Biomechanical evaluation of cell-loaded and cell-free hydroxyapatite implants for the reconstruction of segmental bone defects

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Porous hydroxyapatite (HA) scaffoldings are currently used in tissue engineering for bone reconstruction. When this osteoconductive biomaterial is combined with osteoprogenitor cells, it acquires osteoinductive features which accelerate and improve bone formation *in vivo.* The aim of our study was to assess the mechanical properties of HA–bone complexes undergoing indentation tests, and relate stiffness to composition and structure as examined by micro X-ray. To this purpose, 35-mm tibia diaphyseal resections were performed in sheep. Gaps were filled using porous HA cylinders. Implants were loaded with autologous bone marrow stromal cells (BMSC); cell-free cylinders were used as control. After 8 weeks, bone tissue was found within the internal macropores of cell-loaded HA carriers, and in control implants, bone formation was mostly limited to the outer surface. As assessed by indentation testing the stiffness values of bone–HA composites were halfway between those of HA scaffoldings and tibia bone. Cell-loaded implants were stiffer than cell-free ones. In a cell-loaded implant we also analyzed the variation of stiffness along the main axis of the tibia.

1. Introduction

Bone defect repair still presents a problem of difficult resolution in orthopaedic and reconstructive surgery. Several biomaterial-based approaches have been proposed [1, 2]. Among them, hydroxyapatite (HA)-based bioceramics have been extensively used as bone substitutes in clinical applications. Bone formation can be achieved *in vivo* combining osteogenic cells and bioceramics as described by several authors [3–7]. This integrated biotechnological approach is potentially a significant advancement in the skeletal reconstruction field. We have recently shown that bone marrow stromal cells (BMSC)-loaded HA implants provide satisfactory bone formation as early as 2 months after surgery [8].

It has been shown that bone tissue ingrowth is promoted not only by the chemical and physical features of bioceramics but also by their macroporosity [9, 10]. However, porosity and pore size can reduce the mechanical properties of HA ceramic [11]. When implanted, a HA scaffolding is slowly reabsorbed and replaced by the newly formed bone that fills the pores. As a consequence implants change their structure and composition over time as well as their mechanical properties [12]. Standard tests (such as compressive, bending or torsion tests) when performed on the whole bone–HA complexes [13–16], could not be exhaustive as they yield global features of the implant. A good alternative is indentation testing, a non-destructive technique that evaluates the stiffness of small areas. Indentation was originally designed to test metals but it has been successfully applied to bone tissue (compact and cancellous) in bovine and canine models as reported by many authors [17–19]. The objectives of our study were: (i) to assess the stiffness on transverse sections of the two kinds of implant (cell-loaded versus cell-free); (ii) to compare the stiffness values of BMSC–HA composites, unimplanted HA scaffolding and ovine tibia bone; (iii) to analyze the variation of stiffness along the main axis of a cell-loaded implant.

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2. Materials and methods

Marrow aspirates were harvested in heparin (about 200 units ml^{-1} final) from adult ewes iliac crest under total anaesthesia. BMSC were isolated and *in vitro* expanded, as previously described [20]. The osteogenic potential of *in vitro* expanded BMSC was evaluated by subcutaneous implantation in immunodeficient (ID) mice as described elsewhere [20].

In this study 35 mm diaphyseal segments of four sheep left tibia were surgically removed and replaced with four *ad hoc* shaped bioceramic implants, which were secured by external fixators [1]. The bioceramic carriers were highly porous cylinders, 20 mm in diameter and 35 mm long, with a central bore of 10 mm in diameter. They were 100% hydroxyapatite, 70–80% porosity. Pore size distribution was: $<10\,\mu$m ~ 3 vol %; 10–150 μm ~ 11 vol %; $>150\,\mu$m ~ 86 vol %. The scaffoldings were kindly provided by Fin-Ceramica Faenza (Faenza, Italy).

Two HA carriers were loaded with autologous *ex vivo* expanded BMSCs; the other two implants were cell-free and used as control. The sheep could walk unrestrictedly after surgery. After 8 weeks the implants were retrieved and processed for microradiographic analysis. Undecalcified specimens were dehydrated in ethanol and embedded in an epoxy resin (Araldite^{TM}), or air-dried and used unembedded. We obtained 100 μm thick sections either by grinding or by means of a microtome equipped with a rotating diamond knife (Leica, Germany). Contact microradiographs were prepared using an X-ray generator (XRG 3000, Ital Structures, Riva del Garda, Italy) and Kodak high resolution film.

As proposed by Timoshenko and Goodier [21] Young's modulus (E) can be calculated from stiffness measurements, by the equation $E = s(1 - \nu^2)/d$ (where s is the slope of an indentation test load–displacement curve in its linear region, ν is Poisson's ratio, and d the diameter of the indentor). The indentor should be small in diameter, to have a detailed map of stiffness in transverse sections, and should satisfy the above equation. We performed preliminary indentation tests on slabs of bovine tibia diaphyses to choose the optimal indentor diameter and testing protocol (strain rate, indentation depth, preload). Flat-ended indentors with various diameters (ranging from 1 mm to 4 mm) were used. Compression tests were performed on $3 \times 3 \times 3$ mm cubes of tibia bone to evaluate E. The 2.5-mm flat-ended indentor yielded a strong correlation between bovine compact bone E as calculated by Timoshenko's equation and E estimated from non-destructive compression tests.

For our study we cut out a 3 mm high slab from each HA-bone aggregate orthogonally to the long axis of the tibia. Moreover six consecutive slices (3 mm high and 1 mm spaced) were obtained from a cell-loaded implant; their apparent density was measured as the ratio between weight and volume.

On each slice we tested about 15 sites 3 mm apart. We used a Lloyd testing machine (LR5K) equipped with a 500 N load cell. Indentation depth never exceeded 0.35 mm to preserve the specimen integrity and minimize the effect on adjacent sites. Each site was preloaded at 2 N for 30 s. Strain rate was 0.1 mm min^{-1}. Following the same protocol we tested non-implanted HA cylinders and bone slabs from the same sheep tibia.

The slope of the force–depth curve corresponding to each site was determined and assumed as the local stiffness value. On each slab side the mean stiffness and standard deviation were calculated.

3. Results

Sheep BMSC were isolated from marrow aspirates at a frequency of nearly 120 per million nucleated cells. BMSC colonies were formed by homogeneously thin, elongated fibroblast-like cells. BMSC-loaded porous HA implanted in ID mice resulted in the formation of bone tissue as early as 4 weeks after implantation: bone was never observed in control implants of HA alone.

Morphological analysis of retrievals demonstrated proper bony integration of HA cylinders in all samples. Substantially greater amounts of bone were formed within HA pore space and over the HA cylinder external surface in cell-loaded implants ($\sim 50\%$ versus $\sim 7\%$) (Fig. 1).

Fig. 2 reports mean stiffness values plus standard deviation (SD) of the four sample sets. Bone–ceramic complexes showed intermediate stiffness values between bioceramic alone (556 ± 297 N mm^{-1}) and ovine tibia bone (1653 ± 215 N mm^{-1}). Cell-loaded implants were

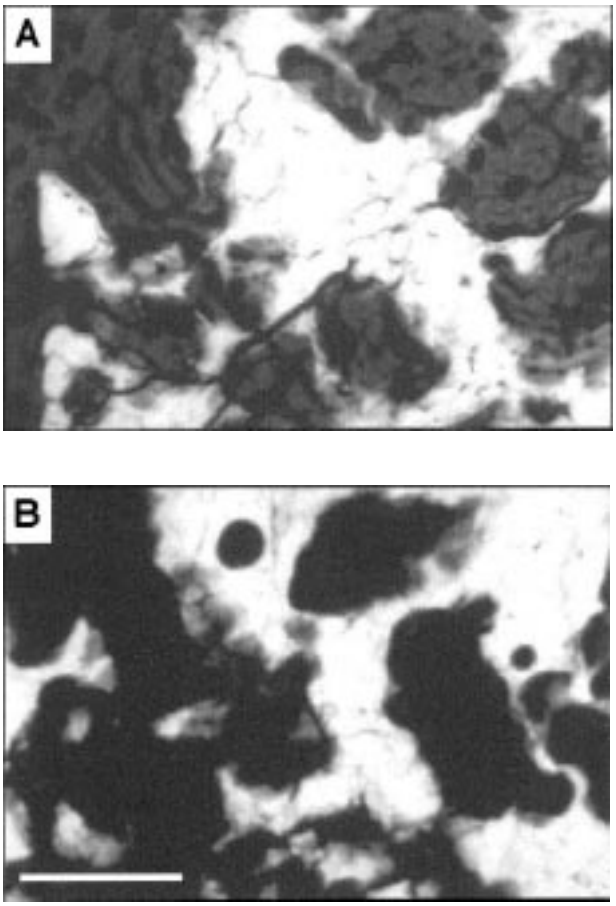

Figure 1 Details of contact microradiographs of resected specimens 2 months after implantation. (A) BMSC-loaded HA cylinder; (B) cell-free HA control cylinder. Bone neoformation is well represented within HA pore space in BMSC-loaded specimens (A) in contrast with control implants (B). Bar: 250 μm.

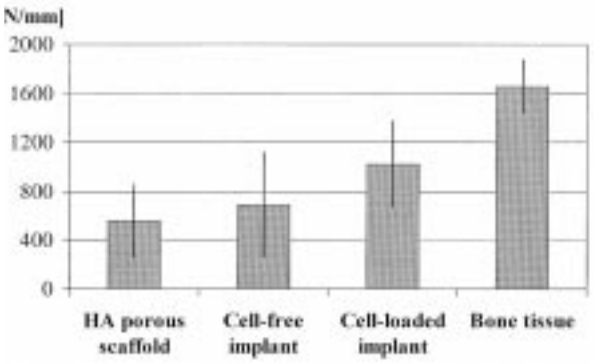

Figure 2 Mean values and standard deviations of the stiffness in unimplanted macroporous ceramics, cell-free implants, BMSC-loaded implants and ovine bone tissue. Bone-HA composites show intermediate stiffness between only bioceramic and bone tissue. Stiffness values are higher in BMSC-loaded implants and standard deviations are lower.

stiffer than cell-free ones, and SD was lower ($1022 \pm 350\,\mathrm{N\,mm^{-1}}$ versus $690 \pm 427\,\mathrm{N\,mm^{-1}}$).

We also evaluated stiffness variation along the main axis of a cell-loaded implant and we observed an increase in mean stiffness and a SD decrease in the implant–bone complex towards the bony end. As shown in Fig. 3 stiffness increases as slice density increases.

4. Discussion and conclusions

Current approaches to the treatment of extensive bone gaps are the use of vascularized autologous bone segments as well as the Ilizarov technique. These two methods require a very long time (12–18 months) for the complete functional recovery and full biomechanical strength to occur. Furthermore, in vascularized autologous bone segments, a significant lesion is created at the donor site. *In vitro* expanded osteoprogenitor cells associated with suitable synthetic biomaterials may lead to the development of integrated bone substitutes, where a biologically controlled osteogenesis is combined with the intrinsic osteoconduction properties of the HA-based bioceramics.

Our results suggest that seeding osteoprogenitor cells into porous HA cylinders improves the mechanical properties of bone–HA composites. Compared to cell-free bioceramic, cell-loaded implants are stiffer. This can be reasonably attributed to a more compact structure due to the large amount of newly formed bone as revealed by micro X-ray observations. While bone grows, the scaffolding is reduced in progressively smaller fragments, thus resulting in a higher integration between neoformed bone and HA ceramic. The lower SD of stiffness values measured on transverse sections of cell-loaded implants shows that they are macroscopically more homogeneous than the controls. This homogeneity minimizes stress concentration that can induce implant rupture. These features make BMSC-loaded implants most valuable bone substitutes in the treatment of long-bone defects.

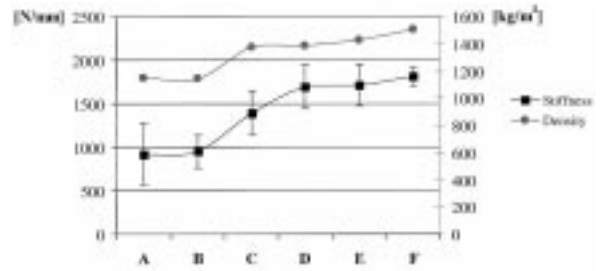

Figure 3 The variation of stiffness and density in six consecutive transverse slabs of a cell-loaded implant along its main axis. Stiffness increases from the portion with the implant (A) towards the bony end (F) while its standard deviation decreases. Slab density grows as stiffness grows.

In a BMSC-loaded implant mean stiffness is related to apparent density, as suggested by the similarity of the curve patterns. Density is a measure of porosity [11], so we can reasonably infer that there is a close relationship between stiffness and porosity. However, porosity is not the only factor to influence the mechanical properties of an implant. As a consequence of bone formation and HA fragmentation/replacement, porosity, pore size and composition change over time and along the main axis. Some authors focus on the effect of porosity and pore size on the mechanical properties of macroporous structures, presenting contradictory data [11, 22]. Other studies emphasize the role of the implant composition [12]. In our opinion all these factors should be taken into account for an in-depth investigation. Further studies will be carried out to establish the weight of each single factor on the mechanical properties of macroporous bone substitute.

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