Relationship between bioceramics sintering and micro-particles-induced cellular damages

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We performed experimental studies to confirm the hypothesis that cellular damages occurring around implanted biphasic bioceramics could be related to a micro-particles release because of an insufficient sintering. First, an in vitro cytotoxicity study was performed on four biphasic ceramic (BCP) samples. Without treatment of the extraction medium, a cytotoxicity was observed, although after centrifugation this cytotoxicity disappeared in all samples. Second, micro-particles of hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and 40% β-TCP/60%HA mixture were used for a cell inhibition study. A decrease of cell viability was observed with the increase in particles concentration. At 10 000 particles per cell, the viability and proliferation were completely inhibited. Third, HA, β-TCP and BCP ceramic granules were implanted in rabbit femoral cavities for 12 weeks. No degradation of HA granules was observed. The degradation was higher for β-TCP (40%) than for BCP (5%). On the other hand, new bone formation was significantly higher for β-TCP (21%) and HA (18%) than for BCP (12%). More micro-particles were formed around BCP granules than around β-TCP, and phagocytised by macrophages.

The release of ceramic micro-particles could be related to the sintering process. BCP ceramic have to be sintered at only 1160 °C. Consequently, HA micro-particles of BCP ceramic are incompletely sintered and easily released after immersion or implantation. The micro-particles could be at the origin of local inflammation and cell damage and could perhaps modify osteogenesis. Attention must be paid to this problem especially with BCP ceramics because of the sintering difficulties of this bioceramic.

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1. Introduction
Hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and biphasic calcium phosphate (BCP) bioceramics are frequently used in orthopaedic and dental surgeries. Although the components of these ceramics are well known for their harmlessness, a local inflammation has been observed in some cases around implants [1–5]. In previous in vitro experiments, we observed a cytotoxicity of BCP ceramic although none was observed for HA and β-TCP ceramics (unpublished results). We hypothesised that these effects could be related to the presence of micro-particles released from BCP ceramics because of an insufficient sintering. We developed in vitro and in vivo experiments to confirm this hypothesis.

2. Materials and methods
2.1. Micro-particles
The HA and β-TCP micro-particles were respectively obtained from Trans-Tech SA (Adamstown, USA) and Tomita (Tokushima, Japan). Their physico-chemical properties are shown in the Table I.

2.2. Porous ceramics
Pure HA, pure β-TCP, 40% β-TCP/60%HA (BCP-1 and BCP-2) and 30% β-TCP/70%HA (BCP-3) powders were used by Biocétis (Berck sur Mer, France) to produce porous ceramics presenting a same porosity and an identical mean size of spherical pores (500–630 μm) and

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TABLE I Properties of calcium phosphate micro-particles

<table>
<thead>
<tr>
<th>Formula</th>
<th>Ca/P ratio</th>
<th>Particle size (µm)</th>
<th>Specific surface (m² g⁻¹)</th>
<th>Crystal structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.667</td>
<td>1.05–5.98</td>
<td>3.29</td>
<td>Hexagonal</td>
</tr>
<tr>
<td>β-TCP</td>
<td>1.50</td>
<td>0.80–4.47</td>
<td>3.0</td>
<td>Rhombohedral</td>
</tr>
</tbody>
</table>

interconnections (100–130 µm). Commercial samples of 40%β-TCP/60%HA (BCP-4) ceramic (60–85% of porosity and 200–500 µm of pores) were obtained from another company. All ceramics were produced by casting with a suspension of β-TCP, HA or BCP micro-particles and using polymer macro-beads as porogen agent. β-TCP as well as BCP ceramics were sintered at 1120 °C and HA ceramics were sintered at 1250 °C. Then, the porous ceramics were elaborated in granules of 0.5–1.5 mm in diameter.

2.3. Cytotoxicity study

BCP-1, BCP-2, BCP-3, BCP-4 samples and Thermaonox® (negative control) (Fisher Scientific, France) were immersed (0.1 g ml⁻¹) in DMEM culture medium (Eurobio, France) during 48 h to prepare an extraction medium. This one was divided in two parts, one was centrifuged at 1500 rpm during 5 min and the other one remained untreated.

A fibroblast (L929) suspension was prepared in complete DMEM to obtain 10⁶ cells ml⁻¹. Two hundred microlitres of the suspension were inoculated in each well of 96-wells culture plates. After 48 h of incubation (37 °C, 5% CO₂, 98% humidity), the culture medium was replaced by 200 µl per well of different concentrations (1%, 10%, 50%, 100%) of the extraction medium for each sample (BCP-1, BCP-2, BCP-3, BCP-4). After 24 h of incubation, the culture medium was eliminated and 0.1 mg per well of MTT (Thiazolyl Blue, Sigma, France) was added. After 4 h, colorimetric reaction in the MTT-treated plates was measured by spectrophotometry (absorbance at 570 and 650 nm). Cell viability was calculated from assay results divided by those of negative control (Thermaonox®).

2.4. Cell inhibition study

The micro-particles of pure HA, pure β-TCP, and 40%β-TCP/60%HA mixture were diluted in DMEM to obtain a suspension of 2.1 × 10⁵ micro-particles per ml. 2 × 10⁴ cells (L929) per well with different concentrations of micro-particles (0, 10, 100, 1000 or 10000 micro-particles per cell) were distributed in each well of a 24-wells culture plate. After one, three and seven days, MTT coloration test was performed to evaluate the cell viability.

2.5. Animal implantation

Under general anaesthesia and in rigorous aseptic conditions, cavities of 5 mm diameter and 10 mm depth, perpendicular to the longitudinal axis of femur, were bilaterally made in femoral condyles of 11 New Zealand rabbits. Each cavity was filled with granules of HA or β-TCP or BCP-1. All animals were sacrificed after 12 weeks of implantation, distal femurs were harvested and fixed in 10% formaldehyde solution for two weeks. The bone segments were dehydrated and embedded in polymethylmethacrylate without decalcification. Sections of 50 µm thick were stained with Picro-Fuchsine van Gieson staining. Porosity, residual material and new bone formation in the implant were measured with a semi-automatic image analysis system (Histolab-Microvision Instruments, France) and degradation ratio was calculated [6].

3. Results

3.1. Cytotoxicity study

An indirect cytotoxicity study was performed with L929 fibroblasts using extraction medium of BCP-1, BCP-2, BCP-3 and BCP-4 samples. The extraction medium before centrifugation showed clearly for all samples a cytotoxicity which disappeared completely after centrifugation (Table II).

3.2. Cell inhibition study

L929 fibroblasts were co-cultivated with micro-particles of pure HA, pure β-TCP and 40%β-TCP/60%HA mixture for one, three and seven days. A decrease of cell viability and cell number correlated with the increase of particles concentration was obtained for each delay. Up to 10 particles per cell, a standard viability and proliferation were observed. Above 100 particles per cell, the viability and proliferation decreased and were completely inhibited at 10000 particles per cell for all samples (Figs. 1–3).

TABLE I Ceramic micro-particles influence upon cell viability

<table>
<thead>
<tr>
<th>Ceramics</th>
<th>Composition</th>
<th>Cell viability Without treatment (%)</th>
<th>After centrifugation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP-1</td>
<td>40%β-TCP/60%HA</td>
<td>57</td>
<td>82</td>
</tr>
<tr>
<td>BCP-2</td>
<td>40%β-TCP/60%HA</td>
<td>57</td>
<td>87</td>
</tr>
<tr>
<td>BCP-3</td>
<td>30%β-TCP/70%HA</td>
<td>52</td>
<td>80</td>
</tr>
<tr>
<td>BCP-4</td>
<td>40%β-TCP/60%HA</td>
<td>51</td>
<td>89</td>
</tr>
</tbody>
</table>

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