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# Microtomographic and morphometric characterization of a bioceramic bone substitute in dental implantology

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**Summary.** In recent years, bone tissue regeneration studies have led to a deeper knowledge of chemical and structural features of the best biomaterials to be used as replacements for lost bone structures, with the autologus bone still today the only graft material able to ostegenerate, osteinduct and/or osteoconduct. The difficulties of the small available amount of autologus bone, together with morbidity of a second surgical operation on the same patient, have been overcome using both synthetic and biologic substitute bones. The possibility of investigating morphometric characteristics of substitute bones makes it possible to evaluate the predictability of regenerative processes and, so far, a range of different methods have been used for the purpose. X-ray microtomography (micro-CT) is a miniaturized form of conventional tomography, able to analyze the internal structure of small objects, performing three-dimensional images with high spatial resolution (<10 micron pixel size). For a correct analysis, samples need not be altered or treated in any way, as micro-CT is a non-invasive and non-destructive technique. It shows promising results in biomaterial studies and tissue engineering. This work shows the potential applications of this microtomographic technique by means of an *in vitro* analysis system, in characterizing morphometric features of human bone tissue, and contributes to the use of this technique in studies concerning biomaterials and bioscaffolds inserted in bone tissue.

*Key words:* X-ray microtomography, biocompatible materials, tissue scaffolds, tissue engineering.

**Riassunto** *(Caratterizzazione microtomografica e morfometrica di un sostituto osseo di origine bioceramica in implantologia dentale)*. La rigenerazione del tessuto osseo è stata oggetto, negli ultimi anni, di numerosi studi che hanno condotto alla conoscenza delle caratteristiche chimiche e strutturali di un biomateriale ideale. Poiché, ad oggi, l'unico materiale da innesto in grado di svolgere, al contempo, azione di osteogenesi, osteoinduzione ed osteoconduzione rimane ancora l'osso autologo, emerge la necessità di superare le problematiche legate alle ridotte quantità disponibili ed alla morbilità associata ad un secondo rientro chirurgico nello stesso individuo, utilizzando dei sostituti ossei sia sintetici che biologici. La possibilità di conoscerne a fondo le caratteristiche morfometriche consente di valutare la predicibilità del processo rigenerativo e diverse sono le metodiche finora sfruttate a tal fine. La microtomografia a raggi X (micro-TC) è una forma miniaturizzata di tomografia convenzionale, in grado di indagare la struttura interna di piccoli oggetti opachi, fornendone immagini tridimensionali ad elevata risoluzione spaziale (<10 micron pixel size). Poiché, ai fini analitici, i campioni esaminati non devono essere alterati o trattati in alcun modo, la tecnica microtomografica risulta essere assolutamente non distruttiva e non invasiva e viene attualmente applicata con risultati proficui sia nello studio dei materiali che in ambito biomedicale. Lo scopo di questo lavoro è quello di confermare le possibilità applicative di tale metodica, attraverso la strumentazione Skyscan 1072, nella caratterizzazione morfometrica del tessuto osseo umano normale e di fornire un ulteriore contributo nella messa a punto della stessa nell'ambito dello studio delle proprietà dell'osso innestato con determinati biomateriali.

*Parole chiave:* microtomografia a raggi X, biomateriali, scaffolds, ingegneria tissutale.

## INTRODUCTION

Studies on biological and synthetic biomaterials have been carried on to restore form and function of lost bone structures. Bone healing is a quite complex and dynamic process that ends with the restoration of anatomical and functional standard conditions

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of the bone and goes backwards through modeling and re-modeling to embryo bone generation.

Different healing processes after a fracture or a dental extraction show two different restoration mechanisms. After a fracture, blood clot forms which, invaded by macrophages, fibroblasts and capillaries, is replaced by granulation tissue. This tissue generates the fibrocartilaginous callus, made of compact fibrous tissue and cartilage, that temporarily links the skeletal fractures. At the same time, because of the activation and differentiation of osteocompetent periosteum cells, osteoblasts generate a fibrocartilaginous callus and cover it with membranous bone outside and around. Then, the bone callus is gradually replaced by compact bone tissue. New-formed bone is gradually replaced by compact bone with a smaller volume than the first callus [1, 2].

After dental element extraction, coagulation appears with blood cells, serum and saliva. A fibrin network is formed in the next 24 hours due to thrombus formation and vase closing, so granulocytes, neutrofiles, monocytes and fibroblasts can attach on it. Within two or three days, starting from the central area, the hemolysis process begins, allowing blood coagulation replacement by granulation tissue. At day 4, fibroblast density in the coagulum increases and epithelium cell generation takes place in the wound. A week after the avulsive event trauma, granulation tissue made of blood vases, fibroblasts and new collagen fibres appears in the alveolus; after three weeks a formation of compact connective tissue and elementary trabecular architecture can be observed. Bone regeneration ends in 2-4 months [3].

Graft biomaterials now available are able to osteoconduct, working as scaffolds, matrix or three-dimensional substrates to support new bone tissue formation [1-6].

By means of X-ray microtomography it is possible to study morphometric features of substitute bones with a non destructive, non invasive analysis, as accurate as other traditional common optic and electronic microscopic techniques [6-18].

This work shows the potential of microtomographic technique applications by means of an *in vitro* analysis system, SkyScan 1072, to study the morphometric characterization of bone tissue.

A qualitative and quantitative analysis has been carried out on human bone tissue of the same patient, harvested from the healing of a bone defect obtained after extraction of total impacted lower third molars; extraction sites have been cured only with blood clot and ceramic biomaterial graft, respectively.

After four months, bone samples from both sites have been extracted to be analyzed by means of microtomography and histology.

## MATERIALS AND METHODS

The following experimental research, performed at the Dipartimento di Tecnologie e Salute (the Health and Technologies Department) of the Istituto Superiore di Sanità (the Italian National Institute of Health, ISS) with the cooperation of the Dipartimento di Scienze Odontostomatologiche e Maxillo Facciali (Department of Odontostomatological and Maxillofacial Science) of Sapienza Università di Roma (Sapienza University of Rome), studied a synthetic substitute bone made of mimetic bioceramic (ENGIpore-Sweden Martina-Calcium, porous Phosphate). Analysing the material by means of micro-CT, the main morphometric features have been observed, especially porosity, in a block sample of 10 x 5 x 5 mm; in a second experimental phase, preliminary evaluations have been carried out on small chips of 0.5-1 mm grafted in a human model bone defect.

We selected a 28-year-old male in good health conditions who needed surgical bilateral extraction of total impacted lower third molars. After patient's agreement, clinical and radiological tests were made in order to confirm the total inclusion of dental elements, which is an important factor for the complete closure of flaps over the graft material.

After the extraction of the two third molars, one post-extractive site was allowed to heal by the coagulation process, and the opposite one was treated with the insertion of bioceramic material. Sixteen weeks after surgery, osseous samples in both test and control site were harvested, using a trephine surgical-steel bur (internal diameter 3 mm, external diameter 4 mm).

Harvested samples were preserved in formalin, then washed in physiological solution before SkyScan 1072 acquisitions. After micro-CT analysis, samples were placed back in formalin then sent to histological testing.

## RESULTS

Once the samples have been placed on the specimen holder of SkyScan 1072, acquisition param-

**Table 1** | *X-ray microtomography acquisition parameters*

| | Block sample | Test bone specimen | Control bone specimen |
|---|---|---|---|
| Magnification | 40X - 7.3 μm | 95X - 3.1 μm | 95X - 3.1 μm |
| Rotation angle | 180° | 180° | 180° |
| Rotation step | 0.45° | 0.45° | 0.45° |
| Source | 100 KV/98 μA | 100 KV/98 μA | 100 KV/98 μA |
| Filter | 1 mm Al | 1 mm Al | 1 mm Al |

**Table 2** | *Morphometric parameters*

| | Block sample | Test bone specimen | Control bone specimen |
|---|---|---|---|
| Bone volume fraction (Bv/Tv) (%) | 23.07 | 23.35 | 39.25 |
| Specific bone surface (Bs/Bv) (1/mm) | 29.02 | 84.13 | 81.06 |
| Trabecular thickness (Tb.Th) (µm) | 128.95 | 73.45 | 61.55 |
| Trabecular separation (Tb.Sp) (µm) | 528.76 | 171.31 | 78.32 |
| Porosity (Po) (%) | 77.04 | 76.65 | 60.75 |

eters are set for each one, as shown in *Table 1*. After bidimensional image acquisition and cross action reconstruction, a region of interest (ROI) has been chosen within the last one, by means of a thresholding process, which makes it possible to distinguish bone phase (indicated by black pixels) from non-bone phase (indicated by white pixels) [19-21].

After the thresholding process, three-dimensional reconstructions are obtained using a 3D creator software, then the main morphometric parameters are calculated [20, 21]. *Table 2* shows morphometric parameters found for the ENGIpore block, osseous sample grafted with ENGIpore, and control samples. Each parameter has a specific value that describes the structural and mechanical properties of each sample.

*Figure 1* and *Figure 2* show the morphometric characterization of bone tissue samples harvested after 4 months from the grafted site and the control site, using different colours to identify the X-ray absorption coefficient, and discriminating, over the total volume, biomaterial, new-formed bone tissue and most mineralized bone areas; different shades of gray indicates the different levels of X-ray absorption coefficient of examined sample structures. Three ranges of value have been chosen *ad hoc*, identified by different colours, to discriminate in each sample structure the different radiopacity.

Yellow indicates the less radiopaque component and less mineralized component, the new-formed bone; orange indicates intermediate radiopaque areas in a first calcification status; red indicates the most calcified component, identifying the biomaterial, as shown in test site, and the most mineralized cores in the newly formed bone.

Different SkyScan reconstruction software X-ray absorption coefficient images have been obtained for each sample; by superimposing them, a single 3D image showing the three different colourings has been obtained.

Once the X-ray microtomographic evaluation was complete, the samples were tested by means of a histological exam: after decalcification and preservation in paraffin, they were cut in 3 micron sections and coloured with Hematoxylin and Eosin (H&E) staining.

## DISCUSSION

Investigated parameters are all important for graft to be successful. Hydroxyapatite is one of the most used biomaterials for regenerative purposes because of its chemical and physical characteristics and biological relations that make it similar to and compatible with human bone tissue [1, 22, 23]; in fact, when graft material is inserted in bone tissue it is possible to determine biological and immunocytological reactions influenced by the following parameters:
- chemical composition;
- presence of impurities or toxic components;
- density;
- porosity;
- chip dimension and surface geometry.

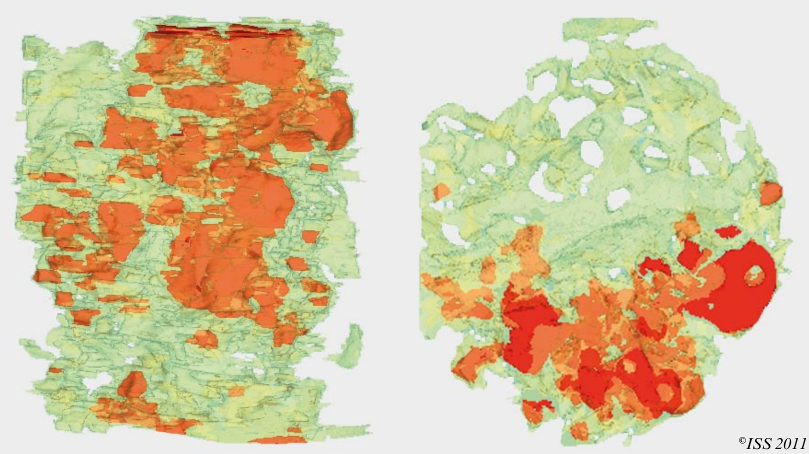



**Fig. 1** | *3D reconstruction colour images of bone sample of test site, after four months of regeneration with ENGIpore (all sample on the left and a slice of the same sample on the right).*

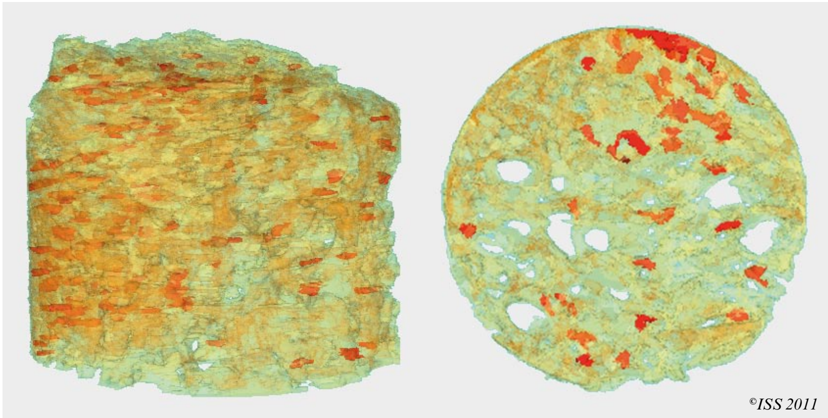


**Fig. 2** | *3D reconstruction colour images of bone sample of control site, after four months of healing with only blood clot (all sample on the left and a slice of the same sample on the right).*

Osteoconductivity and reabsorbility are related to bone structural features; especially particle surface geometry determines different tissue response while porosity and interconnection between pores determine the scaffold performance. A structure with a huge area dedicated to cell adhesion, with high porosity and large interconnection, allows graft to be invaded by new-formed vases and progenitor cells with progressive substitution of bone tissue (well known as "creeping substitution") [1-6, 24-27].

These results have been studied and evaluated by means of micro-CT that identified morphometric parameters and then quantitative properties of each sample *(Table 2)*. Analyzing these values, it is possible to observe that bioceramic material block shows a high porosity with long distances between each trabeculas and a sufficient thickness of individual trabeculas; the tissue sample grafted with ENGIpore shows more porosity than the control sample, due to the presence of more spaces occupied by low-mineralized tissues (fibrous, connective, vascular structures and empty spaces); this structure is confirmed by a low volume percentage and a high trabecula separation.

Observing the three dimensional reconstructions, which located different structures with different X-ray absorption, it is possible to study the qualitative aspects of the two bone specimens. On the one hand, after 4 months, ENGIpore grafted bone tissue sample shows, from site regeneration, high biomaterial persistence compared to the total volume, but also a relevant amount of new-formed bone tissue with a few areas of high mineralization. On the other hand, after the same time, the specimen extracted from coagulation healing site shows a more important bone tissue formation with many mineralized areas. From these evaluations it is possible to suppose a biomaterial slowdown action in bone neoformation process.

In the end, histological examination performed on these samples supported this hypothesis, in that the grafted bone sample showed a high presence of connective fibrous tissue, residual biomaterial particles and thin ossification tissue with histiocytic lacunae. At higher magnification, multinucleated giant cells are visible, due to granulomatous reaction and reabsorption of ENGIpore particles *(Figure 3)*. In the control sample, mature structured bone tissue has been observed with histiocytic lacunae and atrophic marrow spaces containing adipose tissue *(Figure 4)*.

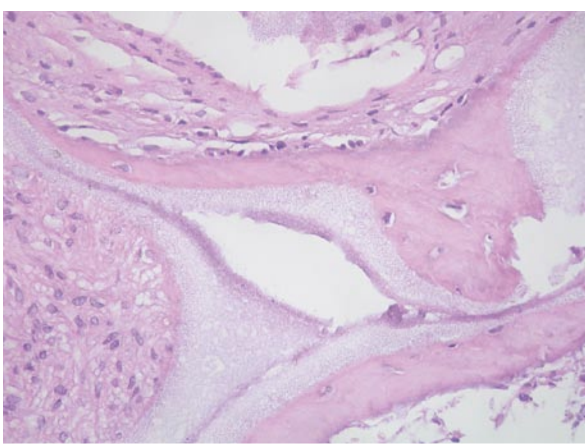

**Fig. 3** | *Histological image of test bone sample (100X, H&E).*

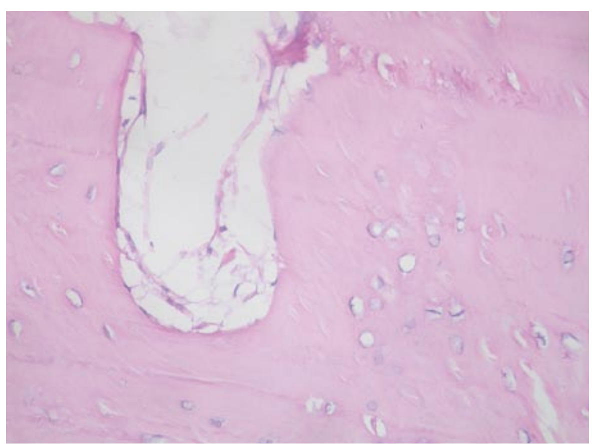

**Fig. 4** | *Histological image of control bone sample (100X, H&E).*

## CONCLUSIONS

The results of this work show the innovation represented by X-ray micro-CT and validate its reliability. The possibility of investigating proprieties and features of a small object without any alteration or corruption demonstrates that micro-CT is a non-invasive and conservative technique in contrast to other traditional but invasive methods like optic and electronic microscopy [22, 23, 28]. These advantages allow microtomography to be used in various fields and suggest that in the near future micro-CT will be used not only for the implementation of biomaterials with defined structural features but also in studies on human bone regeneration characteristics and tissue engineering.

Promising projects about the use and usefulness of this technique in studying 3D biocompatible matrix are increasingly being considered within the scientific community.

### *Conflict of interest statement*

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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