AN ENZYMATIC DEANTIGENATION PROCESS ALLOWS ACHIEVING PHYSIOLOGICAL REMODELING AND EVEN OSTEOPROMOTING BONE GRAFTING MATERIALS

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ABSTRACT

The different bone grafting biomaterials that are marketed nowadays are synthetic or natural. Among natural ones, biomaterials derived from mammal bone are of great interest, since the structure, the chemical composition and the morphology of the mineral part of bone coming from different mammal species is quite similar if not identical. Yet the deantigenation process used to deprive the heterologous bone tissue from the organic antigenic part can alter the kinetic of osteoclastic remodeling of such a mineral part. This is what happens when the common high temperature process is applied: the final product is nearly not resorbable at all.

In order to overcome these limitations, an enzymatic deantigenation method has been devised. It is based on the application of a mixture of enzymes that operate at 37°C, and does not alter the remodeling properties of the mineral bone component, as it is shown also by histomorphometric studies. Moreover, recent developments and refinements of the enzymatic process itself, have allowed developing a new class of animal demineralized bone matrixes that show promising osteoproductive properties in stimulating bone regeneration.

Keywords: bone grafts, enzymatic deantigenation, osteopromoters

Introduction

The recent developments in prosthetic rehabilitation in Orthopedics and osteointegrated implantology in Dentistry have increased the need and the frequency of guided bone regeneration (GBR) surgeries.

GBR surgeries are based on grafting a biomaterial in the site to be augmented, and on covering the graft with a GBR membrane. The primary aim of the biomaterial is to provide mechanical support to newly formed blood vessels and cells that will colonize the grafted site. This mechanical effect is known as the *scaffold* effect, or *osteoconductive* effect, and is necessary to achieve bone regeneration. The GBR membrane is placed over the grafted site in order to prevent epithelial and connective cells, whose mitotic rate is faster than the bone regeneration process, to invade the grafted site. If this happens, bone regeneration fails since the grafted volume will be filled by connective fibrous tissue.

As far as grafting materials are concerned, the *golden standard* – according to current literature – is the autologous bone. The reasons are that a) autologous bone is, obviously, totally biocompatible; b) it gives an excellent osteoconductive effect but also c) it already contains differentiated cells which produce bone tissue (this is called the *osteogenetic* effect) and d) it contains several different growth factors which can stimulate bone regeneration (this is called the *osteoinductive* effect).

Nonetheless, the use of autologous bone is not without risks. A second surgery site is needed for its collection, and this leads to increase both intra-surgical and post-surgical side effects. Even in Dentistry, if the collection site is intra-oral the quantity of bone is small, and big reconstructions cannot be performed. If the collection site is extra-oral (for example, iliac crest), a bigger quantity of autologous bone will be available, but post-surgical effects (pain, etc.) will last for a long time. Moreover, a proper surgery room and personnel are needed, and costs increase.

In order to avoid the collection of autologous bone and the consequent risks, many different osteoconductive biomaterials, either natural or synthetic, have been proposed as an alternative for clinical use. Nonetheless, a detailed analysis of their properties show that their resorption rates (sometimes due to osteoclastic activity, sometimes due to enzymatic digestion or macrophages action) are far from being physiological (7, 13, 14).

Yet the resorption rate of the biomaterial plays a key role in bone regeneration. If, for example, a biomaterial is reabsorbed at a faster rate than the one of new bone formation, part of the grafted site will not be filled by newly formed bone. In this case the success of the regeneration will be only partial. If, on the contrary, the resorption rate is very slow (years) or null, the grafted site will be permanently or nearly permanently filled with a mixture of biomaterial and endogenous bone. As far as Dentistry is concerned, from a theoretical point of view this fact could have negative consequences on the mechanical properties of the regenerated bone and its capacity to support implants (5). As far as Orthopedics, it is obvious that this

clinical result could be accepted only for small, not weight-bearing reconstructions.

The need for a "correct" resorption rate has brought the attention on biomaterials derived from mammal bone. The chemical composition and the morphology of the mineral part of the mammal bone are quite similar among all mammals, comprising Man. This similarity is so close that the mineral bone component of one species should be recognized as endogenous, and remodeled, also by osteoclasts belonging to an individual of another species (12).

Anyway, before being grafted, bone collected from a non-human mammal species must be deantigenated in order to avoid the immune reaction of the host.

A very easy way to eliminate the organic antigenic component of mammal bone is the use of a high-temperature (> 500°C) treatment. Such temperatures are so high that all the organic components of bone (proteins, lipids, sugars), which are responsible for the immune response, are totally eliminated. This method is being applied for many years to achieve one of the most common biomaterial (Bio-Oss, Geistlich, Switzerland – from bovine bone) used in Dentistry. This kind of treatment has the obvious advantage that it is very easy to apply. But, unfortunately, it has been shown that it leads to a dramatic decrease of the osteoclastic remodeling rate of the final product, probably because of a physical modification of the structure of bone apatite caused by heat (11).

In order to overcome this limits of the thermal method an alternative deantigenation method, based on the use of digestive enzymes has been developed and will be shortly described in this paper. This method allows achieving physiologically remodeling bone grafting materials.

Moreover, it will be described also a refinement of the same deantigenation method which allows to achieve mildly stimulating ("osteopromoting") products. The rationale of this application is based on two observations. The first is that type I bone collage, on which mineral salts are deposed, contains some growth factors. This property was discovered by observing that the *demineralized bone matrix* (DBM) is able to stimulate the production of new bone tissue (9), even when grafted in ectopic tissues. This effect is due to growth factors which are present inside the bone collagen matrix, such as IGF II, TGF-beta, IGF I, PDGF, bFGF, BMPs and others (9). The second fact is that these proteins are highly conserved among the different mammal species (6). The amino acidic sequence homology (the percentage of identical amino acids) is so high – when not complete– that *the DBM of one species is stimulating even when grafted in an individual of another species* (2, 4, 10).

So, the second aim of this paper is to describe how the enzymatic deantigenation method had allowed achieving two forms of an equine DBM that are giving promising clinical results.

Materials and Methods

Deantigenation method

At the present time the enzymatic deantigenation method is being applied on equine bones, even if every mammal bone could be deantigenated according to this process. The choice of equine bone is due only to commercial and not to any biological reasons.

Equine femurs are collected in Italy from official slaughters (in Italy, such as in many other countries, equine meat is used for human feeding), where horses are strictly controlled by the State Veterinary Officers. This allows collecting bone coming from healthy animals, homogeneous as far as feeding and weight are concerned.

Bone femurs are then mechanically cleaned from the gross residues and cut in cortical and spongy sections. Such sections are then treated with a proprietary process that, by using a steam jet, eliminates a big fraction of the lipid component of the bone itself.

Partially cleaned bone sections are then treated with the enzymatic deantigenation process. Bone pieces are immersed in water-based enzymatic solutions at 37°C. Such enzymatic solutions are fine calibrated by choosing enzymes whose substrates are not only families of compounds but also specific target molecules. The details of the composition of the solutions cannot be given here, for obvious industrial secret reasons.

The deantigenation process for each single bone section lasts about 7 days. During this time enzymatic solutions are periodically renewed and their composition is adjusted according to the step of the process. Each deantigenation step is therefore composed of three phases: enzyme activity – inactivation of enzymes – washing. Intermediate and final washings are performed with highly purified osmotic water.

The only organic molecule that is not eliminated from the sections is type I bone collagen, which forms the internal mesh of the mineral bone component (Osteoplant, Bioteck, Italy).

Deantigenated bone sections are then processed according to the type of the final product that has to be prepared. They can be grinded to produce granules, or they can be shaped to achieve particular pieces. Solid sections are suitable for orthopedic applications since, given the presence of the collagen network inside, they can still bear the mechanical load due to the patient's weight.

A further process has also been devised to produce flexible bone layers (Osteoplant Flex, Bioteck, Italy), through an acidic electrolytic treatment. This process is not described in the present paper.

For dental applications, bone sections are deprived also of bone collagen through a high-pressure wet treatment (120°C, 7 atm) that permits to achieve totally non-collagenic products (Biogen, Bioteck, Italy).

Selective deantigenation

The enzymatic deantigenation method can be applied also *selectively*. By adapting properly the enzymatic mixture, in

fact, it is possible to choose the molecules that will be digested preserving, at the same time, some other ones. This process has been refined in order to produce two equine DBM-based osteopromoting compounds: after bone blocks have been partially cleaned with steam jets, they undergo *selective* deantigenation and then they are *totally demineralized* by acidic treatment. The final product is type I bone collagen still containing the desired factors inside. Because of deantigenation and further beta-ray sterilization the factors are broken in peptides which, nonetheless, still have the biological function of the whole factor.

Two osteopromoting products, that have to be applied together with a standard osteoconductive graft have been created: an activator of angiogenesis (Osteoplant Angiostad, Bioteck, Italy) and an activator of morphogenesis (Osteoplant Activagen, Bioteck, Italy).

Osteoplant Angiostad is a water-based gel containing the DBM granules. It has to be spread on the bone of the patients, where the graft will be placed, or mixed in a 1:10 proportion to granular graft. Osteoplant Activagen is made of collagen granules that have to be mixed in 1:1 proportion to grafting osteoconductive granules.

Deantigenation effectiveness

The effectiveness of deantigenation has been tested directly. The first direct test is based on the measurement of total lipid content according to the procedure described in the ISO 1443 standard (the method consists in boiling a test portion with hydrochloric acid to free the occluded and bound lipid fractions, filtrating the resulting mass, drying, and extracting with n-hexane or light petroleum, the fat retained on the filter, which is then weighted).

Direct visual examination was performed with Scanning Electron Microscope (SEM) analysis on similar deantigenated bone blocks to assess porosity size in equine bone. Finally, deantigenated blocks were cut in thin sections that were analyzed after hematoxilin-eosin staining (the sample is decalcified with a decalcifying solution containing EDTA and included in paraffin. The sample is then cut in 6-7 micron slices that are stained with hematoxylin-eosin and observed through an optical microscope).

Osteoclastic remodeling properties

Osteoclastic remodeling properties were assessed through histological tests following dental surgeries. When bone height or thickness is not sufficient to place dental implants, grafting surgery is performed and implants are placed during a second surgery some months later. At this time a bone core can be collected and hematoxylin-eosin stained as described in the previous point. Such a test can show how much the bone substitute has been remodeled and the amount of the residual graft. All results presented in this review paper refer to maxillary sinus lift surgeries performed with enzyme treated, non collagenic, cortical-spongy bone granules (Biogen Mix – Bioteck – Italy).

Osteopromoting effect

Osteopromoting effect was assessed both through *in vitro* and clinical studies. *In vitro* studies were performed on cultured human endothelial cells (for the angiogenesis activator) and on human osteoblasts (for the morphogenesis activator). Human endothelial cells were used to assess a) if the angiogenesis activator was capable to induce cell proliferation and b) if the angiogenesis activator was capable of inducing cell migration. Osteoblasts were used to assess if the morphogenesis activator affected their mitotic rate and the activity of their alkaline phosphatase (the marker of the osteogenetic activity).

Osteopromoting effect, as far as clinical studies are concerned, was assessed through standard histological tests, as described previously.

Results and Discussion

Deantigenation effectiveness

a) Total lipidic content

The total lipidic content, measured according the current ISO 1443 standards is null (<0.01 g over 100 g of the sample – non detectable according to the standard itself).

b) SEM analysis

The porosity of the samples analyzed is greater than 100 μm, which is the lower limit of pore diameters to obtain a correct vascular proliferation. Pore size is in the range 435 ± 58 to 750 ± 80 μm (1).

c) Histological analysis of deantigenated samples

Samples are totally deantigenated. The deantigenation process was able to remove also osteocytes (osteocytes lacunae are empty). (**Fig. 1a** and **Fig. 1b**).

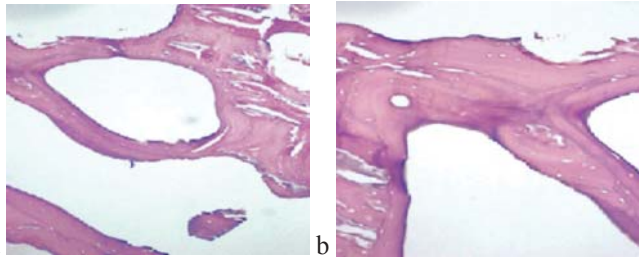

Fig. 1. a) Equine bone treated with the enzymatic total deantigenation process. Hematoxylin-eosin staining. No cellular residue can be observed. b) A detail at greater magnification

Osteoclastic remodeling properties

Histological tests of patients who underwent maxillary sinus lift surgeries showed that the enzyme-processed biomaterial had undergone total osteoclastic remodeling after 12 months (**Fig. 2**). The same results were achieved at 6 months if the bone graft was previously mixed with some autologous bone (1:1) mixture (**Fig. 3**). Such results, concerning enzyme-treated non-collagenic granules, confirm the histological tests regarding enzyme-treated bone blocks (3).

Osteopromoting effect

The angiogenesis activator was capable to induce both proliferation and migration of human endothelial cells when compared to control; the morphogenesis activator induced a significant increase both in the mitotic rate of osteoblasts and in the alkaline phosphatase activity. Such results are in press at the present time. Adding osteopromoting compounds to bone granules, in maxillary sinus lift surgeries, allowed to achieve, at 6 months from grafting, results that are similar to the one observed when the mixture of the graft and autologous bone was used (**Fig. 4**).

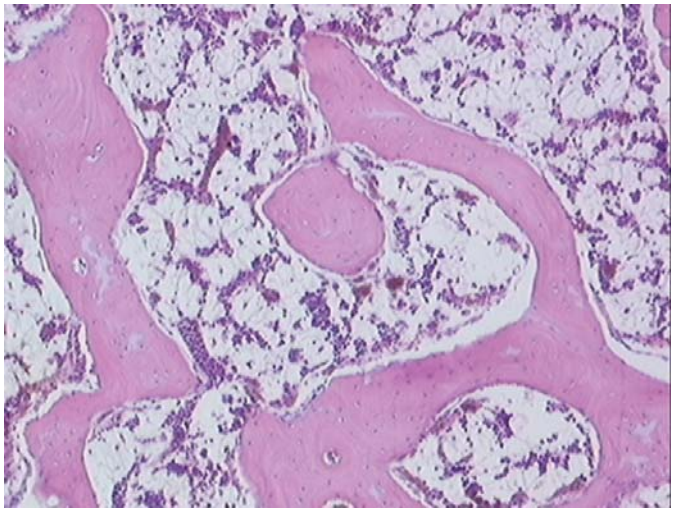

Fig. 2. Bone sample collected at 12 months after grafting with enzyme treated, non-collagenic equine derived bone. Hematoxylin-eosin staining. The material has been totally remodeled

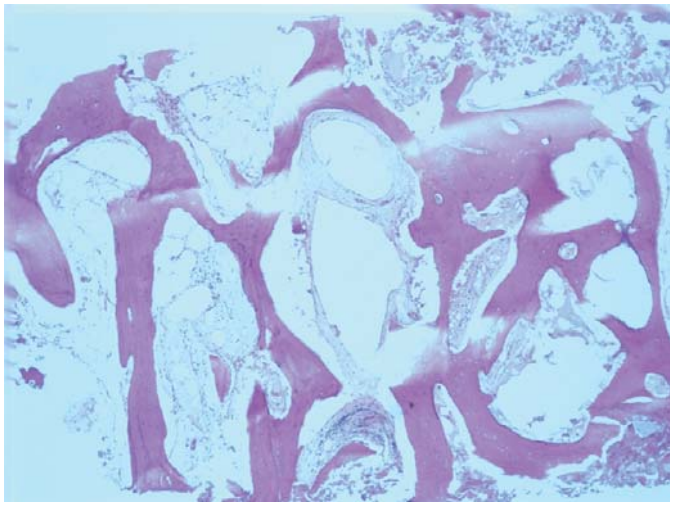

Fig. 3. Bone sample collected at 6 months after grafting with a 1:1 mixture of enzyme treated, non-collagenic equine derived bone and autologous bone. Hematoxylin-eosin staining. Remodeling is complete

All these results show that it is possible to apply an enzyme-based deantigenation method to make mammal bone totally biocompatible for GBR surgeries, when bone grafts must be performed. Such a method allows achieving complete deantigenation, making the mineral part of mammal bone coming from different species totally biocompatible, and suitable to be grafted in humans.

Beyond total deantigenation, a further advantage in applying this method is that osteoclastic remodeling rates of the mineral bone component are not altered. Therefore, once such enzyme-treated mammal bone is grafted, it responds physiologically to the biological surrounding environment, and is remodeled according to the remodeling rate of the site where graft is performed. This is particularly interesting from a clinical point of view because such materials allow achieving, as a result of the regeneration process, only endogenous bone. These results have already been published in literature, as case reports (3). The *restitutio ad integrum* is therefore complete, and regenerated bone can respond physiologically to all the following stimulation (mechanical load of implants in Dentistry, mechanical load of weight of the body in large grafting surgery in Orthopaedics).

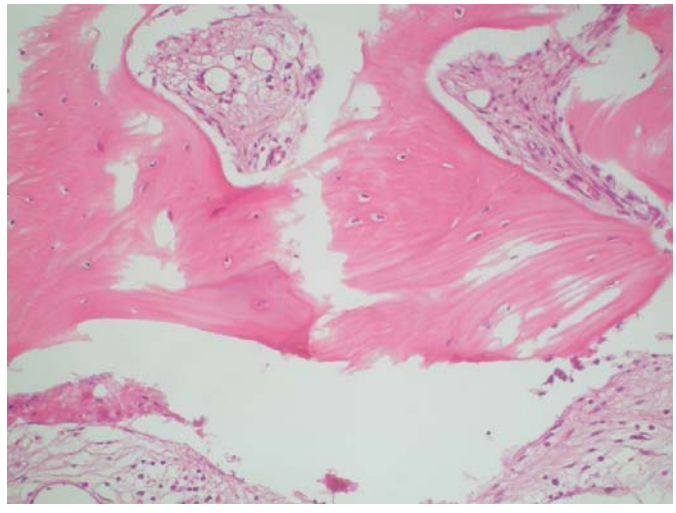

Fig. 4. Bone sample collected at 6 months after grafting with a mixture of enzyme treated, non-collagenic equine derived bone, an angiogenesis activator and a morphogenesis activator. Hematoxylin-eosin staining. Remodeling is complete

Finally, these results show that the enzymatic deantigenation method can be adapted to achieve stimulating preparations, based on the application of animal DBM. Such results are now undergoing thorough study, even if some first results have already been published (8). If such studies should confirm the promising data observed these osteopromoting preparations would be extremely useful in the clinical practice, since they would minimize the collection of autologous bone, with all the consequent obvious advantages. Moreover the advantage in applying such products would be double: from one side they would accelerate the regeneration time of grafted site but, more important, they would increase the rate of success when difficult anatomical situation are encountered.

Conclusions

In this paper we presented a novel process, based on the application of enzymes that has been developed in order to make the mineral part of mammal bone biocompatible and

suitable to be grafted in humans. The method was shown to allow complete deantigenation and, what's more important, to preserve the physiological kinetic of osteoclastic remodeling. The clinical importance of this feature has been discussed. Moreover, a refinement of the same method allowed achieving mild osteoinductive preparations, defined as "osteopromoting", which can be a promising advancement in bone regeneration surgeries.

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