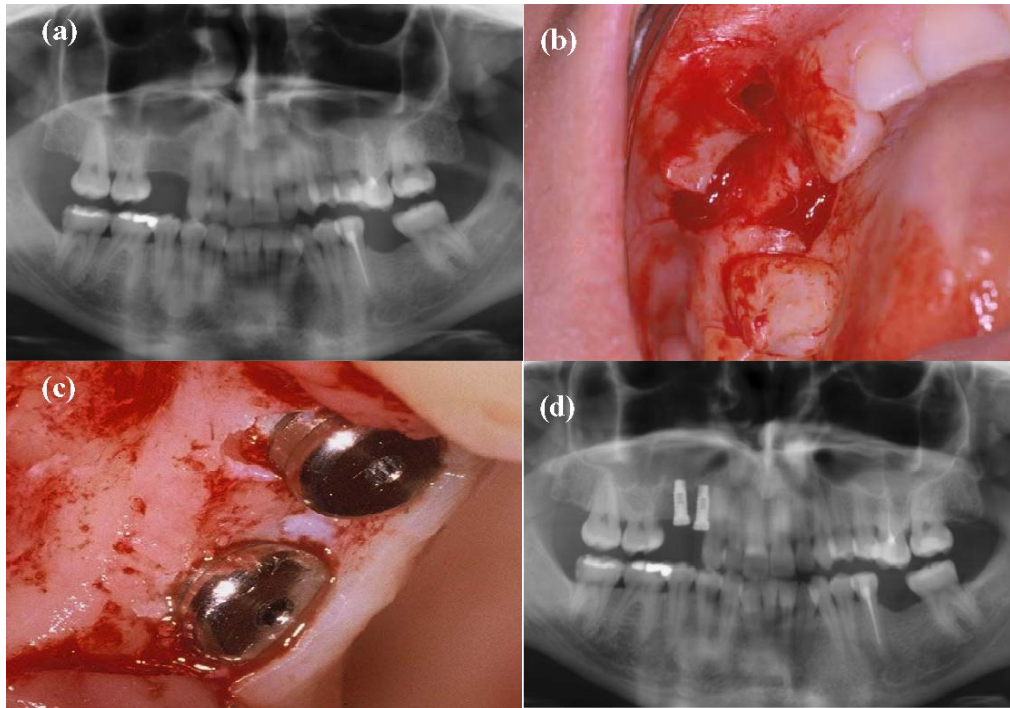


Fig.3. Clinical application: (a) pre-operative orthopantomogram image b) an operative image in the maxilla, (c) two implanted (cp Ti) dental implants coated with Bonelike in the maxilla d) post-operative orthopantomogram image shows dental implants after 6 months implantation.

Fig.4 depicts histological analysis (Haematoxylin-Eosin staining) assessed by the biopsy of one patient containing mineralised matrix in the grafted area and mature lamellar bone that could be detected close to and in contact with Bonelike® interface. From the histological images, Fig.4 (a) it was clear that the new bone formation occurred in intimate contact with the surface of the implants. It was possible to identify the presence of some vessels and lamellae of bone tissue with spaces filled by bone marrow, which indicates angiogenesis and extensive osteogenesis. No signs of inflammatory cells or other adverse effects like fibrous tissue formation were observed. In Fig. 4 (b, c) new bone formation strongly attached to Bonelike® is observed at 400x of magnification optical microscopy picture and it is shown also that osteocytes have an

important role in the mediation of the local response of bone to stress and mechanical deformation.

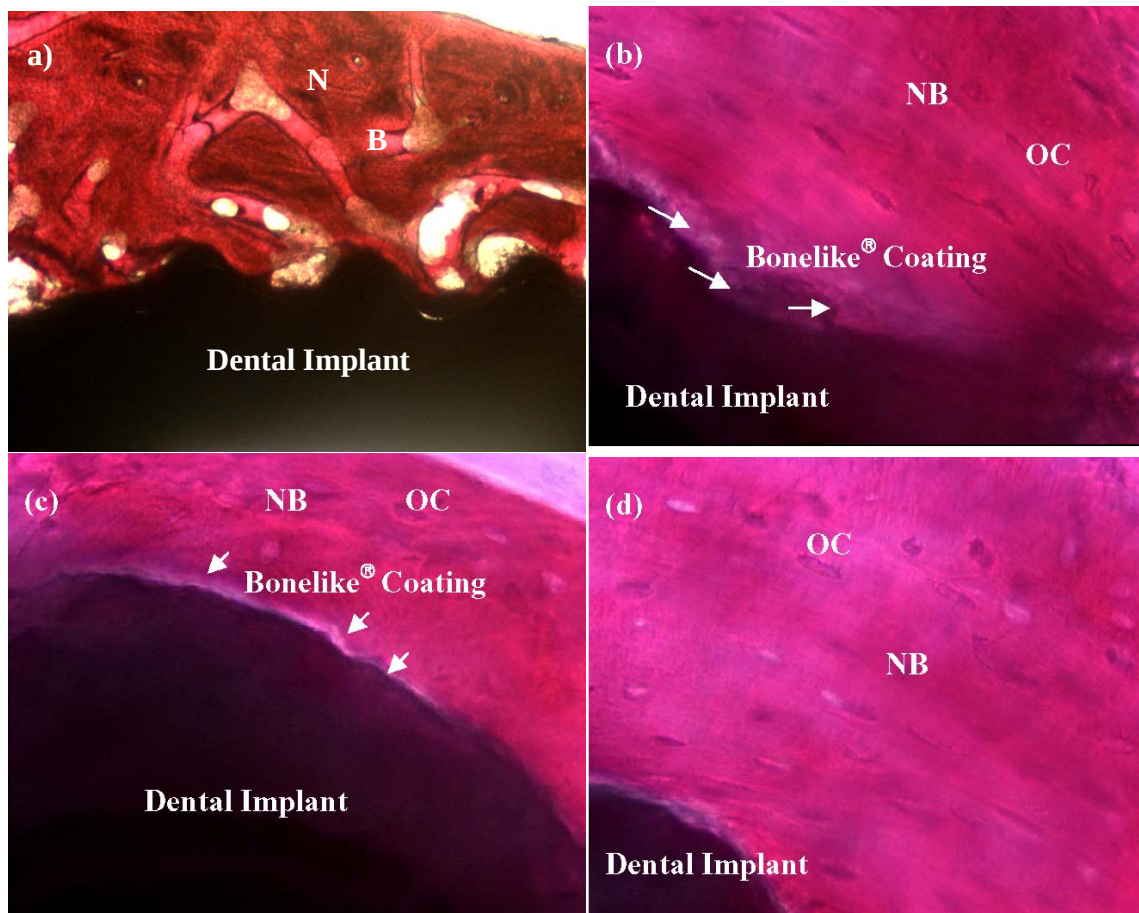


Fig.4. Histological images obtained by Hamotaxiline-Eosin staining show a perfect bone regeneration and Ti- implant bone interface with new bone at magnifications (a) 25x (b-d) 400x. New bone with different degree of maturity may be observed. (NB - New Bone and BV – Blood vessel, OC - Osteocytes).

The structure of new formed bone and its interface with the coated implants were also evaluated using back-scattering scanning electron microscopy. *In vivo* microstructure observations of Bonelike[®] coated implants demonstrated that they had extensive bone remnants on its surface, which indicates an improved primary stability of the coated implants as seen in Fig. 5. A well-bonded Bonelike[®] coating to the cp Ti dental implant and extensive new bone formation with different degree of maturity may

be observed in Fig. 5 (a). After 3 months of healing, the Bonelike[®] coating was still well attached to the cp Ti substrate and also the new bone was apposed to the coating (see Fig. 5 (b, c)).

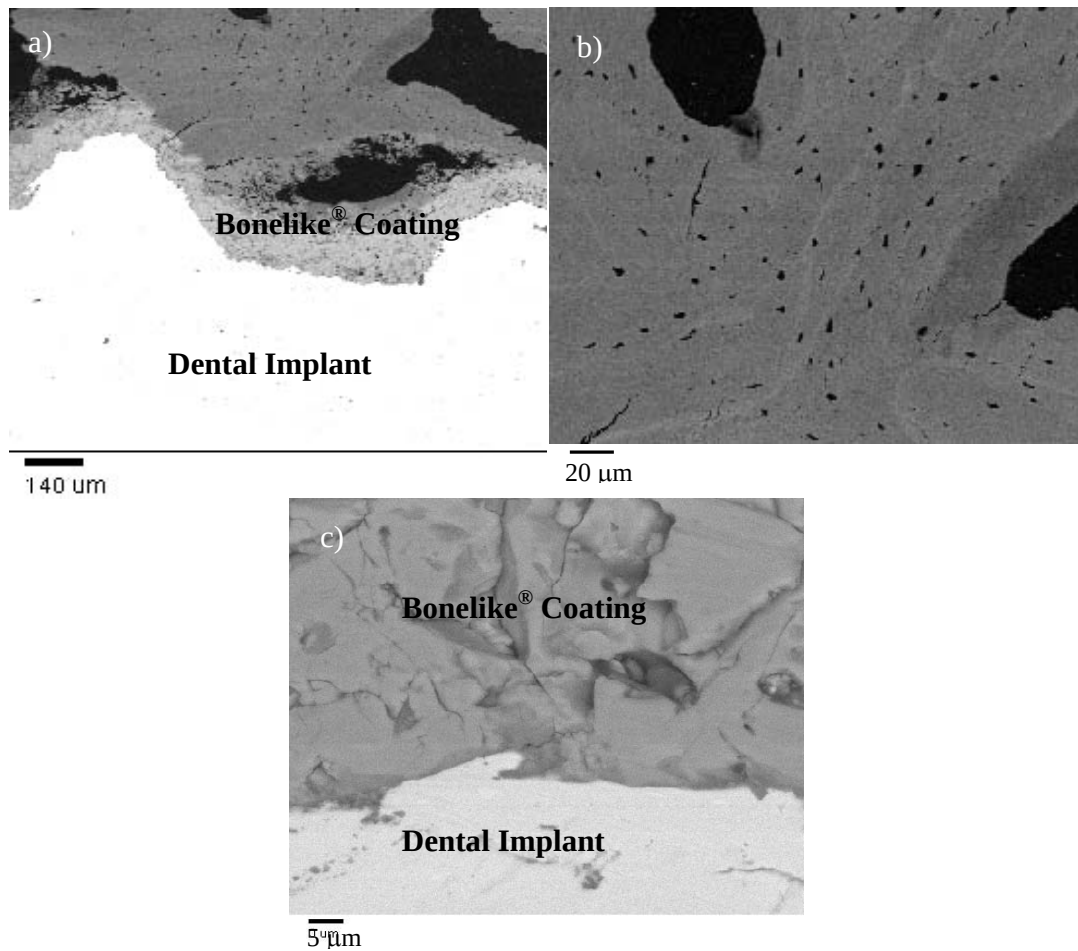


Fig.5. Back-scattered SEM images show a perfect osteointegration of Bonelike[®] coated cp Ti implants at different magnifications (a) 400x (b) 2000 x and (c) 5000x. Extensive new bone formation may be observed with different degree of maturity.

As a final summary the results of this study suggest that the Bonelike[®] played a significant role in the osteointegration of the Bonelike[®] coated implants and that this synthetic graft is an excellent coating material to promote bone regeneration process.

4. Conclusions

A clinical study of a total of 27 (#18 maxilla, # 9 mandible) Bonelike[®] coated cp Ti dental implants showed that excellent primary stability of the coated implants and new bone growth without any bone loss was achieved. The good functionality observed is a consequence of the enhanced osteointegration induced by Bonelike[®] coating.

Acknowledgements

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Chapter 4

Clinical Reports

The previous results demonstrated the high bioactivity of Bonelike® *in vivo* and in several clinical cases as a coating material for titanium implants.

Therefore, the results of the Bonelike® led to its application in different areas of the Regenerative Medicine, such as maxillofacial surgery and implantology.

In this chapter it is described the application of Bonelike® in the granular form in maxillofacial surgery procedures. And also it is discussed the secondary effect of the use of an osteoclasts inhibitory molecule such as zoledronate, a bisphosphonate, in patients suffering from multiple myeloma. These patients usually develop osteonecrosis of the maxilla or/ and mandible.

Bonelike® was implanted in the mandible or maxilla of 11 patients aged between 24 to 53 years, that presented benign cysts previously removed. According to the standard follow-up protocols, radiological examinations were performed and Bonelike®/bone retrieved samples have been analysed histologically using non-decalcified sections obtained perpendicular to bone length axis.

The radiographic examination and histological results clearly demonstrated an extensive new bone formation apposed on Bonelike® granules with a significant degree of maturation. A perfect bone bonding between new bone formed and Bonelike® granules was visible, along with partially surface biodegradation. This quick and effective osteoconductive response from Bonelike® reduces the time required to reconstruct the bone defected area of patients and also allows a future application of dental implants, in order to restore the functionality and the aesthetic appearance of the patients' mandible or maxilla.

Another key factor to further decrease the time required for the total reconstruction of a bone defect is the association of a therapeutical molecule to Bonelike®. Bonelike® can function as a controlled drug-release system, as it was discussed in *Chapter 2*. Although, the selection of a therapeutical molecule, must be done very carefully, according to the specific clinical profile of the patient. Multiple myeloma is the second most common haematopoietic cancer and its major clinical manifestation is related to the loss of bone through osteolysis. Bisphosphonates are specific inhibitors of osteoclastic activity, and are currently used to prevent bone complications and to treat malignant hypercalcemia in patients with multiple myeloma, or bone metastases from breast and prostate cancers. Osteonecrosis of the jaw has been reported in patients with multiple myeloma treated for over 18 to 48 months with intravenous bisphosphonates, like zoledronate. This clinical

report alerts clinicians about the potential complication of bone necrosis in patients receiving bisphosphonates therapy; many questions remain concerning the underlying pathogenesis of this process.

The application of bone grafts like Bonelike® associated to raloxifene hydrochloride, a molecule that inhibits osteoclasts, to restore the bone lesions observed in the maxilla and the mandible of patients suffering from multiple mieloma and treated with bisphosphonates, should be considered a good option.

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A Clinical Report of Bone Regeneration in Maxillofacial Surgery using Bonelike[®] Synthetic Bone Graft

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Abstract

The objective of this study is to evaluate the osteoconductivity and bioactivity of the Bonelike[®] graft in repairing surgical cystic bone defects. Bonelike[®] was implanted in 11 patients, aged between 24 to 53 years with a mean age of 36 years, consisting of 5 men and 6 women. According to the standard follow up protocols, radiological examinations were performed and Bonelike[®]/bone retrieved samples have been analysed histologically using non-decalcified sections obtained perpendicular to bone length axis. Radiographic examination and histological results clearly demonstrated an extensive new bone formation apposed on Bonelike[®] granules with a significant degree of maturation. These clinical applications in maxillary bone defects indicated perfect bone bonding between new bone formed and Bonelike[®] granules, along with partially surface biodegradation. This quick and effective osteoconductive response from Bonelike[®] may reduce the time needed to reconstruct the bone defected area of patients.

KEY WORDS: Bonelike[®], Bone regeneration, Maxillofacial surgery, Histological studies.

Introduction

Osteoconductive materials refer to scaffolds that provide the appropriate framework for bone to grow in sites where bone naturally occurs and therefore, they function as substrates on which locally residing osteoblasts can attach. These materials rely on the presence of sufficient inorganic and organic species in the local environment to direct the bone formation process and depend on direct physical contact with exposed surfaces of viable bone¹. Often, sufficient autogenous bone is not available or would require an additional time for surgery and these factors may be unacceptable in some clinical situations. Allogenic bone, obtained from another individual of the same specie provides an alternative to autogenous grafts but the fear of disease transmission persists^{2,3}. This fear has driven the market to produce clinically beneficial alternatives to human allograft tissue⁴. Doron *et al*⁵ reports an overview of the basic concepts of bone grafting and discussed the most commonly used bone-graft substitutes and their potential indications. Synthetic bone-graft substitutes are available in different forms including blocks, granules, cements, gels and strips⁶. Examples of osteoconductive scaffolds include materials such as β -tricalcium phosphate (β -TCP), β - $\text{Ca}_3(\text{PO}_4)_2$, or hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Although these materials were found to be useful as bone fillers, HA and β -TCP have specific drawbacks⁷. Produced to date crystalline forms of HA undergo osseointegration but have a low solubility compared to the rate of new bone formation^{8,9}. On the other hand, β -TCP's rate of bio-resorption has proved to be too rapid and in an unpredictable way⁷. In order to design a scaffold that supports bone formation while gradually being replaced by bone, an optimum balance between a more stable phase like HA and a more soluble phase like TCP is essential¹⁰. Therefore, the incorporation of a CaO-P₂O₅ based glass in the HA matrix was envisaged as an easier way to produced a material to achieve this goal.

Santos *et al* developed a new biomaterial by the incorporation of glass based on a P₂O₅ system into the HA structure by a liquid sintering process. This glass reinforced HA (GR-HA) was recently patented and registered as Bonelike^{®11-14}. This system allows the incorporation of different ions into the HA structure, such as magnesium, sodium and fluoride, resulting on a bone graft with a closer chemical composition to the mineral phase of human bone¹¹⁻¹³. Additionally, it has been shown that the incorporation of a glass into the HA structure enhances its mechanical properties¹³. Another advantage of this system developed by Santos *et al* is the ability to control the percentage of secondary phases (α and β -TCP) present by the incorporation of different percentages of glass.

The improved *in vitro* biological performance of Bonelike[®] has been reported by the use of human bone marrow osteoblastic cells, and this behaviour is related to its chemical composition. Several animal studies have also been performed using Bonelike[®], and push-out tests and histological analysis demonstrated a good osseointegration of Bonelike[®]¹⁵⁻¹⁷. Furthermore, histomorphometric studies indicated that the rate of new bone formation was higher when compared to control samples of HA.

In order to facilitate the use of Bonelike[®] in different clinical applications (eg. sinus elevation), the Bonelike[®] can be associated to a resorbable matrix. Animal studies showed that the use of a resorbable matrix does not influence the bioactivity of Bonelike[®]¹⁷. Bonelike[®] has already been used with great success in several medical applications like implantology as coated dental implants and orthopaedics surgery^{18,19}. For example, a very recent study¹⁹ on histomorphometric measurements, histological and scanning electron microscopy (SEM) analyses of bone/implant interface of retrieved samples have proved the highly osteoconductive properties of Bonelike[®] in orthopaedic applications.

Eleven patients presented large bone defects resulting from the removal of bone cysts, so that in order to enhance bone regeneration and preserving the jaw volume and contour, the Bonelike[®] granules were used. Radiographic and histological analyses were performed to evaluate new bone formation and to study the interface between Bonelike[®] and the new bone.

Patients and methods

Bonelike[®] preparation and characterization

In the present study, Bonelike[®] granules size ranging from 250-500µm were prepared as follows, firstly a CaO-P₂O₅ based glass with the composition of 65P₂O₅-15CaO-10CaF₂-10Na₂O in mol% was obtained from reagent grade chemicals by using a platinum crucible at 1450°C for 2 h. The glass was crushed in an agate mortar and sieved up to a granule size less than 75µm. The Bonelike[®] was prepared by mixing 2.5% (w/w) of glass with laboratory prepared pure phase HA in iso-propanol. The mixed powders were dried for 24h at 60°C and sieved to a particle size less than 75µm and then isostatically pressed at 200 MPa. The Bonelike[®] was sintered at 1300°C for 1 hour, crushed and then sieved to the desirable particle size range. Finally, sterilisation of Bonelike[®] granules was performed by autoclave at 121°C for 35min.

X-ray diffraction (XRD) and Rietveld analysis was performed to identify and quantify the percentage of crystalline phases present in the microstructure of Bonelike[®] using a Siemens D 5000 diffractometer with Cu-K α radiation ($\lambda=1.5418\text{\AA}$). The scans were performed between 24 - 42 ° (2 θ) with a step size of 0.02° and a count time of 2 sec/step.

Clinical features

For repairing surgical maxillary cystic bone defects, patients were selected strictly on the basis of their clinical needs and according to the following criteria. The including criteria were: patients of any age, any sex, any weight, patients without any systemic disease, or infection, non-characterized maxillar or mandibular cystic lesion up to 12 cm long and the cyst removed is a true bony cyst. The exclusion criteria were: systemic unhealthy patients, infected cystic cavities, acute or chronic infection at local bone defect, bone inflammatory diseases, particularly osteomyelitis, malignant tumours, severe renal dysfunctions, and patients with non-controlled bone metabolism.

In the present study, 11 patients of both sexes, being 5 male and 6 female, ranging from 24 to 53 years with a mean age of 36 years have been considered. Cavities size varied from 3 cm in diameter in the minor lesion up to 12 cm in the largest lesion (Table 1). All cases were operated under general anaesthesia, according to a head and neck surgical protocol, in a main hospital theatre. Through a trans-gingival surgical approach making up a wide muco-periosteal flap, was the main surgical access to the bony lesion. Once attained its entire exposition, osteotomy was done to isolate the “cystic lesion wall”, by scrapping and drilling all the pathological tissues were carefully removed. In the teeth bearing areas of both maxilla and mandible, whenever a tooth route was involved a previous endodontic treatment was performed, followed by a suitable apicectomy. To determine the cyst volume a balloon (Solycil[®]) was inserted into the bone defect. The balloon was filled with saline solution (NaCl 0.9%, Braun[®]) through a catheter. The volume of saline solution that could fill the balloon located inside the cyst was the volume of the cyst. Cysts with a volume larger than 10 cm³ were measured by computer tomography (CT)-scan images. After the completely removal of the cystic tissue, the remaining bone cavities were firmly packed with Bonelike[®] granules, blood and crushed bone remnants, to completely fill the bony cavities and “to sculpt” the cortical bone contour. Primary closure of the mucosa was performed using a reabsorbable suture and care was taken to ensure sufficient mobility of the mucosal flap to cover the granules. Pre-operative CT-scans and post-operative radiological examinations were performed according to the standard follow-up protocol.

Among the 11 studied cases, histological analyses have been performed for only one patient (referred to as case n#2). Operating on a second time surgery for dental implant placement and under formal consent, a bone block ($1.0 \times 0.5 \times 0.5 \text{ cm}^3$) was taken from implant/patient bone transition. In this case, the selected biopsy was retrieved after 48 weeks of implantation and placed immediately in a neutral formaldehyde fixative solution (6%) for one week period, followed by dehydration in a series of alcohol solutions and finally embedded in resin. Non-decalcified sections of $40 \pm 10 \mu\text{m}$ were obtained from the resin blocks, after cutting these forms in the perpendicular direction of the bone length axis using a diamond saw. These sections were stained with haematoxylin/eosin and Solo-ChromeR and examined using an Olympus BH-2 transmitted light microscope and scanning electron microscopy (SEM).

Results

Figure 1 depicts an X-ray diffraction pattern of Bonelike[®]. Rietveld analysis previously reported showed that Bonelike[®] is composed by 68.4 % of HA, 24 % of α -TCP and 7.6% of β -TCP¹¹⁻¹³. For a successful regeneration, it is essential that the implant material is degraded or resorbed at a similar rate to the rate of bone formation. As it has been widely demonstrated in the literature²⁰⁻²² the phases present on Bonelike[®] can be degraded *in vivo*, HA has a slow degradation rate and TCP has a faster degradation rate. The combination of the two phases, allows the degradation rate of Bonelike[®] to be controlled to reflect the rate of bone regeneration.

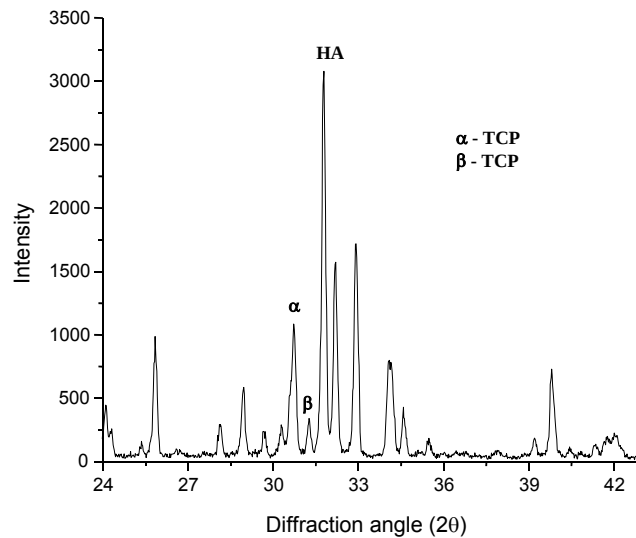


Fig.1. X-ray diffraction of Bonelike[®] graft, which is composed of HA, β - and α -TCP phases.

Most cysts of the oral and facial regions under treatment were located with in the jaws as an intra bony lesion. Figure 2 shows a non-odontogenic cyst in the midline aspect of the mandible. Large and multi loci lesions may considerably complicate the post-operative treatment. In some clinical cases the resection of a large segment of the jaw was necessary to insure complete removal of the lesion, so the pathological “tissue destruction” process and its suitable surgical removing led to a massive bone lose.

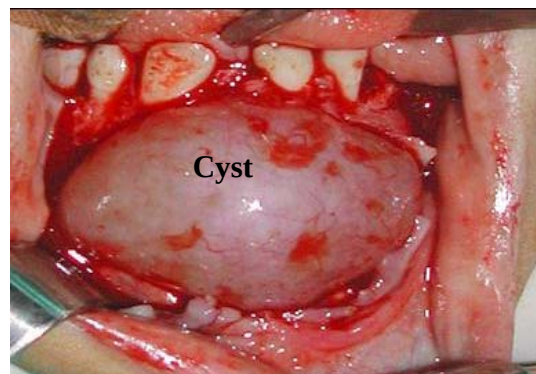


Fig.2. Frontal view showing symphysis mandibular cyst that was excised and later filled with Bonelike[®] granules in order to regenerate the bone defect.

It has been reported that the use of filling material substantially decreases the time required for the healing of a bone defect in comparison with the traditional technique (no

filling material). Additional, there is no record of post-operative infection, foreign body reaction or tear of the mucosa, independent of the cysts size, when a filling material was used. Table 1 presents the cysts diameter and volume in each clinical case, where it is possible to observe that the smallest and larger cyst as 2.4 and 12 cm in diameter, respectively. Similarly, the cyst volume varied from 9 to 152 cm³. An in-depth radiological follow-up was performed to all patients. It is noteworthy that in 10 out of 11 patients the first control was carried out immediately after surgery, the second control was performed 6 to 12 weeks after surgery (8 out of 11 patients), the third and fourth control were done after 24 to 36 weeks and 36 to 48 weeks, in 10 and 7 patients, respectively.

Table 1. Cyst volume and dimensions in each case.

Case No.	Largest Cyst Dimension (cm)	Cyst Volume (appr.) (cm³)
1.	2.4	9.6
2.	12.0	152.0
3.	7.0	52.0
4.	2.5	18.7
5.	3.0	21.0
6.	4.0	36.0
7.	3.0	11.2
8.	3.0	9.0
9.	3.0	18.0
10.	4.0	24.0
11.	3.0	12.0

One representative clinical case of the use of Bonelike[®] is demonstrated in Figure 3. Figure 3A, shows the gingival and periosteal tissue covering the entire maxillary cystic lesion. After 12 weeks of implantation, the post-operative radiological analysis showed excellent granules adaptation to the host cavity without material dislocation accompanied and partial regeneration the bone defect (Figure 3B). After 48 weeks of implantation a complete restoration of the local biofunctionality was achieved (Figure 3C).

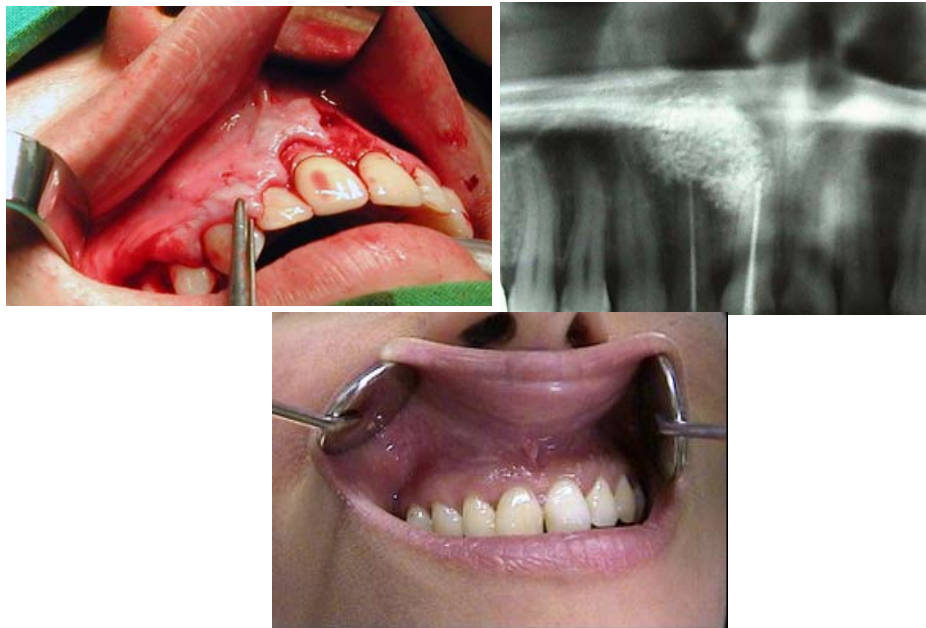


Fig.3. (A) A gingival and periosteal tissue covering the entire maxillary cystic lesion. (B) Post-operative radiograph shows the excellent Bonelike[®] granules adaptation to the bone cavity without any sings of material dislocation after 12 weeks and (C) Complete restoration of the bone defect and biofunctionality may be seen 48 weeks after implantation.

The histology analysis of the biopsy performed after 48 weeks, showed a mineralised matrix in the grafted area and mature lamellar bone was observed close to and in contact with Bonelike[®] granules (Figure 4A). Active angiogenesis, with a large number of blood vessels throughout the osteoid matrix was also seen (Figure 4A). Some degradation of Bonelike[®] granules can also be seen as shown in Figure 4B.

the case of not filled cystic cavities, the healing process was slower and normally it is observed a regenerated bone volume default^{23,24}.

In the present study, the histology showed that Bonelike[®] granules were being resorbed and that they were surrounded by new bone. The new bone presented a lamellar-like structure and filled spaces between implanted granules, as well as established direct contact between the surface of the biomaterial and the bone matrix. No inflammatory cells or fibrous tissue was seen surrounding the implant. The osteoid matrix presented several blood vessels indicating an active angiogenetic process.

The formation of a vascular network simultaneous to the formation of new bone is extremely important; this network is vital for cellular viability. It is through this network that oxygen and nutrients reach the cells, and this network is also responsible for the removal of waste products²⁵. Another important function of this network is the transport of progenitor cells and several cytokines and growth factors required for balanced bone regeneration. The angiogenesis process, formation of a vascular network from pre-existent blood vessels and the osteogenesis process, formation of new bone tissue at the defect site is interconnected from a structural, biochemical and functional away²⁵. Therefore, the ability of a bone graft to induce or allow the formation of this network is very important.

In this report it has been shown that Bonelike[®] not only has the ability to stimulate bone regeneration, but also has the ability to stimulate the formation of a vascular network. So, Bonelike[®] is an excellent scaffold for developing of bone regeneration process.

The results presented in this clinical report corroborate previous results in different clinical applications such as in implantology, and orthopaedics where histomorphometric measurements, histological and SEM analyses of bone/implant interface demonstrated the high osteoconductive properties of this bone graft^{18,19,26}. It has been shown that after 6 months of implantation in a human tibia, the contact between Bonelike[®]/ *de novo* bone was approximately of 67% and after 12 months this value can reach 84%.

Conclusions

After 48 weeks of implantation with Bonelike[®] all the patients showed high bone regeneration, they are recovering from their bone lesions and none of the patients presented any symptoms of rejection or infection. The controlled biodegradation of Bonelike[®] strongly enhances new bone formation and stimulates the revascularization of the bone tissue; therefore it may be used in a large spectrum of surgical applications. Other sites of implantation involving a large number of clinical studies with the long-term biocompatibility are underway to further insight the medical use of Bonelike[®].

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Jaw Avascular Osteonecrosis after Treatment of Multiple Myeloma with Zolendronate

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Abstract

Purpose: Multiple myeloma, a second most common haematopoietic cancer, which represents the collection of plasma-cell neoplasms that invariably, becomes fatal when self-renewing myeloma cells begin unrestrained proliferation. The major clinical manifestation of multiple myeloma is related to the loss of bone through osteolysis. This can lead to pathologic fractures, spinal cord compression, hypercalcemia, and pain. It is also a major cause of morbidity and mortality in these patients, who frequently require radiation therapy, surgery and analgesic medications. Bisphosphonates are specific inhibitors of osteoclastic activity, and are currently used to prevent bone complications and to treat malignant hypercalcemia in patients with multiple myeloma, or bone metastases from breast and prostate cancers. Hence, osteonecrosis of the mandible has been reported in 3 patients from Centro Hospitalar de Vila Nova de Gaia (CHVNG) with multiple myeloma treated for over 18 to 48 months with intravenous zoledronate, commonly prescribed for multiple myeloma therapy. Although, this report alerts clinicians about the potential complication of bone necrosis in patients receiving bisphosphonates therapy, many questions remain concerning the underlying pathogenesis of this process.

Patients and Methods: The medical and dental records of 3 patients with multiple myeloma, who were treated in CHVNG in the past 4 years, were reviewed. These 3 patients presented exposed bone and osteonecrosis of the mandible, and shared one common clinical feature: all of them were treated with bisphosphonate zoledronate, administered intravenously for long periods. Sequential orthopantomogram (OPGs) and histological evaluation have been analyzed from biopsies of the non healing dental extraction sites of these patients.

Results: After a routine dental extraction, these patients developed avascular osteonecrosis of the mandible and secondary bone infection with *Actinomyces israelii* (actinomycotic osteomyelitis), with no evidence of metastatic disease evaluated by biopsy. In these 3 described clinical cases, surgical debridement without flap elevation, intensive antibiotherapy and the suspension of the zoledronate allowed a partial recovery of the patients.

Conclusion: The purpose of this clinical report is to point out that patients suffering from multiple myeloma can develop bone osteonecrosis induced by treatment with bisphosphonates. Research to determine the mechanism of this dental phenomenon is needed to fully validate and substantiate the possible link between bisphosphonates treatment of multiple myeloma or other cancer diseases with avascular osteonecrosis of

the jaw. Until then, clinicians involved in the care of patients at risk should consider this possible complication.

Keywords: Bisphosphonates, multiple myeloma, avascular osteonecrosis, jaw.

Introduction

Multiple-myeloma (MM) constitutes a group of plasma-cell neoplasms sharing two prominent features: elevated production of monoclonal antibodies and loss of bone through osteolysis¹. Even patients responding to chemotherapy may have progression of skeletal disease, with rare occurrence of recalcification of osteolytic lesions^{2,3}. Bone loss either from direct tumoral involvement or from generalized osteoporosis is a major cause of morbidity and mortality in these patients, resulting for example, in pathologic fractures, spinal cord compression, hypercalcemia, and pain⁴. Also, these patients frequently require radiation therapy, surgery, and use of analgesics. These complications result from an asynchronous bone turnover wherein increased osteoclastic bone resorption is not accompanied by a comparable increase in bone formation. This increase in osteoclastic activity is mediated by the release of osteoclast-stimulating factors, which are produced locally in the bone-marrow microenvironment by cells of both tumour and non-tumour origin⁵. The enhanced bone loss results from the interplay between the osteoclasts, tumour cells and other non-malignant cells in the bone marrow microenvironment⁶. The bisphosphonates are non-metabolized analogues of endogenous pyrophosphates (PP_i) that can be localized in bone and inhibit osteoclastic function. These molecules bind avidly to exposed bone mineral around reabsorbing osteoclasts, resulting in very high levels of bisphosphonates in the resorption lacunae. Because bisphosphonates are not metabolized, high concentrations are maintained within bone for long periods of time. Bisphosphonates are then internalized by the osteoclast, causing disruption of osteoclast-mediated bone resorption^{7,8}. Their potential for strong inhibition of osteoclastic bone resorption and high affinity for hydroxyapatite crystals have progressively extended the field of their clinical indications⁹⁻¹². Such compounds are able to chelate Ca²⁺ ions very effectively, and its high affinity for Ca²⁺ crystals permits its binding to hydroxyapatite crystals in the mineralized bone matrix¹³. Although, the exact mechanism of this bisphosphonates-mediated osteoclast inhibition has not been completely elucidated, but it has been established that these compounds affect bone turnover at various levels^{7,8}. At tissue level, bisphosphonates inhibit bone resorption and decrease bone turnover as assessed by biochemical markers^{7,8}. On a cellular level, the bisphosphonates clearly target the osteoclasts and may inhibit their function in three possible ways: (1) inhibition of osteoclast recruitment¹⁴, (2) reduction of the osteoclast life span¹⁵, and (3) inhibition of osteoclastic activity at the bone surface¹⁶. At a molecular level, it has been postulated that bisphosphonates modulate osteoclast function by interacting with a cell surface receptor or an intracellular enzyme¹⁷. Several structurally related bisphosphonates have been

synthesized by changing the two lateral chains on the carbon or by sterifying the phosphate groups¹⁸. The resulting analogues vary extensively in their anti-resorptive potency, with analogues such as etidronate being the weakest, alendronate being stronger, and the new analogue, zoledronate, being the most potent^{18,19}. Intravenous bisphosphonates are the current standard for the treatment of hypercalcemia of malignancy (HCM) and of prevention of skeletal complications associated with bone metastases^{1,20,21}. Currently, zoledronic acid (2-[imidazol-1-yl]-1-hydroxyethylidene-1,1-phosphonic acid, known as Zometa, 4 mg via a 15-min infusion) and pamidronate (Aredia[®], 90 mg via a 2-hour infusion) are the only agents recommended by the American Society of Clinical Oncology (ASCO) for the treatment of bone lesions derived from breast cancer and multiple myeloma^{22,23}. Furthermore, zoledronic acid is approved by both the U.S. Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products for the prevention of skeletal complications in patients with multiple myeloma, bone metastases secondary to a variety of solid tumours, (breast, prostate and lung cancer) and malignant hypercalcemia²⁴⁻²⁷. These intravenously administered bisphosphonates significantly reduced the development of skeletal complications and improved the survival of patients²⁴⁻²⁷. Recent studies have demonstrated the efficacy and increased convenience of the newer, more potent imidazole-containing bisphosphonate zoledronic acid in the management of the skeletal complications of myeloma^{6,28} and also provides long-term reduction of bone pain in patients with bone metastases secondary to prostate cancer^{6,28}. If tolerated, it is common for these patients to be maintained indefinitely on bisphosphonates therapy⁷. The oral bisphosphonate preparations (alendronate and risedronate) are also potent osteoclast inhibitors, but are not as effective in the treatment of malignant osteolytic disease, and therefore are only prescribed for the treatment of osteoporosis⁷. Bisphosphonates-associated osteonecrosis of the jaws (ONJ) is currently a very topical subject. Initially it was thought to be an extremely rare condition but in a retrospective chart review of multiple myeloma and breast cancer, ONJ was reported in 10.5% of those who received intravenous bisphosphonates at the Memorial Sloan-Kettering Cancer Center in 2003²⁹. Osteonecrosis has not been seen at any other skeletal site in these patients. Bisphosphonates-associated ONJ is characterized by dehiscence of the oral mucous membranes, with exposure of the underlying mandible or maxilla where it can be observed bone necrosis. More than 50% of the cases have been diagnosed after surgery procedures, like extractions, implants and periodontal procedures. In some clinical cases, ONJ does not respond to any form of treatment that has yet been attempted, like interruption of the chemotherapy and bisphosphonates administration. According to the literature the use of hyperbaric oxygen does not induce

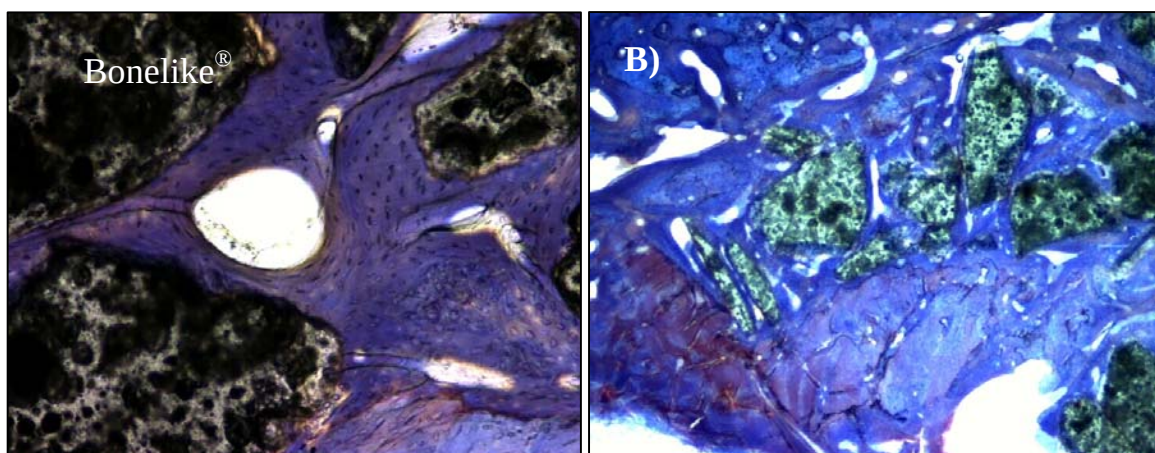


Fig.4. Histological image shows the implantation of Bonelike[®] granules in the range of 250-500 μm after 48 weeks. New bone formation and surface resorption have been observed around granules (A), the formation of blood vessels due to active angiogenesis (B), osteoid matrix (C) and Bonelike[®] resorption could also be observed, which indicates that this novel bone graft shows controlled biodegradation *in vivo* by SEM (D), (Original magnification 400x, Solo-Chrome R staining).

Discussion

The present clinical report shows that the use of Bonelike[®] aids the recovery of the patients restoring the biofunctionality of the affected area. After 48 weeks of implantation there is significant bone regeneration and all patients are recovering from their bone lesions and did not present any symptoms of rejection or infections.

As mentioned previously Bonelike[®] has a similar composition to the mineral phase of bone, the presence of controlled biodegradable β - and α -TCP phases into its structure results in a local enrichment in Ca^{2+} , P^{5+} , Na^{+} and F^{-} into the physiological environment, which stimulate new bone formation. The presence of a more stable phase like HA provides a scaffold for the attachment of bone cells that will support bone formation. It has been widely demonstrated in the literature that β - and α - TCP can be degraded *in vivo* and that HA is a bioactive material²⁰⁻²².

In this clinical report, the ratio of remnant bone to synthetic graft varied from case to case and in accordance with cavities volume and amount of collected bone. Nevertheless about 1/3 of total filling was patient's own bone. Therefore, bone healing was occurred from the periphery of the cystic cavity to the centre with a controlled process in which the filling material served as a matrix to conduct bone cells as it could be observed in X-rays images. In

any effect on these patients³⁰. Antibiotics cannot penetrate the necrotic tissue, being only used to manage cellulites in adjacent tissues. By default, a conservative and symptomatic treatment is the current recommendation. Patients receiving bisphosphonates infusions are asked to avoid oral surgery^{31,32}. The mechanism underlying the reaction is unknown, but it has been postulated that bisphosphonates inhibit new vessel formation. In many cases, dental extractions and other oral surgeries have been identified as precipitants. Cancer diagnosis, concomitant therapies (chemotherapy, radiotherapy and corticosteroids) and morbid conditions (anaemia, coagulopathies, infection, and pre-existing oral disease) are documented risks factors³³.

Patients and Methods

The first reported case in the CHVNG hospital was a 71-year-old man who was originally diagnosed an IgA multiple myeloma in 2002 (Fig.1 a - d). This patient was simultaneously treated with chemotherapy (cyclophosphamide by intravenous administration, 1 mg/day, and every month), eritropoetin (30000 U/day, by subcutaneous administration, every month,), zoledronic acid (4 mg during 15 minutes per month, by intravenously administration), dexamethasone (40 mg, *per os*, during 4 consecutive days, and every month), and thalidomide (100 mg/ day, *per os*) during 3 years. In July of 2003, it was performed a routine dental extraction of tooth 4.5 and a devitalisation of tooth 4.4. After the tooth extraction, the patient developed symptoms of a more intensive mandible pain. These procedures did not solve the patient clinical symptoms, and were followed by a routine dental extraction of tooth 4.4 in September of 2003. In April of 2004, the presenting symptoms were still mandible pain, being already visible exposed bone at the site of the previous teeth extractions. The orthopantomogram taken at that time, revealed a circumscribed area of osseous necrosis of the right mandible.

The second clinical report refers to a 66-year-old man who was diagnosed an IgA multiple myeloma, in May 2001 (Fig. 2 and Fig. 3). This patient received treatment during 3 years with intravenously zoledronic acid (4 mg during 15 minutes per moth) associated with chemotherapy (cyclophosphamide, by intravenous administration, 1 mg/day, and every month,) and eritropoetin (30000 U/day, by subcutaneous administration every month,). The patient was also being treated with dexamethasone (40 mg, *per os*, during 4 consecutive days, and every month), filgastrin (30000000 U/day, by subcutaneous administration, every month), and thalidomide (100 mg/ day, *per os*). In March 2004, the dental extraction of the tooth 4.6 was performed. The tooth 4.5 had been extracted 6 months before. At that time the patient started to complain of jaw pain, difficulty in

masticating and in brushing teeth. The clinical appearance simulated dental abscesses or osteomyelitis. In July 2005 a biopsy of the involved area showed the presence of necrotic lacunae, bacterial debris, and granulation tissue with infiltration of lymphocytes and histiocytes. Culture results revealed a secondary infection with *Actinomyces Israelii* (actinomycotic osteomyelitis). The teeth extraction resulted in a painful, and nonhealing bone lesion in the mandible. Examination revealed an area of exposed and necrotic bone, resulting in a jaw avascular osteonecrosis diagnosis. The secondary infection by *Actinomyces israelii* was treated with amoxicillin (500 mg, *per os*, every 8 hours, during 3 months) and the bisphosphonate treatment was immediately interrupted. Superficial debridement of the osseous necrosis area was attempted under local anaesthesia, without elevating a gingival flap.

The third case reported is a 40-year-old woman with a medical history of IgA multiple myeloma diagnosed in 2003 (Fig. 4 and Fig. 5). She had been receiving chemotherapy (cyclophosphamide, by intravenous administration, 1 mg/day, and every month), zoledronic acid (4 mg infusion during 15 minutes per moth) and dexamethasone (40 mg, *per os*, during 4 days, every month) during 18 months. In November 2004 the dental extraction of the mandible tooth 4.7 was performed. In August 2005, a panoramic radiography revealed that there wasn't regeneration of the bone tissue and that a process of osteonecrosis with reactive osteosclerosis was present. An area of exposed, necrotic bone was observed, and the diagnosis was jaw avascular osteonecrosis. The biopsy of the involved area was performed, revealing the presence of necrotic lacunae, bacterial debris, and granulation tissue with infiltration of lymphocytes and histiocytes. Evidence of metastatic bone disease was not detected in any of the biopsied jaw lesions from the three patients reported. Minor debridement procedures under local anaesthesia were also attempted, however it was required a major surgery to remove all of the involved bone. The patient is presently receiving treatment with cyclosporine (15 mg / kg / day, *per os*) in order to be performed a bone tissue auto transplantation without subsequent rejection.

Results

Fig. 1(a) shows an orthopantomogram taken to the patient from the first clinical report previously described, and it was obtained in July 2003, before extraction of tooth 4.5 and devitalisation of tooth 4.4. By that time, the patient was receiving the zoledronate infusion treatment. The orthopantomogram taken to the same patient mandible in April 2004 is represented in figure 1(b). At that time, the dental extraction of both teeth 4.4 and 4.5 from the right side had already been performed and a slight bone necrosis with

sequestered tissue could be observed in the right mandible. At this point, the patient had been receiving intravenous zoledronate for a period of 2 years. Figure 1(c) is the orthopantomogram obtained in July 2005, after 3 years of zoledronate administration, and shows the presence of an extended zone of the bone necrosis in the exact region of the extraction site. Figure 1(d) is an image taken to the patient's right mandible in September 2005 where an exposed necrotic mandibular bone is clearly observed, being correlated with the diagnosis of jaw avascular bone necrosis. The biopsy taken at that time consisted in removing a sample of the overlying tissue from the dental extraction site. The histological exam showed a necrotic bone with associated bacterial debris and granulation tissue. Culture results revealed normal oral flora and a secondary infection with *Actinomyces israelii*, which caused an actinomycotic osteomyelitis. This infection was treated with amoxicillin (500 mg, *per os*, every 8 hours, during 4 months) and the bisphosphonate administration was immediately interrupted. In November 2005, a superficial osteotomy under local anaesthesia, of the necrotic bone was performed, but it was interrupted by profuse intraosseous haemorrhage.



Fig.1(a). Orthopantomogram obtained in July of 2003 before dental extraction of the teeth 4.4 and 4.5. The patient was receiving intravenous zoledronic acid treatment. The right mandible bone was apparently normal.



Fig.1(b). Orthopantomogram control obtained from the patient mandible in April of 2004, 6 to 8 months after the teeth 4.5 and 4.4 extractions, respectively, from the right mandible.



Fig.1(c). Orthopantomogram obtained in July of 2005. An extended zone of the bone necrosis is present in the right mandible, probably associated to the zoledronic acid treatment (red circle).



Fig.1(d). Exposed necrotic mandible bone in a patient receiving intravenously zoledronic acid for a long period of time, following a routine dental extraction of teeth 4.4 and 4.5 from the right mandible. This image was taken to the patient's right mandible in September 2005 and is correlated with the diagnosis of jaw avascular bone necrosis.

Fig. 2 shows an orthopantomogram obtained in March 2004, just before the extraction of tooth 4.6 from the right mandible of the second patient. The tooth 4.5 had been removed 6 months before.



Fig.2.Orthopantomogram obtained in March 2004, immediately before dental extraction of tooth 4.6 from the right mandible of the second patient. The tooth 4.5 was extracted 6 months before. The patient received zoledronic acid intravenously for 3 years after the diagnosis of an IgA multiple myeloma. It was already present a local area of bone necrosis in the site of the first dental extraction (red circle).

Fig. 3 shows another panoramic radiography from the second clinical case, taken in June 2005. In this X-Ray exam is visible a more extended area of bone destruction involving the right mandible in the region where the dental extraction had been performed 15 months before. Fig. 4 shows a panoramic radiograph of the third patient obtained in November 2004 of the mandible, immediately before the extraction of tooth 4.7.



Fig.3. Orthopantomogram obtained in June of 2005, showing a more extensive area of bone destruction (red circle) involving the right mandible due to bisphosphonates - associated osteonecrosis in the local where the dental extraction was performed.



Fig.4. Orthopantomogram of the mandible before the dental extraction in a patient suffering from IgA multiple myeloma who had previously received intravenously zoledronate for 18 months.

Figure 5 shows the orthopantomogram from the previous patient, taken in August 2005, 9 months after the dental extraction, where it can be seen an exuberant osteonecrosis of the mandible in the region of the non-healing extraction site.

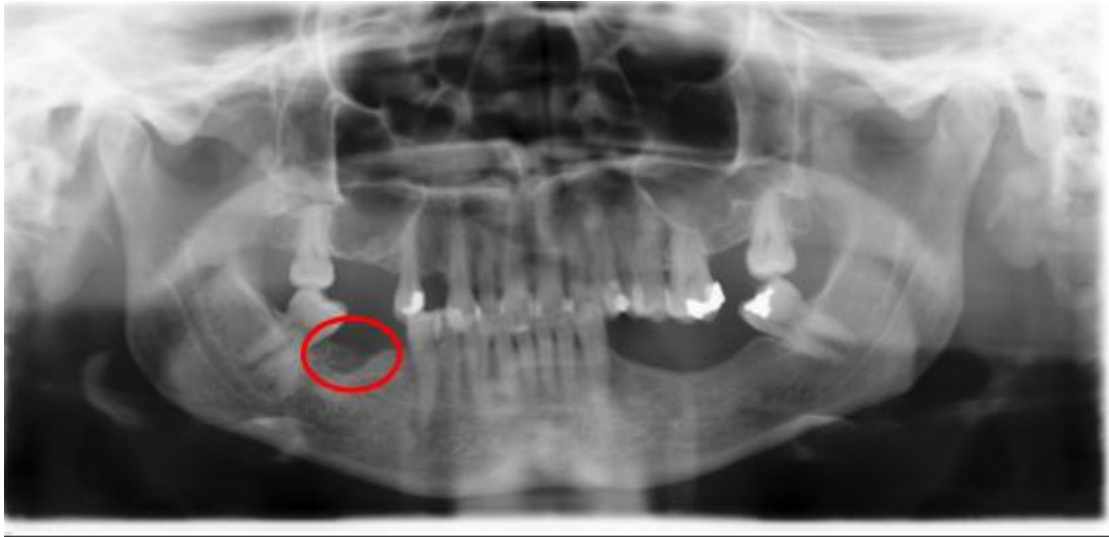


Fig.5.Orthopantomogram of the mandible 9 months after the dental extraction of tooth 4.7. The mottled bone is observed in the region of the non healing extraction site, due to jaw bisphosphonate-induced osteonecrosis (red circle).

Discussion

In multiple myeloma patients the major clinical problems that arise are related to the enhanced bone loss that commonly occurs in these patients. Even patients responding to chemotherapy may have progression of skeletal disease, with rare recalcification of osteolytic lesions^{2,3}. The treatment protocols include the administration of thalidomide, which is a radiosensitizing agent. In order to safely reduce bone complications in myeloma patients, bisphosphonates like zoledronic acid or pamidronate can be intravenously administered monthly in combination with chemotherapy.

Bisphosphonates are effective inhibitors of bone resorption and reduce the risk of skeletal complications. Osteoclasts and osteocytes functions are part of the bone turnover cycle, which is critical to maintain bone reserves and bone viability. If the osteoclastic function is severely impaired, the osteocytes are not replaced, and the bone capillary network is lost, resulting in avascular bone necrosis⁴. The mechanism underlying the reaction is unknown but it has been postulated that bisphosphonates inhibit new vessel formation, leading to avascular bone necrosis³⁴. It is believed that bisphosphonates related osteonecrosis results from altered bone homeostasis, to such extent that the bone's ability to heal after minor lesions is compromised. In certain conditions fungi and bacteria may also secondarily infect the bone. Osteonecrosis of the jaws can remain asymptomatic for many weeks or months and may only be recognized by the presence of exposed bone in the oral cavity. Also, these lesions are frequently symptomatic, when

secondarily infected or there is trauma to the soft tissue due to sharp edges of the exposed bone. Osteonecrosis may occur spontaneously or, more commonly at the site of previous tooth extraction. Some patients may present typical complaints such as a numbness feeling of a heavy jaw and various dysesthesias. The signs and symptoms that may occur before the development of a clinical osteonecrosis include a sudden change in the health of periodontal or mucosal tissue, failure of the oral mucosa to heal, undiagnosed oral pain, loose teeth, or soft-tissue infection. Studies involving larger numbers of patients have shown that nearly 80% of cases were initiated by tooth removal³⁰. It is not clear at the time of osteonecrosis appearance whether discontinuing bisphosphonates would significantly alter the risk or the course of osteonecrosis of the jaw. Bisphosphonates are not metabolised and have a strong affinity to bind to osteoclasts, persisting in bone tissue for months and sometimes years after the drug withdrawal therapy, which does not seem to hasten recovery of the osteonecrosis³³.

In the reported clinical cases, the treatment with zoledronate was suspended, associated to surgical procedures and intensive antibiotherapy, allowed a partial recovery of the patients. If osteonecrosis is suspected, panoramic and tomographic imaging may be performed to rule out other causes like alveolar dental cysts or impacted teeth. Smaller intraoral films can also be used to demonstrate subtle bone changes. Tissue biopsy should be performed only if metastatic disease is suspected, and microbial cultures (aerobic and anaerobic) may provide identification of pathogens causing secondary infections^{33,34}. Potential risk factors for the development of osteonecrosis of the jaws may include: concomitant therapy with steroids, chemotherapy, and bisphosphonates therapy by intravenous administration, dental extraction, infectious disease, and/or trauma, head and neck radiotherapy, chemotherapy, immunotherapy, or other cancer treatment protocols, coagulopathies, periodontal disease, bone exostosis, previous invasive dental procedures, dental prostheses, vascular disorders, alcohol abuse, and malnutrition^{35,36}. A potential preventive measure prior to the initiation of intravenous bisphosphonates therapy will avoid any elective jaw procedure that requires bone heal. It is recommend a routine clinical dental exam that may include panoramic jaw radiography to detect potential dental and periodontal infections^{31,37}. If bisphosphonates can be briefly delayed without the risk of a skeletal-related complication, teeth with a poor prognosis or in need of extraction should be extracted and other dental surgeries should be completed prior to the initiation of bisphosphonate therapy^{31,37-39}. Bisphosphonate treatment must be performed together with the oncologist and the oral maxillofacial surgeon or another dental specialist. Preventive dentistry procedures should be performed before the chemotherapy, immunotherapy, and/or bisphosphonate therapy (removing abscessed and nonrestorable

teeth and involved periodontal tissues, functional rehabilitation of the teeth, and oral self-care hygiene education)³⁹. The efforts should focus on preventing the progression of lesions and limiting complications related to secondary infection. In established cases, the primary goals are palliative treatment and control of osteomyelitis. Oncologists should perform a brief visual inspection of the oral cavity at every follow-up visit. As a matter of fact, patients should be monitored every 3 months or sooner (if symptoms continue or worsen), cessation or interruption of bisphosphonate therapy may be considered in severe cases, osteointegrated dental implants are contra-indicated and may result in further osteonecrosis. The objective of antibiotic therapy is to prevent secondary soft-tissue infection, pain and osteomyelitis.

Although the report of these 3 clinical cases alerts clinicians about the potential complication of bone necrosis in patients receiving bisphosphonate therapy, many questions remain, concerning the underlying pathogenesis of this process. Further research is needed to elucidate the precise relationship between bisphosphonates and jaw osteonecrosis. It can be hypothesized that a number of factors might intervene in raising the risk of this complication: (a) taxanes are increasingly used to treat patients affected by several types of tumours, including MM; (b) thalidomide, a drug with an antiangiogenic mechanism, is widely used to treat MM patients who are also receiving bisphosphonates; (c) due to the prolonged survival of cancer patients, they have to receive bisphosphonates for longer periods of time, without interruption; (d) a wider use of bisphosphonates specially the most powerful ones like zoledronic acid is being observed; (e) the availability of potent oral bisphosphonates, such as ibandronate, while rendering more convenient the administration of the drug, might make this pathology pass unnoticed or delay its diagnosis. It becomes important to adopt appropriate preventive dentistry with control of dental caries and periodontal disease, and it seems prudent to make health care professionals and patients aware of the potential risk associated to the referred treatment. In the 3 described clinical cases, surgical debridement without flap elevation, intensive antibiotherapy and the suspension of the zoledronate treatment allowed a partial recovery of the patients. We propose this type of clinical approach in patients suffering from MM and jaw osteonecrosis induced by bisphosphonate treatment.

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Chapter 5

**General Discussion and
Final Conclusions**

General Discussion

During lifetime, bone mass is continuously involved in bone remodelling, which is responsible for the renewal of the skeleton, necessary for the maintenance of bone tissue integrity and mineral homeostasis. The remodelling process is also important to replace dead or damaged tissue, to give bone the capacity to adapt to load variations and to respond to nutritional and/or metabolic changes. It is an orderly sequence of events, achieved by the concerted actions of different bone cells– the basic multicellular unit (BMU)¹, that the remodelling processes occurs. There are four different phases – activation, resorption, reversal and formation – the ARRF sequence, which takes about 3 – 6 months for completion. In these phases there are mainly two distinct cell lineages involved, the osteoblasts and the osteoclasts, which form and resorb the mineralised tissue, respectively. Living bone is continuously undergoing remodelling and the turnover rate is around 10 % a year in adult bone. However, when a substantial amount of bone has already been lost, inhibition of bone resorption may not be sufficient to remove the risk of fracture.

Regenerative graft procedure refers to technologies that repair or replace any defective diseased tissues or organs by trauma, ageing, etc. Bone grafting is commonly used in the reconstruction of defect areas in several surgical procedures, mainly to swift *de novo* bone formation *in vivo* with the aim of providing a rigid structure, in which the host bone can regenerate and heal in a proper way at defined time periods².

Generally, there are four types of bone grafts, namely autograft, allograft, xenograft and synthetic graft that have been widely used in regenerative surgery³. Autografts are those where the bone to be grafted is from another site in the body of the same individual. They are immunologically safe and thus limiting rejection concerns. The harvest of an autograft implies extra and invasive surgical procedure coupled with the post-operative pain. Another disadvantage is the limited quantity of bone available for harvesting. Allografts are taken from human donors such as organ, tissues or cells donated from genetically distinct individual of the same specie. The use of this graft can solve some of the drawbacks related with autologous bone grafting since the second surgical procedure is eliminated and the quantity of tissue is available in large amounts. However, the risk of postoperative infection and disease transmission etc, are higher than with autograft. As a matter of fact, for bone allograft, the risks of immunological rejection and of contamination, in particular by virus and prions, like HIV, hepatitis, and Bovine Spongiform Encephalopathy (BSE), respectively, should be considered as important contra-

indications, nowadays. Xenografts involve the harvest of animal tissue and its implantation in humans. The animal bone, most commonly bovine (cow) is especially processed to make it biocompatible and sterile. It acts like filler, which in time, the body will replace by natural bone. Synthetic graft substitutes have been developed to provide an alternative to autografts, allografts²⁻⁶ and xenografts. Synthetic bone graft substitutes offer many advantages compared to autografts including a lower probability of rejection or risk of morbidity, patient pain and recovery time. Therefore, a number of different materials such as metals, ceramics, polymers and composites are now commonly used for repair human bone. Nowadays, many of the bone grafts under clinical evaluation use synthetic materials as an alternative to the bone-derived grafts. For successful bone grafting, there are three basic criteria namely, osteogenesis, osteoinduction and osteoconduction³. Osteogenesis is the process by which bone is formed by the transplanted living cells (osteoblast precursors and osteoblasts). To date, the only graft that displays true osteogenic properties is the autograft. Osteoinduction is the process, that stimulates new bone formation by bone-forming cells². Blood derived proteins, peptides, growth factors and a specific group of molecules named cytokines provide this stimulation. Osteoconduction is the process, which provides a structural framework and environment that supports the attachment, proliferation, and differentiation of osteoprogenitor and osteoblasts cells into the graft. Autografts, allografts and mineral bone graft substitutes such as, hydroxyapatite (HA) and bioactive glass present this property.

Osteoconductive materials, such as synthetic calcium phosphate ceramics are of especial interest for bone repair due to its similar chemical composition to biological apatites in normal calcified tissues, e.g. enamel, dentine and bone. The calcium phosphates grafts can be used in dense, granular or porous form as well as coatings of metal prosthesis and implants. The benefits of synthetic grafts include availability, sterility, cost-effectiveness, and reduced morbidity. However, the selection of a grafting procedure is purely dependent on the nature and pathology of the bone defect, as well as the choice of available bone grafts. Ideally a synthetic bone graft should be biocompatible, show minimal fibrotic reaction, undergo remodelling and support ossification.

Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is a biocompatible material and it has been used as bone graft for a long time. HA is osteoconductive, however its resorption rate is slow. Hence, different approaches have been used in order to overcome this disadvantage, like modifying and combining HA with other materials to improve its functionality and faster resorption. Tricalcium phosphate (TCP) $\text{Ca}_3(\text{PO}_4)_2$, in their allotropic forms β and α -TCP, have a higher solubility and resorption rate than HA. Due to their relative solubility, TCP is generally used in circumstances where structural support is less important. Glass-based materials are considered as a surface reactive ceramics.

These types of materials when implanted undergo dissolution and release ions into the surrounding environment with consequences to the local pH environment. The composition of the materials influences surface reactivity and some are known to bond to the surrounding living bone tissues. The form of the synthetic graft can be adapted to the defect, e.g. blocks are normally used in situations of trauma, interbody spinal fusion and non-union and granules are generally used for posterior/lateral spinal fusion, filling cystic voids as well as for hip and knee revisions. Granules have a significant price advantage over the other forms of bone grafts. Among other indications, cement is used in the augmentation of pedicle screw fixation, whereas gels can be used percutaneously and injected into closed fractures. Strips are less commonly used, but could be utilised in acetabular reconstructions⁶. The use of a bone graft depends on the bone defects, for instance, if the defect is minor, bone has the capability to self remodel within a few weeks, but in the case of large defects with loss of bone volume the bone cannot heal by itself, therefore, grafting is required to restore function without damaging the living bone tissue. So, new bioactive bone grafts can be used, instead of allo- and autografts, which opens-up enormous possibilities for reconstructive surgery.

According to the statistical and published data on bone graft substitutes, it is estimated that 500,000 to 600,000 bone grafting procedures are performed annually in the United States. Approximately half of these surgeries involve spinal arthrodesis whereas 35% - 40% are used for general orthopaedic applications^{2,7}. In Europe the number of grafting procedures was reported to be 287,300 in the year 2000, with a predicted increase to 479,079 in the year 2005^{6,8}. Synthetic bone graft substitutes currently represent 10% of the bone graft market, but their share is increasing day-by-day as experience and confidence accumulated⁷.

As mentioned previously, synthetic bone grafts have been developed to provide an alternative to autografts and allografts. Nowadays, it is possible to prepare synthetic bone substitutes that have very similar composition to the mineral osseous tissue. This is an important aspect to enhance the regeneration of bone, since bone graft should promote an ideal microenvironment where it is possible for cellular adhesion, proliferation and differentiation to occur. Some of these biomaterials can be resorbed by physiologic mechanism in a time controlled way, in order to permit the correct process of natural reconstruction of bone tissue. There are several medical applications for this synthetic bone grafts, for instance, dentistry, maxillofacial surgery and orthopaedics, aiming at the regeneration of bone defect areas which resulted from a bone disease, trauma or ageing.

Therefore, in order to design a scaffold that supports bone formation while gradually being replaced by bone, an optimum balance between a more stable phase like HA and a

more soluble phase like TCP, is essential. J. D. Santos and co-authors developed⁹⁻¹² a glass-reinforced HA (GR-HA) composite based on the incorporation of a CaO-P₂O₅ glass into the HA matrix. This patented material has been recently registered and marketed as Bonelike[®]. This bone graft displays two distinctive advantages: (a) enhanced bioactivity, by reproducing the inorganic phase of bone which contains several ionic substitutions, modulating its biological behaviour, (b) improved mechanical properties, due to its innovative manufacture procedure, which used a liquid phase sintering process reducing the porosity and grain size of the material¹³. The controlled release of several ions such as: fluoride, magnesium and sodium, from Bonelike[®] to the surrounding medium can stimulate bone formation.

In the biomaterial field there is a need to promote biological tests in order to assure the benefit to the patient. Biocompatibility testing is concerned with *biosafety* and *the ability of the material to perform with an appropriate host response in a specific application*. Therefore, *in vitro* and *in vivo* studies form an integral part of tests to assess the potential of implant materials, before clinical trials.

The biological performance of Bonelike[®] was previously assessed by using human bone osteoblastic cell cultures, namely the osteosarcoma cell line MG63 and osteogenic-induced bone marrow cells. The cell response was evaluated by a direct assay, i.e. culturing the cells on the material's surface, and also using an indirect assay, with the cultures being performed in the presence of Bonelike[®] extracts¹⁷⁻¹⁹. Results regarding the response of human bone marrow osteoblastic cells to Bonelike[®] showed, in general, that, Bonelike[®] has a positive effect regarding cell proliferation, synthesis of alkaline phosphatase and the formation of a mineralised matrix. The improved biological performance of Bonelike[®] it is probably related with its chemical composition. As mentioned previously, this biomaterial is composed of an HA matrix with more soluble phases like β - and α -TCP and also several ions, such as, fluoride that has a positive effect on bone cells, as reported by the literature²⁰. Previous animal studies with Bonelike[®] demonstrated its osteointegration and high bioactivity²¹.

The first part of this thesis, chapter 2, is focused in *in vivo* test of Bonelike[®] associated to a resorbable matrix and some therapeutic molecules using a rabbit model. Afterwards, the clinical trials performed with Bonelike[®] granules or dental implants coated with Bonelike[®] in maxillofacial surgery procedures, are extensively described and discussed in *Chapter 3* and *Chapter 4*.

After the promising results obtained in the acute screening using several *in vitro* techniques, it is essential to perform *in vivo* animal tests of the implantable materials. The *in vivo* testing has nowadays important ethical issues that should and must be followed by the researchers and, of course, were carefully followed during the experimental part of this thesis. All animals were housed in a temperature and humidity controlled room with light/dark cycles appropriate to their physiology. The animals were fed properly, with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking in account human endpoints for animal suffering and distress. The *in vivo* test of Bonelike® and all the procedures were performed with the approval of the National Veterinary authorities in accordance with the European Communities Council Directive 86/609/EEC, since the research group works in the European Community. Experimental animals such as rabbits have short life span than humans, so they have higher metabolic rates, which obviously include the rate of bone tissue regeneration. In this work, we used New Zealand White rabbits (Charles River Laboratories, Barcelona, Spain) with a weight between 2.5-3.5kg as our animal model for bone tissue regeneration evaluation.

The report of *in vivo* studies with rabbits, of a user-friendly version of Bonelike® with two granulometries (150-250µm and 250-500µm) is included in *Chapter 2*. The Bonelike® granules were associated to resorbable matrixes (FloSeal® or Normal Gel 0.9% NaCl®) and to a therapeutical molecule, the raloxifene hydrochloride. FloSeal® is easily used and it can be extruded from a syringe and applied topically to the bleeding area. According to the literature, this haemostatic agent has the ability to acquire irregular shapes fitting the wounded site^{22,23}. When FloSeal® is in contact with blood; the collagen particles are hydrated and swell. The thrombin present converts the patient fibrinogen into a fibrin polymer, originating a clot around the granules^{22,23}. Normal Gel 0.9% NaCl® (Moneylycke, Portugal) is a polymeric vehicle²⁴ and the raloxifene hydrochloride is a known selective estrogen receptor modulator (SERM) and acts as an estrogen agonist on bone and liver, it can also increase bone mineral density²⁵⁻²⁷, therefore it is used for prevention of osteoporosis in postmenopausal women²⁵. It is also known that *in vitro* raloxifene hydrochloride inhibits mammalian osteoclast differentiation and bone resorption in the presence of interleukin-6 (IL-6)²⁵⁻²⁷.

As mentioned previously, for the *in vivo* testing of Bonelike® associated to several vehicles, healthy skeletally mature male New Zealand White rabbits were used as experimental models^{24,28}. For surgery, rabbits were placed prone under sterile conditions and under deep anaesthesia a longitudinal incision was made on the lateral surface exposing the femur. In each femur, several holes were drilled through the cortex and into medulla using a micro-burr continuously flushed with a saline solution (NaCl 0.9%, Braun)

to minimize thermal damage and to remove any residual bone^{24,28}. The two vehicles (FloSeal[®] and Normal Gel 0.9% NaCl[®]) were tested in association with Bonelike[®] granules, and implanted into the holes. A therapeutic molecule, raloxifene hydrochloride^{24,28}, was associated to these vehicles.

Through sequential x-ray images, it was possible to follow the healing process every week in the operated rabbits. X-ray analysis of rabbit femurs revealed high osteointegration and defect healing for all experimental conditions. During the healing period, rabbits easily recovered and no rejection symptoms were observed in the implantation site for all implanted samples^{24,28}. Rabbits were sacrificed 12 weeks after implantation, and the retrieved samples analysed by scanning electron microscopy (SEM) and Solo Chrome R/Haematoxylin-Eosin stain was used for histological evaluation. SEM characterisation of unstained slices was performed to quantify the contact percentage of new bone formed within the implanted granules and to assess the *in vivo* degradation process. The interface layer implanted material/new bone formed was evaluated by SEM-EDX (energy dispersion x-ray microanalyser)^{24,28}. Both SEM and histological analyses confirmed the osteointegration of Bonelike[®] granules and the new bone formation, with almost complete regeneration of the bone defects^{24,28}.

Bonelike[®] associated with FloSeal[®] and raloxifene hydrochloride showed that new bone was rapidly apposed on implanted granules after 12 weeks of implantation in rabbits. Bonelike[®] granules were completely surrounded by *de novo* mature bone and it was possible to observe a complete osteointegration of the Bonelike[®] granules with bone tissue forming among them with the presence of new osteon. Additionally, an extensive surface dissolution of Bonelike[®] granules could be observed with both matrixes^{24,28}. No evidence of osteoclasts activity was observed which may be explained by the presence of raloxifene hydrochloride that is known to inhibit osteoclast activity^{25,27}. The Bonelike[®] granules were completely surrounded by new bone (fibroreticular) with vascular structures and cement lines indicating active bone regeneration. The formation of several blood channels without any sign of inflammation was observed throughout the osteoid matrix. The presence of blood vessels was due to active angiogenesis process that is an extremely important process for bone regeneration²⁸.

In conclusion, the Bonelike[®] graft associated to FloSeal[®] or Normal Gel[®] matrix seemed to serve as an excellent scaffold for bone regeneration^{24,28}. In addition, the association of Bonelike[®] to a resorbable vehicle can act as a controlled release system to osteoinductive molecules and therefore enhancing the osteointegration of Bonelike[®]. This system is also easier-to-handle and can be considered as an injectable osteoconductive synthetic bone graft.^{24,28} For several medical applications that require bone regeneration, the use of a vehicle to carry the bone graft is considered a very relevant issue. In fact, this

association not only facilitates the medical application of the bone graft but also opens-up new areas of application in medicine, namely those related to: (a) minimal invasive surgery and (b) the possibility of associating therapeutic molecules that have crucial function in bone regeneration^{24,28}.

Bonelike[®] graft has been successfully applied in several areas of Reconstructive Medicine namely in oral and maxillofacial surgery, implantology and orthopaedics²⁹⁻³³. In oral surgery Bonelike[®] has been used for the regeneration of bone defects after cyst removal and retained tooth extraction, in maxillofacial surgery for the reconstruction of maxillar and mandible, in implantology for bone augmentation around implants, ridge augmentation for later implantation and sinus floor elevation, in periodontology furcating and intraosseous defects, and in orthopaedic for the regeneration of bone defects caused by trauma, ageing and for the correction of *valgus* knee using open wedge high tibial osteotomies (HTO)²⁹⁻³³.

The previous results demonstrated the high bioactivity of Bonelike[®] *in vivo*. Therefore, the outstanding results of the Bonelike[®] in the rabbit and sheep animal models, led to its application in different areas of the Regenerative Medicine, such as maxillofacial surgery. The second part of this thesis reports the clinical application of Bonelike[®] in its granular form in maxillofacial surgery procedures and in other clinical cases as a coating material for titanium implants.

In oral and maxillofacial surgery³¹, Bonelike[®] was used to regenerate bone defects after cyst removal in 11 patients, aged between 24 to 53 years, consisting of 5 men and 6 women. Most cysts of the oral and facial regions under treatment were located within the jaws as an intrabony lesion with a median mandible cyst, referred to as a non-odontogenic cyst in the midline aspect of mandible. Sometimes, in this type of clinical cases, resection of a large segment of the jaw is necessary to insure complete removal of the lesion. Thereby, the pathological “tissue destruction” process and its suitable surgical removal leads to a significant bone loss. After the complete removal of the cysts, the remaining bone cavities were firmly packed with Bonelike[®] granules mixed with blood and crushed bone remnants compound in an attempt to completely fill bony cavities and “to sculpt” the cortical bone contour. According to the standard follow-up protocols, radiological examinations were performed and Bonelike[®]/bone retrieved samples have been histological analysed using non-decalcified sections obtained perpendicular to bone length axis. Radiographic examination and histological results clearly demonstrated an extensive new bone formation apposed on Bonelike[®] granules with a significant degree of maturation. These clinical applications in maxillary bone defects indicated perfect bonding

between new formed bone and Bonelike[®] granules, along with partially surface biodegradation. This quick and effective osteoconductive response from Bonelike[®] reduced the time required to reconstruct the bone defected area of patients³¹.

The study of biomedical implant surface and the effects of surface modifications have become popular in recent years because surface characteristics directly influence the biomaterial–tissue interactions³⁴. Metals such as pure titanium, tantalum, niobium, zirconium, cobalt–chromium alloy, Ti–6Al–4V alloy, and ceramic materials such as aluminium oxide, HA, or β -tricalcium phosphate have been used for oral implants^{35,36}. The mostly used biomaterial in oral implantology is commercially pure titanium (cpTi) because of its strength, comparatively low stiffness, light weight and bioinertness^{35,36}. When metals are used as an implant material, their biocompatibility and osteointegration is lower when compared to coated metal implants with bioceramic materials³⁴⁻³⁶. Therefore, in order to improve the osteointegration of titanium implants, there are different coatings that have been applied by a variety of methods³⁷. Among them, plasma spraying appears to be the most favourable one in terms of chemical control, bio-corrosion resistance, process efficiency and the degree to which the substrate fatigue resistance is reduced³⁷. *In vitro* and *in vivo* biocompatibility testing of titanium alloy with and without plasma-sprayed hydroxyapatite coating have been studied^{38,39}. Earlier, HA and double-layer HA-P₂O₅/CaO glass (i.e. Bonelike[®]) coatings showed to have a positive effect on human bone marrow cells, increasing osteoblasts differentiation^{40,41}. The glass reinforced HA composites (Bonelike[®] coating) present better characteristics for bone cell growth and function when compared with HA ones. In another *in vitro* study⁴², the bioactive testing using simulated body fluid (SBF) shown that during the immersion of Bonelike[®] coated implants, dissolution of the coating surface occurred and an apatite layer formed on its surface faster than on pure HA coatings. Hence, these results are a strong indication that Bonelike[®] coatings are more bioactive than HA coatings⁴³. An implant elicits a biological response in the surrounding tissue, which determines its acceptance and long-term function. Bone-anchored titanium implants *ad modum* Bränemark have been in clinical use for several years⁴⁴. Adverse tissue reactions ranging from mild reactions to those leading to the removal of the implant are few⁴⁴ and were, in a latter follow-up, reported in about 10% of the observations. Various factors, including an operation technique minimizing tissue injury and the use of implants of titanium, probably contribute to the good clinical performance. This clinical performance maybe even more improved, when the implants are coated with Bonelike[®], which proved its capacity to bond new formed bone.

Chapter 3 includes a case report and a clinical study performed with Bonelike[®] coated titanium implants. The aim of these clinical trials was to evaluate the direct bone bonding and osteointegration of the commercial pure (cpTi) implants coated with Bonelike[®] by a plasma-sprayed method for dental oral applications^{45,46}. SEM morphological characterisation of Bonelike[®] coated dental implants showed that the coating presented a microstructure composed of partially melted particles, characteristics of plasma spraying process^{45,46}. For the structure and chemical mechanism of bone physiology, it is essential to provide substances that are endogenous to the body for successful bone regeneration. Hence, to create the natural bone structure, these substances must be present on the interface at the same rate at which bone formation occurs. Therefore, in Bonelike[®] coated dental implants, there is a local enrichment in Ca^{2+} and P^{5+} in the physiological environment, which stimulates new bone formation¹⁰. The histological analysis of the biopsy samples showed new bone ingrowth surrounding the Bonelike[®] coated dental implants with a mature lamellar-like structure and a direct contact between the surfaces of the coating and also the bone matrix was established. No inflammatory cells and fibrous tissues have been found and mature bone was clearly the major bone type observed around the retrieved samples^{45,46}. Due to intimate bonding between new bone and Bonelike[®] it was almost impossible to distinguish any discontinuity at the Ti-implant/bone interface, which indicated a complete osteointegration. The radiological follow-up exams confirmed the osteointegration of the Bonelike[®] coatings. The interfaces between the dental implant/Bonelike[®] coating, and Bonelike[®] coating/new bone were also evaluated using SEM analysis. Microstructural observations of Bonelike[®] coated dental implants demonstrated that they had excellent bone remnants on its surface and an improved primary stability^{45,46}. The results of both studies suggested that the Bonelike[®] played a significant role in the new bone formation process around the dental implants. Implants coated with Bonelike[®] showed a high osteointegration after 3 months of healing period and therefore these dental implants maybe clinical used when primary stability is needed^{45,46}. Excellent primary stability of the coated implants and new bone growth without any bone loss was achieved. The good functionality observed is a consequence of the enhanced osteointegration induced by Bonelike[®] coating. Hence, Bonelike[®] proved to be an excellent coating for bone regeneration and therefore it maybe used in implantology^{45,46}.

In *Chapter 4*, the case report of 3 patients with multiple myeloma, who were treated in CHVNG in the past 4 years, is included. These 3 patients presented exposed bone and osteonecrosis of the mandible, and shared one common clinical feature: all of them were treated with bisphosphonate zoledronate, administered intravenously for long periods. Sequential orthopantomogram (OPGs) and histological evaluation have been performed

from biopsies of non healing dental extraction sites of these patients. After a routine dental extraction, these patients developed avascular osteonecrosis of the mandible and secondary bone infection with *Actinomyces israeli* (actinomycotic osteomyelitis), with no evidence of metastatic disease evaluated by biopsy. In these 3 described clinical cases, surgical debridement without flap elevation, intensive antibiotherapy and the suspension of the zoledronate allowed a partial recovery of the patients⁴⁷.

Multiple myeloma, a second most common haematopoietic cancer, which represents the collection of plasma-cell neoplasms that invariably, becomes fatal when self-renewing myeloma cells begin unrestrained proliferation⁴⁸. The major clinical problems that arise in myeloma patients relate to the enhanced bone loss that commonly occurs in these patients. These complications resulted from an asynchronous bone turnover wherein increased osteoclastic bone resorption is not accompanied by a comparable increase in bone formation⁴⁸⁻⁵¹. This increase in osteoclastic activity is mediated by the release of osteoclast-stimulating factors, which are produced locally in the bone-marrow microenvironment by cells of both tumour and non-tumour origin⁴⁸⁻⁵¹. The enhanced bone loss results from the interplay between the osteoclasts, tumour cells and other non-malignant cells in the bone marrow microenvironment⁵²⁻⁵⁴. Recent improvements in radiologic techniques have enhanced our ability to detect bony involvement more accurately. With the development of minimally invasive surgical procedures such as kyphoplasty that effectively treat vertebral compression fractures, it becomes increasingly useful to find these fractures in myeloma patients⁵⁵. Recent advances in the use of bone-seeking radiopharmaceuticals make these attractive therapeutic candidates to combine with the new anti-myeloma drugs (thalidomide, bortezomib) since these latter agents are also radiosensitizing⁵⁵. The results of two large phase III clinical trials show the benefit of adjunctive use of intravenously administered monthly bisphosphonates (zoledronic acid or pamidronate) in addition to chemotherapy in safely reducing bone complications in myeloma patients^{52,53,56,57}. Bisphosphonate treatment should now be reconsidered for all myeloma patients due to evident bone loss. Although, preclinical studies suggest the potential anti-myeloma effects of especially more potent nitrogen-containing bisphosphonates, clinical trials - probably at higher doses given more slowly - will be necessary to establish their anti-tumour effects clinically^{52,53,56,57}. Bisphosphonates-associated ONJ is characterised by dehiscence of the oral mucous membranes, with exposure of the underlying mandible or maxilla where bone necrosis can be observed. Most cases occur after surgery procedures, like extractions, implants and periodontal procedures^{49,58-64}. The mechanism underlying the reaction is still unknown, but it has been postulated that bisphosphonates inhibit local angiogenesis^{49,58-64}. Dental extractions, other oral surgeries, cancer therapies like chemotherapy or radiotherapy, corticosteroids, and

morbid conditions like anaemia, coagulopathies, infection, and pre-existing oral disease have been identified as precipitants and, are documented as risks factors^{49,58-64}. A number of promising new agents, including RANK-Fc, are in early clinical development for the treatment of myeloma bone disease^{49,58-64}.

The purpose of this clinical report was to point out that patients suffering from multiple myeloma can develop bone osteonecrosis induced by the treatment with bisphosphonates. Research to determine the mechanism of this dental phenomenon is needed to fully validate and substantiate the possible link between bisphosphonates treatment of multiple myeloma or other cancer diseases with avascular osteonecrosis of the jaw. Until then, clinicians involved in the care of patients at risk should consider this possible complication. The use of bone substitutes like Bonelike[®] associated to a resorbable matrix and to molecules like raloxifene hydrochloride can be used to restore the bone tissue of the mandible or maxilla, of patients suffering from ONJ, being this an attractive treatment for these typical clinical cases. As a matter of fact, therapeutic molecules like raloxifene hydrochloride inhibit osteoclast differentiation and bone resorption in the presence of interleukin-6 (IL-6) which is increased in multiple mieloma. In these cases there is an asynchronous bone turnover wherein increased osteoclastic bone resorption is not accompanied by a comparable increase in bone formation⁴⁸⁻⁵¹. This increase in osteoclastic activity is mediated by the release of osteoclast-stimulating factors, which are produced locally in the bone-marrow microenvironment by cells of both tumour and non-tumour origin, as it was referred previously⁴⁸⁻⁵¹.

Final Conclusions

Bonelike[®] represents a new concept for synthetic bone grafts. It has the ability of mimic the inorganic chemical composition and structure of natural bone tissue, thus enhancing osteointegration. With the increasing demand for new alternatives to autograft and allograft, the research on synthetic grafts field has exponentially increased, due to their advantages when compared to autografts and allografts. This particular synthetic bone graft, Bonelike[®], can be used in several areas of Regenerative Medicine including maxillofacial surgery and implantology therefore it should the benefit a vast number of patients.

Bonelike[®] associated with FloSeal[®] or Normal Gel 0.9%, acting as a vehicle, and a therapeutic molecule like raloxifene hydrochloride, showed that new bone was rapidly apposed on the bone graft after 12 weeks of implantation in rabbits. Bonelike[®] granules were completely surrounded by *de novo* mature bone and it was possible to observe a complete osteointegration of the Bonelike[®] granules with bone tissue formed among them with the presence of new osteon. The bone tissue formed was fibroreticular, with vascular structures and cement lines indicating active bone regeneration. Several blood channels without signs of inflammation throughout the osteoid matrix have been observed and no inflammatory cells and fibrous tissue have been found. The presence of blood vessels was due to active angiogenesis process that is an extreme important process for bone regeneration. Additionally, an extensive surface dissolution of Bonelike[®] granules could be observed in the presence of both matrixes. No osteoclasts activity seemed to have taken place, which may be explained by the presence of raloxifene hydrochloride that is known to inhibit osteoclast activity.

The association of a resorbable matrix like FloSeal[®] or Normal Gel 0.9% NaCl[®], with therapeutic molecules, to a precise size of Bonelike[®] granules is one step-forward for the clinical applications of Bonelike[®], since it is easier-to-handle during the surgery procedures, when compared to other granular materials, and can be used as a controlled-released system of therapeutic molecules in the defect site.

The *in vivo* testing with animals like rabbits and sheep demonstrated the high bioactivity of Bonelike[®]. In oral and maxillofacial surgery³¹, Bonelike[®] was used to regenerate bone defects after cyst removal in 11 patients. Most cysts of the oral and facial regions under treatment were located within the jaws as an intrabony lesion with a median mandible cyst, referred to as a non-odontogenic cyst in the midline aspect of mandible. Sometimes, in this type of clinical cases, resection of a large segment of the jaw is necessary to insure complete removal of the lesion. Thereby, the pathological “tissue destruction” process and its suitable surgical removal leads to a significant bone loss.

After the complete removal of the cysts, the remaining bone cavities were firmly packed with Bonelike[®] granules mixed with blood and crushed bone remnants compound in an attempt to completely fill bony cavities and “to sculpt” the cortical bone contour. Radiographic examination and histological results clearly demonstrated an extensive new bone formation apposed on Bonelike[®] granules with a significant degree of maturation. These clinical applications in maxillary bone defects indicated perfect bonding between new formed bone and Bonelike[®] granules, along with partially surface biodegradation. This quick and effective osteoconductive response from Bonelike[®] reduced the time required to the regeneration of the bone defected area of these patients.

Bonelike[®] was also tested in implantology as a coating material for titanium dental implants. Titanium is the most common material used in oral implantology due to its strength, comparatively low stiffness, light weight and bioinertness, although its osteointegration is poor. One way to improve the osteointegration and biocompatibility of the titanium implants is to coat them with a bioactive ceramic. It has been reported in previous studies that a double-layer HA-P₂O₅/CaO glass (Bonelike[®]) coating has a positive effect on bone cells proliferation and function and also it was shown that Bonelike[®] has an enhanced bioactivity in comparison with HA. So, Bonelike[®] proved to be an excellent coating material for dental implants, stimulating the local bone regeneration and therefore it maybe used in implantology.

The clinical report regarding patients suffering from multiple myeloma that developed jaw osteonecrosis induced by prolonged treatments with bisphosphonates, points out to the possible clinical use of bone grafts like Bonelike[®] associated to a resorbable matrix and to molecules like raloxifene hydrochloride. This might permit to restore the necrotic bone tissue of the mandible or maxilla, which developed avascular osteonecrosis of the jaw. As a matter of fact, the raloxifene hydrochloride inhibits osteoclast differentiation and bone resorption in the presence of IL-6 which is increased in multiple myeloma. When associated to a bone graft like Bonelike[®], the molecule can be released in a controlled way, in the defect site. Nevertheless, research to determine the mechanism of this dental phenomenon is needed to fully validate and substantiate the possible link between biphosphonates treatment of multiple myeloma or other cancer diseases with avascular osteonecrosis of the jaws. Until then, clinicians involved in the care of patients at risk should consider this possible complication.

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Execução Gráfica:

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