Biocompatibility and osseointegration in osteoporotic bone

A PRELIMINARY IN VITRO AND IN VIVO STUDY


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We implanted nails made of titanium (Ti6Al4V) and of two types of glass ceramic material (RKKP and AP40) into healthy and osteopenic rats. After two months, a histomorphometric analysis was performed and the affinity index calculated. In addition, osteoblasts from normal and osteopenic bone were cultured and the biomaterials were evaluated in vitro.

In normal bone the rate of osseointegration was similar for all materials tested (p < 0.5) while in osteopenic bone AP40 did not osseointegrate (p < 0.0005).

In vitro, no differences were observed for all biomaterials when cultured in normal bone-derived cells whereas in osteopenic-bone-derived cells there was a significant difference in some of the tested parameters when using AP40.

Our findings suggest that osteopenic models may be used in vivo in the preclinical evaluation of orthopaedic biomaterials. We suggest that primary cell cultures from pathological models could be used as an experimental model in vitro.

Materials and Methods

For the in vivo study we used 36 cylindrical nails (2 mm in diameter and 3 mm in length) made of RKKP, AP40 and Ti6Al4V. AP40 and RKKP are silicophosphates of Ca, K and Na with similar composition and differ only in regard to the presence of amphoteric network-formers, La$^{3+}$ and Ta$^{5+}$ in RKKP, which contribute to the stabilisation of their molecular network. They were prepared by melting the starting products in platinum crucibles at 1450°C for 60 minutes. Glass nails were manufactured by casting the synthesised melted products from the crucible into a cylindrical graphite die. In order to allow for glass thermal shrinkage the diameter of the die was slightly larger than the required final diameter of the nail. The height was set to the desired value by cutting and polishing.
The Ti6Al4V rod had a concentration of C < 0.08% and of Fe < 0.25%.

For the in vitro tests, plates were made of the same materials measuring 8.5 ± 0.5 mm in diameter and 1 mm in thickness.

The materials were sterilised by autoclaving at 120°C for 20 minutes.

**Experimental model.** We used 36 female, healthy Sprague-Dawley rats aged ten months and weighing between 390 and 420 g. They were randomly divided into two groups of control (C) and ovariectomised (OVX). In the latter group we performed a bilateral ovariectomy by lumbar access after anaesthesia by a subcutaneous injection of ketamine (Ketavet 100, 87 mg/kg; Farmaceutici Gellini, Aprilia Lt, Italy) and xylazine (Rompun, 44 mm/kg; Bayer AG, Leverkusen, Germany). Four months later, the left distal femur of all the animals was exposed through a lateral skin incision. A cylindrical nail of either Ti6Al4V, AP40 or RKKP was implanted into six rats each of both groups. Two months after the implants had been inserted the animals were killed. The left distal femur was then removed immediately from each animal for histomorphometric studies and specimens of trabecular bone were obtained from the right femur, to isolate osteoblasts for the in vitro study.

All the experimental procedures were performed according to the International and European Laws on animal experimentation.

**Osteoblast culture.** Under aseptic conditions, the right distal femur from randomly selected rats of both groups was cleaned of soft tissues and the condyles removed from the cortical area to expose the trabecular tissue and to cultivate osteoblasts from normal (NB-OST) and osteopenic (OVX-OST) bone. Cell cultures were obtained according to the method of Halstead et al. Cells were then seeded (1 × 10^5 cells/ml) onto 24-multiwell plates and cultured in Dulbecco’s minimum essential:F12 medium to which ascorbic acid (50 g/ml) and β-glycerophosphate (10 M) had been added. Characterisation of the phenotype of the osteoblasts was done by adding 1,25 (OH 2)D3 (10^−9 M) to a part of the cultures and then measuring osteocalcin in the supernatant (OC; Novocalcin Kit; Metra Biosystem, California) and the activity of alkaline phosphatase (ALP; Sigma Kit 104, UK). These cells were then fixed, tested for type-I collagen (monoclonal immunofluorescence, Sigma) and stained by the Von Kossa method (BiOptica, Milan, Italy). These tests confirmed that the cells had the capacity to differentiate and to mineralise and exhibited osteoblast behaviour.

The residual cells were seeded as above and sterile specimens of all materials were placed in contact in cultures of NB-OST and OVX-OST. Cultures of both groups incubated without biomaterials were used as negative controls. The cultures were maintained at 37°C in a 5% CO2 atmosphere for six days and the medium was changed on days two and four. At the end of the experiment, all the cultures were tested in triplicate for ALP, lactate dehydrogenase (LDH; Boehringer Mannheim, Germany), OC and the tetrazolium salt test (MTT; Sigma, UK).

**Histomorphometric analysis.** The distal femora containing the implants were fixed for 24 hours in 4% buffered paraformaldehyde, dehydrated in a graded series of alcohols and embedded in methylmethacrylate resin. After polymerisation, blocks were sectioned along a plane perpendicular to the bone surface using a Leitz 1600 microtome (Leica SpA, Milan, Italy), yielding undecalcified sections of 70 to 80 µm in thickness. The sections were stained with Fast Green and then observed by a blinded investigator with a Zeiss Axioskop optical microscope (Carl Zeiss GmbH, Jena, Germany). Histomorphometry was performed using a semi-automatic Kontron KS image analysis system (Kontron Electronic GmbH, München, Germany).

In order to determine the quality of the host bone in which the biomaterials had been implanted, we calculated the following parameters in a defined area of the femur: trabecular bone volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), trabecular separation (TbSp) and the connectivity index (N.Nd/N.Tm). We then calculated the affinity index (AI) which is the ratio obtained by dividing the length of the region where the bone directly faces the implant, without the presence of the fibrous membrane, by the total length of the bone-implant interface, multiplied by 100.

**Statistical analysis.** We used the software package SPSS/PC + Statistics 7.1 (SPSS Inc, Chicago, Illinois). Data are given as the mean ± standard deviation (SD) and the significance level was set at p < 0.05. After evaluating the homogeneity of variance and the normality of the data, ANOVA and Scheffe’s multiple comparison tests were done to determine differences among the groups.

**Results**

Table I gives details of the histomorphometric data and shows the development of an osteopenic state in the femora which were used both for the osteoblast cultures and the implants.

In vitro, there was no difference between the NB-OST and OVX-OST cultures.

In NB-OST cells, no significant differences were found for the three types of biomaterial (Table II). In OVX-OST cells, however, significant differences were observed for levels of MTT and OC (Table III). In vitro results showed that the three biomaterials behaved differently when placed in normal and osteopenic osteoblast cultures. AP40 caused a significant decrease in MTT and OC only when cultured in OVX-OST cells indicating damage to mitochondrial respiration and protein synthesis. Scheffe’s test revealed that these decreases were significant when AP40 was compared with Ti6Al4V (∆MTT = -19.3, p = 0.027; ∆OC = -11.9, p = 0.005) and control cultures (∆MTT = -19.0, p = 0.007; ∆OC = -12.6, p = 0.003).

Our in vivo results confirmed that in osteopenic bone the
rate of osseointegration of biomaterials is different from that in normal bone and that AP40 osseointegrates only if implanted in normal bone.

Two-way ANOVA showed an interaction between ovariectomy and the type of implanted biomaterial on Al results (F = 56.2, p < 0.0005). Biomaterials implanted in healthy rats showed similar values of Al without any significant differences (p < 0.05). Al results changed slightly when biomaterials were implanted in osteopenic rats. In particular, AP40 had the worst result (p < 0.0005) and did not osseointegrate in osteopenic bone (Fig. 1).

**Discussion**

In a study of the osseointegration of biomaterials the quality of the host bone bed in which biomaterials are implanted should be one of the main factors to be considered. In the presence of osteoporosis, the biomaterial of the implant should compensate for the limitations of osseointegration in osteopenic bone.

Our aim was to evaluate the possibility of improving preclinical knowledge of orthopaedic biomaterials by ‘pathological’ in vitro and in vivo models. We therefore

**Table I.** Histomorphometric data of the distal femur

<table>
<thead>
<tr>
<th>Group</th>
<th>BV/TV (%)</th>
<th>Tb Th (µm)</th>
<th>Tb N (1/mm)</th>
<th>TbSp (µm)</th>
<th>N.Nd/N.Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>44.2 ± 3.8</td>
<td>116.6 ± 13.7</td>
<td>3.8 ± 0.6</td>
<td>148.7 ± 29.7</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>OVX</td>
<td>31.2 ± 4.2</td>
<td>96.9 ± 9.6</td>
<td>3.2 ± 0.6</td>
<td>218.9 ± 55.7</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>t</td>
<td>5.56</td>
<td>2.88</td>
<td></td>
<td>2.72</td>
<td>3.49</td>
</tr>
<tr>
<td>p value*</td>
<td>0.0005</td>
<td>0.05</td>
<td>NS</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Student’s t-test

**Table II.** In vitro results in NB-OST cells with biomaterials

<table>
<thead>
<tr>
<th>LDH (IU/l)</th>
<th>ALP (IU/l)</th>
<th>MTT (OD)</th>
<th>OC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti6Al4V</td>
<td>61.0 ± 2.6</td>
<td>14.0 ± 1.0</td>
<td>1.69 ± 0.2</td>
</tr>
<tr>
<td>RKKP</td>
<td>59.3 ± 0.5</td>
<td>13.2 ± 0.8</td>
<td>1.62 ± 1.3</td>
</tr>
<tr>
<td>AP40</td>
<td>58.0 ± 2.0</td>
<td>12.8 ± 4.1</td>
<td>1.52 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>62.3 ± 4.5</td>
<td>13.8 ± 2.2</td>
<td>1.67 ± 2.0</td>
</tr>
<tr>
<td>F</td>
<td>1.361</td>
<td>0.143</td>
<td>1.778</td>
</tr>
</tbody>
</table>

**Table III.** In vitro results in OVX-OST cells with biomaterials

<table>
<thead>
<tr>
<th>LDH (IU/l)</th>
<th>ALP (IU/l)</th>
<th>MTT (OD)</th>
<th>OC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti6Al4V</td>
<td>61.3 ± 1.5</td>
<td>13.6 ± 1.5</td>
<td>1.78 ± 0.02</td>
</tr>
<tr>
<td>RKKP</td>
<td>58.0 ± 0.0</td>
<td>13.9 ± 0.7</td>
<td>1.69 ± 0.13</td>
</tr>
<tr>
<td>AP40</td>
<td>63.0 ± 4.0</td>
<td>11.8 ± 2.0</td>
<td>1.58 ± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>60.6 ± 4.0</td>
<td>13.8 ± 2.4</td>
<td>1.77 ± 0.04</td>
</tr>
<tr>
<td>F</td>
<td>1.455</td>
<td>0.884</td>
<td>6.950</td>
</tr>
<tr>
<td>p value</td>
<td>NS</td>
<td>NS</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Fig. 1

Affinity index variations of different implanted biomaterials in control and osteopenic (OVX) rats. Scheffe’s multiple comparison test: AP40, OVX v control groups, p < 0.0005.
used an in vivo model of osteopenia and an in vitro model with cells derived from osteopenic bone to test certain biomaterials and to evaluate whether these studies could influence the selection of such materials in the clinical situation. The ideal biomaterial should have the same biocompatibility characteristics and rate of osseointegration when tested in normal and pathological conditions. In this study attention was not focused on the biomaterials themselves but on the methods for testing them.

The ovariectomised rat was used as an animal model for osteopenia because of the large amount of data available. Histomorphometric analysis at the site of the implants showed the effectiveness of ovariectomy on the distal femur. In addition, osteopenia had been confirmed by densitometric, histomorphometric and biomechanical tests (unpublished data). Since this was a preliminary study six animals in each group were considered to be adequate for the histomorphometric analysis. In order to reduce the interanimal variance and thus limit the number of animals needed, the study group was as homogeneous as possible for strain, gender, age and weight.

For the in vitro study we used cells cultured from the same animals, thus reducing intersubject variability. It has been shown that using human cells in studies of bio-

- Histomorphometric analysis at the site of the implants showed the effectiveness of ovariectomy on the distal femur. In addition, osteopenia had been confirmed by densitometric, histomorphometric and biomechanical tests (unpublished data). Since this was a preliminary study six animals in each group were considered to be adequate for the histomorphometric analysis. In order to reduce the interanimal variance and thus limit the number of animals needed, the study group was as homogeneous as possible for strain, gender, age and weight.

21.22 Varying results have been obtained by different authors when comparing healthy osteoblasts with cells from osteoporotic patients. Even if the phenotype characterisation did not show variations between normal and osteopenic-bone-derived cells, differences in the rate of proliferation or in the response to cytokines, hormones or mechanical stimuli have been observed.

In our study, no differences were observed in control cultures between NB-OST and O VX-OST cells. Our hypothesis is that, even if OVX-OST cells do not differ from normal cells under basal conditions, they behave differently in the presence of an external stimulus such as a biomaterial as shown by cytotoxicity (MTT) or activity (OC).

Our findings in the in vivo study showed that osteopenia was associated with a decrease in osseointegration for all the materials tested and confirmed the necessity for a preclinical in vivo study of biomaterials in pathological animals.

Martin et al. implanted Ti6Al4V cylinders into the proximal metaphysis of the humerus of dogs which had been either ovariectomised or sham operated four months before. After two months, they observed that the deficiency of oestrogen had significantly affected ingrowth of tissue into the implant, the strength of the bone-implant interface and the relationship between these variables. Hayashi et al. implanted hydroxyapatite (HA) and alumina (Al2O3) cylinders into the tibiae of both healthy rats and those with bone rarefaction due to disuse osteoporosis. According to their results, HA showed good osseointegration both in healthy animals and in osteopenia while Al2O3 significantly changed its behaviour if implanted in pathological bone. They then compared HA and Ti6Al4V cylinders implanted in the tibiae of three groups of rats: normal, ovariectomised and ovariectomised plus neurectomised. While the rate of osseointegration of Ti6Al4V in normal bone was greater than in osteopenic bone in the case of HA and in the absence of fibrous encapsulation, a rapid osteogenic response was also observed in ovariectomised plus neurectomised rats. In rabbits in which a Ti screw implant had been inserted in the tibia, Mori et al. studied the effects of ovariectomy and ovariectomy plus a low calcium diet. Formation of new bone in osteopenic rabbits was delayed even if considerable bone contact was observed at 12 weeks. Fini et al. implanted HA and Ti6Al4V cylinders into the femoral condyles of normal and osteopenic rats. At two months, both materials produced a decrease in the rate of osseointegration in osteopenic animals in comparison with normal animals and this value was significant for HA. Fujimoto et al. used a rabbit model of steroid-induced osteoporosis and implanted Ti into the tibia and mandible of normal and osteopenic rabbits. The mechanical attachment of Ti to the tibial bone was significantly lower in prednisolone-treated animals, while no significant differences were observed in the removal torque data of the implants placed in the mandible. Also osteopenia related to degenerative arthritis was observed to be one of the causes of poor bone ingrowth around materials.

In our study, in osteopenic bone only fibrous tissue was found around AP40 and, surprisingly, when comparing in vitro and in vivo data, the most significant decreases in MTT and OC levels were seen only with this biomaterial which did not osseointegrate in vivo.

It is important to stress that the negative effects of AP40 on bone osseointegration arose only in the ‘pathological models’ and would have remained undiscovered in tests performed on normal cells or healthy bone.

In conclusion, in order to give better characterisation of biomaterials before clinical application, pathological animal models may be used, not only for in vivo osseointegration studies but also for in vitro evaluation. Primary cell cultures from pathological animal models could represent a new experimental in vitro model and complement in vivo studies. For these reasons, in vitro strategies with ‘pathological’ cells should be further investigated and understood.

Financial support of this research was partially given by the Italian Minister of Health, Special Project n. G1890079 and by the ‘Fondazione Cassa di Risparmio’, Bologna, Italy. Moreover, the authors wish to thank Patrizio Di Denia, Claudio Dal Fiume, Giovanna Caligiuri, Nicola Corrado and Franca Rambaldi of the Department of Experimental Surgery, Rizzoli Orthopaedic Institute, for their technical assistance.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.
References


