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Human osteoclast formation and activity on an equine spongy bone substitute

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Abstract

Objectives: The aim of the present study was to evaluate the *in vitro* formation and activity of human osteoclasts (OCLs) generated on a new type of xenograft for bone substitution, an equine spongy bone.

Material and methods: Peripheral blood mononuclear cells from healthy volunteers were used to generate OCLs *in vitro* in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) on bovine bone slices (positive control) and equine spongy bone. Morphological and biochemical methods were used to assess OCLs formation and activity.

Results: Cells generated after 21 days of culture on equine spongy bone showed similar morphology to those on the positive control and displayed typical OCL markers and features, indicating that this material supported OCL formation. Moreover, these cells were functionally active on equine spongy bone with statistically significant differences compared with the control in the release of tartrate-resistant acid phosphatase (TRAcP5b) at days 14 and 21 of culture. With regard to the resorption, on equine bone, OCLs formed smaller discontinuous island-like lacunae rather than the typical lobulated, tracking resorption lacunae observed on the control.

Conclusions: This study enables clinicians to tailor the usage of equine spongy bone and presents a model, which can be applied to the preclinical assessment of bone substitute material's resorbability and resorption rates.

Bone substitutes are currently applied after bone fracture (Rajan et al. 2006) and tumour resection (Ogose et al. 2005). They are used in bone augmentation procedures to support dental implants (Esposito et al. 2006), and are used as filling materials in severe osteoporosis (Monchau et al. 2002). Autologous bone offers the greatest potential for success in regenerative procedures (Herold et al. 2002). However, the availability, the tendency to undergo partial resorption, the need for an additional surgery and the associated morbidity (limping, anaesthesia, paraesthesia, residual defects) represent significant limitations to the use of autogenous graft materials in clinical practice (Valentini & Abensur 1997; Valentini et al. 1998; Wang 2003). The application of natural substitutes such as allografts and xenografts seems to be a valid alternative (Jensen et al. 1998; Merkx et al. 2003; Scarano et al. 2006). For a natural material to be suitable as a bone substitute, it should be biocompatible, non-infectious and non-antigenic (Yamada et al. 1997; Taylor et al. 2002). In addition, it should ideally be osteogenic, osteoinductive and

osteoconductive; although most materials are usually only osteoconductive (Taylor et al. 2002). Moreover, the material should not inhibit the normal activity of bone cells on its surface and disturb the natural bone remodelling process occurring around it (Yamada et al. 1997). Specifically, as osteoblastic bone formation is associated with osteoclastic bone resorption during the bone remodelling process, the surface of the implanted material should be conducive to osteoblast and osteoclast (OCL) activity (Hayden et al. 1995; Yamada et al. 1997). Although it might seem desirable for a complete healing of bone defects to have a fast remodelling process, that disposes the body of the biomaterial as soon as possible, stability is the most important factor for the patient (Schilling et al. 2004), particularly when an implant rehabilitation has been planned. In those cases, the resorption rate of the biomaterial should be balanced by the patient's ability to form new bone (Schilling et al. 2004).

Among biomaterials, xenografts are very popular (Milthorpe 1994; Sogal & Tofe 1999), especially for sinus elevation procedures. They are highly attractive because they carry a small risk of contamination from infectious diseases, do not compromise the patient's remaining tissues and may have the 'correct' structure as the component being replaced (Milthorpe 1994). In addition, due to their chemical–physical characteristics similar to those of human bone, these natural materials show great osteoconductive properties (Yildirim et al. 2000; Traini et al. 2007). Xenogenic materials are very slowly resorbed, which appears not to influence survival rates of implants placed in grafted sites when compared with those placed in non-grafted areas (Yildirim et al. 2000; Maiorana et al. 2006). The most common source of xenograft for bone replacement is bovine bone; as an alternative, materials of porcine origin have been used (Barone et al. 2005; Orsini et al. 2006). To our best knowledge, there are no studies on bone substitutes of equine origin, apart from few papers on an equine bone protein extract, which was capable of inducing osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells (El-Sabban et al. 2007) and ectopic bone formation in a rat model (Li et al. 2006). A clinical, histological and immunohistochemical study aimed at evaluating the suitability of a new equine spongy bone graft in alveolar ridge augmentation procedures has been conducted in our laboratory (Di Stefano et al. 2007). This study demonstrated that equine bone was biocompatible and osteoconductive, and its usage was associated with new blood vessel ingrowth.

The baseline hypothesis connected to the usage of xenografts is the assumption of temporary structural support, integration in the surrounding bone, bioresorption and replacement with vital bone. However, while the graft material's characteristics relevant for osteoconduction and bone formation have been extensively investigated, little is known about bioresorption (Yamada et al. 1997; Herold et al. 2002; Taylor et al. 2002; Schilling et al. 2004; Detsch et al. 2008). A better understanding of the osteoclastic degradation of a bone substitute material would be of interest, because osteoclastic resorption may affect bone formation during the coupled activity of bone cells in the remodelling process. *In vitro* studies are one means of clarifying cells–substrate interaction and determining which material is more suitable for use as a bone substitute relative to the activities of bone cells (De Bruijn et al. 1994).

The aim of the present study was to evaluate OCL formation, attachment, morphology and resorptive activity on a new xenogenous bone-grafting material of equine origin.

Material and methods

Bovine bone and equine spongy bone preparation

Bovine bone and equine spongy bone blocks (10 mm × 10 mm × 20 mm) (Osteoplant®, Bioteck Srl, Arcugnano, Vicenza, Italy) were cut into slices at a thickness of approximately 200 μm using a Buehler low-speed saw equipped with a diamond wafer blade (Buehler Isomet, Lake Bluff, IL, USA). The slices were cut into small squares, processed through washes in distilled water (d-H_2O), sonicated, and then further processed through washes in d-H_2O and acetone before sterilization in 70% ethanol. Substrates were dried, labelled and then re-sterilized using ethanol.

Generation of human OCLs from peripheral blood mononuclear cells

Human peripheral blood was obtained from healthy adult volunteers in syringes containing 1000 U/ml of preservative-free heparin. Blood was diluted 1 : 1 in phosphate-buffered saline (PBS) (Oxoid Ltd, Basingstoke, UK) containing 2 mM glutamine (G), 100 IU/ml benzyl penicillin and 100 mg/ml streptomycin (P/S) (Invitrogen, Gibco, Paisley, Scotland), gently layered over Ficoll-Plaque (Sigma-Aldrich, Poole, UK) and centrifuged. The lymphocyte fractions of mononuclear cells were collected into tubes containing PBS, centrifuged again and resuspended in minimal essential medium eagle (MEME) (Sigma-Aldrich), supplemented with 15% heat-inactivated foetal calf serum (Sera Lab, Crawley, UK), G and P/S (concentrations as above). Cells were then seeded at a concentration of 3×10^5 cells on bovine bone and equine spongy bone slices in 96-multi-well plates, incubated for 4 h in 5% CO_2, 95% air at 37°C. Unattached cells were removed by washing the substrates in PBS and then transferring them to a new culture dish where the cells attached were then cultured in MEME supplemented as above in the presence of 25 ng/ml of human macrophage colony stimulating factor (M-CSF) (Genetic Institute, Cambridge, MA, USA) for 3 days in 5% CO_2, 95% air at 37°C. Cultures were fed every 3–4 days by replacing half of the medium with fresh medium containing M-CSF and 30 ng/100 μl of receptor activator of NF-κB ligand (sRANKL) (Amigen, CA, USA), while collecting supernatants for enzyme-linked immunosorbent assay (ELISA) analysis (Fujikawa et al. 1996; Matsuzaki et al. 1998; Massey & Flanagan 1999; Lader et al. 2001).

At day 21, cells were fixed and processed for confocal, light and reflection microscopy analysis. All the experiments were performed in quintuplicate and repeated four times using cells from different volunteers. Bovine bone was used as positive control, and both substrates, cultured under the same conditions without cells, were used as negative controls to monitor the occurrence of non-cell-mediated hydrolytic degradation.

Immunofluorescence for confocal laser scanning microscopy

Cells on the substrates were fixed in a 40 : 60 mixture of MEME with fixation buffer (3.5% paraformaldehyde and 2% sucrose in PBS), rinsed in PBS and then placed in ice cold permeabilizing buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100

and 0.5% sodium azide in PBS), before being rinsed again in PBS. Substrates were incubated with monoclonal antibodies to human $\alpha_v\beta_3$ subunit of the vitronectin receptor (VNR) (supplied by Prof. Michael Horton, UCL, UK), and to human native type I collagen (Sigma-Aldrich). The antibodies were recognized by applying either rabbit anti-mouse conjugated to fluorescein isothiocyanate (FITC) (Dako, Glostrup, Denmark) or goat anti-mouse conjugated to Alexa 647 (Molecular Probes Inc., Eugene, OR, USA) secondary antibodies, respectively. Finally, they were incubated with rhodamine-phalloidin (Molecular Probes Inc.) to stain for actin and then examined by scanning laser confocal microscopy on a Leica TCS NT System (Heidelberg, Germany). Fluorescent images were collected in sequential 1 μm steps through the OCLs for FITC, tetramethyl rhodamine isothiocyanate (TRITC) and CY5 fluorochromes at 500, 575 and 601 emission wavelengths, respectively. OCLs were defined as multinucleated cells expressing VNR ($\alpha_v\beta_3$) and F-actin rings or patches, or as cells exhibiting F-actin rings with positive staining for type I collagen in resorption pits. Images were displayed in *XY* orientation plane.

Staining for light and reflection microscopy

OCL formation was evaluated by the presence of tartrate-resistant acid phosphatase (TRAP)-positive cells. At day 21, the substrates were washed in d-H_2O, fixed in 10% neutral-buffered formalin, further washed in d-H_2O before being histochemically stained for TRAP, according to the manufacturer's protocol (Sigma-Aldrich; KIT 387-A). TRAP positive cells, containing three or more nuclei, were examined and imaged by light microscopy. Preparations were then counterstained using 0.1% toluidine blue to assess the presence of resorption pits using reflective microscopy.

ELISA

Supernatants collected at days 7, 14 and 21 were used in the ELISA analysis to assess both the release of TRAcP5b (Immuno-Diagnostics, Bedford, MA, USA), which reflects the number of OCLs, and of C-terminal type I collagen fragments (Serum CrossLaps® ELISA; Nordic Bioscience, Herlev, Denmark), a marker of bone resorption. ELISAs were performed according to the manufacturer's protocol.

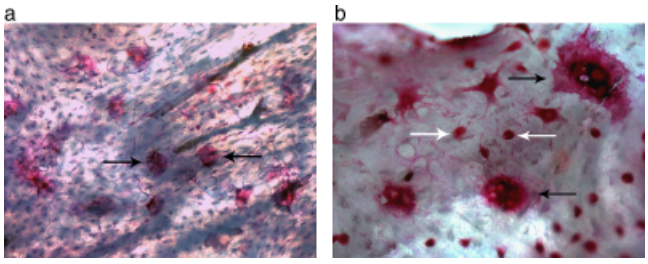

Fig. 1. Light microscopy images of osteoclasts generated from peripheral blood mononuclear cells on bovine bone (a) and equine spongy bone (b) at day 21 of culture. Red indicates tartrate-resistant acid phosphatase (TRAP)-positive cells (black arrows). Small, round-shaped cells were observed on equine bone (white arrows). TRAP staining, magnification ×200.

Statistical analysis

Data presented in this work are the representative results of four separate experiments in cell cultures established from four different donors. All the experiments were carried out in quintuplicate. Comparison was carried out using the non-parametric Mann–Whitney *U*-test, for independent samples. Results are presented as means ± standard errors (SE), and differences at $P \leq 0.05$ were considered statistically significant.

Results

Many cells were readily visible on all the substrates after 21 days of culture. To establish that the generated cells were OCLs, we analysed cell morphology and the expression of specific OCL markers.

Light microscopy revealed that the cells generated on both substrates were TRAP-positive, multinucleated and covered the material's surface, exhibiting a range of cytoplasmatic extensions among cells (Fig. 1a and b). The cell size and appearance did not differ substantially between test and control, although on equine spongy bone, clusters of small and round-shaped cells without projections extending from the cell surface, possibly undifferentiated OCL precursors, were observed (Fig. 1b).

Scanning laser confocal microscopy revealed cells on both materials showing positive staining for F-actin and VNR. In particular, actin staining revealed the typical adhesion contact ring (Fig. 2a–c). On equine bone, cells were irregularly distributed and exhibited a stellate polygonal morphology with multidirectional spreading and projections interconnecting among cells. Immunofluorescence staining also confirmed that clusters of small, round-shaped cells were present and that these cells were not positive for VNR (Fig. 2d); these data support the light microscopy observation of the likelihood that these cells represented not fully differentiated OCLs.

The activity of the generated cells was also assessed. TRAcP5b released in the culture medium increased in both control and equine bone in a time-dependent fashion (Table 1). Specifically, it was detected from day 7 of culture, reaching a maximum up to day 21. Equine bone exhibited a greater release of TRAcP5b when compared with that of bovine (positive control) with no statistically significant differences between negatives ($P = 0.0635$) and at day 7 ($P = 0.3095$), while significant differences were found at day 15 ($P = 0.0082$) and day 21 of culture (0.0493) (Fig. 3).

Resorption lacunae were observed on equine bone by means of toluidine blue staining, demonstrating that this bone substitute material was actively resorbed by human OCLs. On control substrates, OCLs produced the typical lobulated, tracking resorption lacunae (Fig. 4a), while on equine bone, OCLs formed smaller discontinuous island-like lacunae with well-defined margin clearly distinguishable from the non-resorbed surface (Fig. 4b).

In addition, staining for collagen was detected in resorption pits generated on bovine bone and equine spongy bone, along with the formation of the characteristic actin ring, further indicating the presence of OCLs actively resorbing this substrate (Fig. 5a and b).

Discussion

The ideal bone substitute material should be osteoinductive, osteoconductive and only remains in the body as long as necessary to replace the defect by newly formed

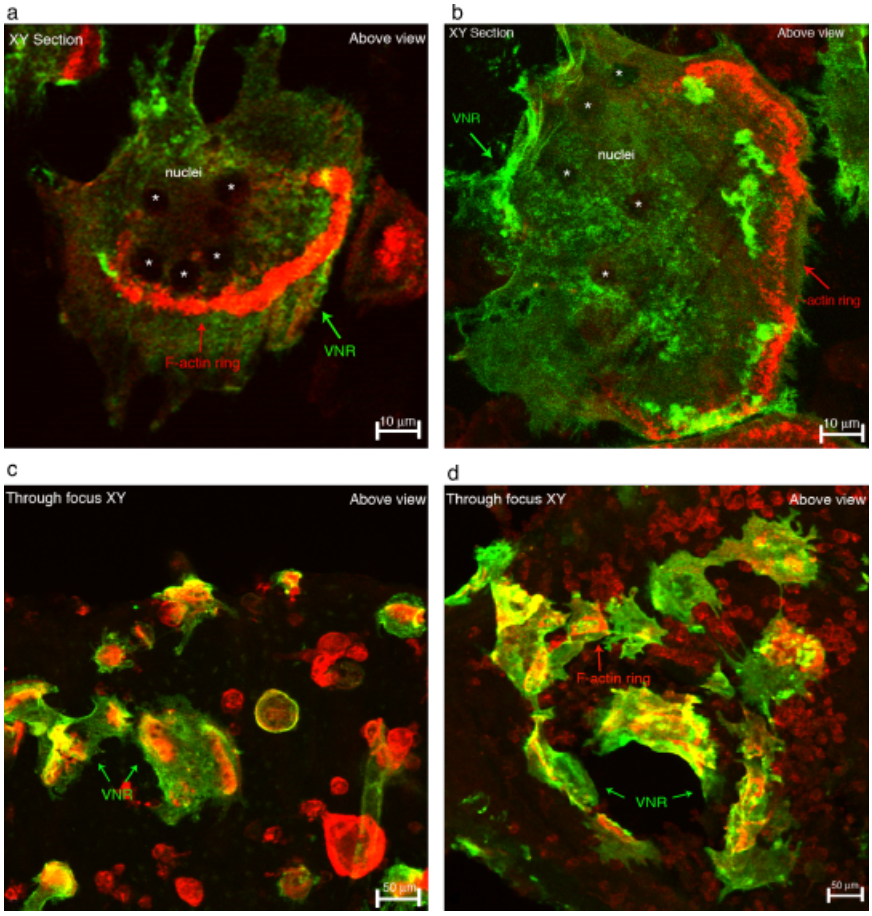

Fig. 2. High power *XY* view of a multinucleated (asterisks) osteoclast (OCL) generated in peripheral blood mononuclear cells cultures on bovine bone (a) and equine spongy bone (b). Red indicates positive staining for F-actin enriched patches and rings with phalloidin-TRITC indicating activated OCLs (red arrows). Green indicates positive staining for monoclonal antibody 23C6 to human $\alpha_v\beta_3$ complex of vitronectin receptor (VNR), scale bar = 10 μm. Low-power through-focus confocal photomicrographs of OCLs on bovine bone (c) and equine spongy bone (d), indicating positive staining for VNR (green arrows) and F-actin (red arrow). Yellow indicates a co-localization of VNR and F-actin, scale bar = 50 μm.

***Table 1.* Levels of tartrate-resistant acid phosphatase (TRAcP)-5b fragments released by osteoclasts on bovine bone and equine spongy bone at days 7, 14 and 21, and assessed by ELISA**

	Negative	Day 7	Day 14	Day 21
Bovine bone	0.0128 ± 0.014	0.2358 ± 0.058	0.4963 ± 0.051	0.9941 ± 0.057
Equine spongy bone	0.1185 ± 0.048	0.2673 ± 0.025	1.0015 ± 0.139	1.6232 ± 0.297

Results are given as means ± SE.
ELISA, enzyme-linked immunosorbent assay.

bone (Dorozhkin & Epple 2002). As every patient varies in bone structure, type of injury and illness, this goal can only be achieved if the clinician has a variety of biomaterials in order to tailor an individual treatment (Schilling et al. 2004) and if the resorption rates of available materials are known before use. So far, investigations on biomaterial's resorbability have been predominantly performed on animal models, which present limitations such as ethics, costs and transferability to human (Schilling et al. 2004). On the contrary, by cultivating human OCL precursors directly on biomaterials, the physiologic conditions leading to resorption are very closely mimicked. Therefore, the culture system employed in the present study is an excellent model for the evaluation of biomaterials for consideration as bone substitutes. This system has been used to generate OCLs for different purposes (individual testing of cell–substrate interaction in patients with specific disorders, i.e. OCL defects) (Nicholls et al. 2005); however, it has only rarely been applied for preclinical testing of resorbability and resorption rates of biomaterials used for regeneration in orthopaedics and dentistry (Schilling et al. 2004). Comparisons of degradation caused by OCLs on different bone graft materials is one way of determining a desirable material with the appropriate resorption timing and rates to fulfil clinical needs.

It seems that the OCLs-resorptive behaviour on graft materials depend on factors such as solubility (Yamada et al. 1997) and surface rugosity, as well as physico-chemical features (Herold et al. 2002; Taylor et al. 2002). OCL attachment and resorptive activity involves the formation of cellular attachments to proteins, within either normal bone matrix or proteins adsorbed onto biomaterials' surface. In particular, VNR is an integrin with a major role in mediating OCL attachment to bone matrix and creating an isolated, sealed microenvironment where acid and enzymes are released, with the consequent degradation of both organic and inorganic phases of bone (Helfrich et al. 1996). However, when proteins are absent from bone matrix (i.e. anorganic bone graft materials), the quality of OCL attachment is compromised, and resorption activity is restricted to only minimal etching of these materials surface (Taylor et al. 2002). The equine bone graft tested in the present study has been subjected to a low temperature (37°C), deantigenation process, without altering its original inorganic composition. Specifically, mineral content and composition is the same as in normal equine bone (Ca: 385 ± 2 mg/g of ash of limb bone and P: 163 ± 3.2 mg/g of ash of limb bone) (Schryver et al. 1974). The extracellular matrix of bone is composed mainly of type I collagen (Helfrich et al. 1996); in the equine bone, type I collagen represents about 70% in weight (unpublished data). These features might explain the very close similarities of the morphology of OCLs generated on equine spongy bone with those formed on the control. Moreover, this observation is supported by the positive expression of VNR in these cells.

In addition, the present study clearly showed that OCLs resorbed equine spongy bone. In our condition, OCLs formed small discontinuous island-like lacunae, but not

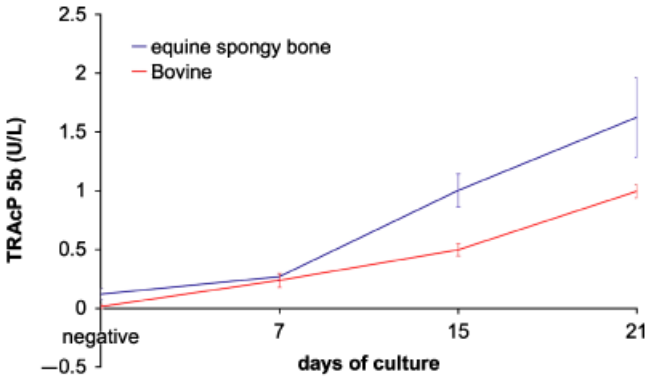

Fig. 3. Levels of tartrate-resistant acid phosphatase (TRAcP)-5b fragments released by osteoclasts generated from peripheral blood mononuclear cells on bovine bone and equine bone into cell culture supernatants collected at days 7, 14 and 21, and assessed by enzyme-linked immunosorbent assay. Results are given as mean ± SE.

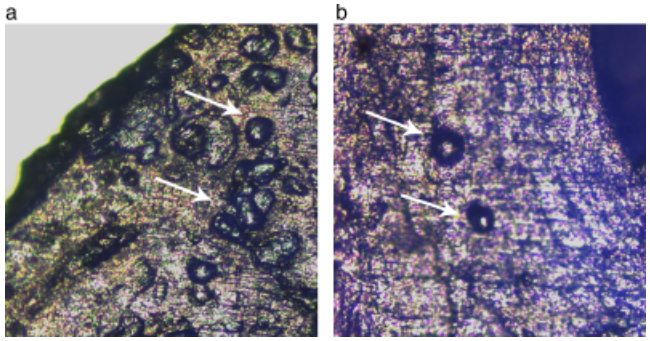

Fig. 4. Reflection microscopy images of osteoclasts generated from peripheral blood mononuclear cells on bovine bone (a) and equine bone (b) at day 21 of culture. Resorption lacunae (white arrows) with well-defined margin, clearly distinguishable from the non-resorbed material surface, were observed on both substrates. Toluidine blue staining, magnification ×100.

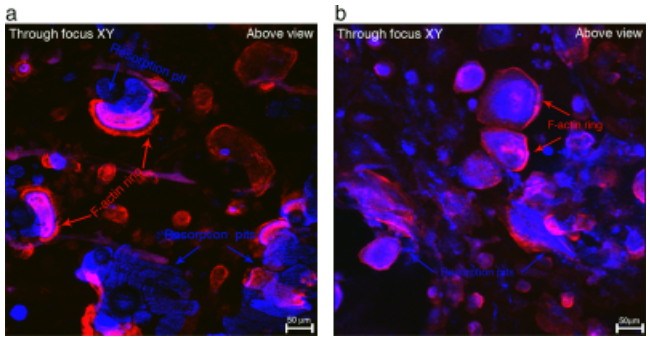

Fig. 5. Low-power through-focus confocal microscopy overview of resorbing osteoclasts (OCLs) generated from peripheral blood mononuclear cells on bovine bone (a) and equine bone (b), stained with monoclonal antibody to native type I collagen (blue). Red indicates staining for F-actin, pink staining indicates a co-localization of type I collagen and F-actin, representing resorption pits underneath active OCLs; scale bar = 50 μm.

resorption tracks on equine spongy bone. Presumably, OCLs resorbed the material's surface only little by little during each resorption phase, which was then followed by a migration phase. The discontinuity of this resorption pattern on equine bone may be explained by the complex three-dimensional structure of this material, which is not uniform, but presents voids and peaks, that could be an obstacle for a continual resorption. The same resorption pattern has been found on pure β-TCP (Yamada et al. 1997). On the other hand, on controls, OCLs produced typical lobulated track-like resorption lacunae, possibly due to the smoothness and flatness of their surface topography. It is known that porosity, pore size and pore structure are important factors that influence the supply of nutrients to cells. It has been found that a pore size of 200–400 μm was optimal to maximize bone ingrowths, but it is poorly understood whether this pore size could be optimal for osteoclastic development and activity as well. The equine bone substitute used in the present study has a porosity ranging from 430 to 750 μm; hence, it is likely that the porosity ideal for OCL development and function is within this range. The occurrence of a more efficient osteoclastogenesis on porous surfaces is supported by a study where OCLs grown on sandblasted surfaces developed at higher rates than they did on polished surfaces (Sommer et al. 2005).

In the present study, although resorption pattern on equine bone graft does not perfectly resemble that of ordinarily observed on bone, the activity of such cells does not seem to be impaired. Actin rings are OCL-specific structure, involved in sealing the OCL to mineralize matrix; they form just before initiation of bone resorption and are present during the resorptive process (Lakkakorpi et al. 1989; Lakkakorpi & Väänänen 1991). On equine bone, it was possible to observe many cells displaying clear F-actin rings. Moreover, the greater release of TRAcP5b from cells generated on equine bone when compared with control suggests that this equine substrate allows OCLs to act in a natural way. The positive expression for type I collagen in resorption pits further supports the occurrence of OCL-mediated resorption and not only an etching of the material's surface. Indeed, collagen fibrils were exposed, which indicate the presence of distinct resorption lacunae and not only superficial erosions of the material's surface.

Because remodelling is a coupled process, the osteoclastic resorption can lead to the assumption that it will be followed by osteoblastic bone formation, which can be promoted by the cell to cell contact, cell-matrix interaction, cytokines released from OCLs, etc. This issue is of pivotal importance, especially when bone grafting is associated at a one-stage implant placement. In such a clinical condition, it is desirable that graft materials undergo remodelling for the formation of a biomechanically strong tissue at the bone–

implant interface, necessary to limit micro damage during implant function. Although favourable survival and success rates for implants inserted in sites augmented with a variety of grafting materials has been reported (Jensen et al. 1998; Nevins et al. 1998; Shulman & Jensen 1998), it is reasonable to use resorbable materials in a one-stage procedure and materials with varied resorption, i.e. resorbable, slowly resorbable or non-resorbable with a staged approach.

In conclusion, the present study has demonstrated that human OCLs can differentiate and function on the equine spongy bone-grafting material. Such findings enable clinicians to tailor the usage of equine spongy bone and present a model which can be applied to the preclinical assessment of bone substitute material's resorbability and resorption rates. However, further *in vivo* experiments aiming at verifying the clinical significance of the present findings should be carried out.

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