# 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.


## 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*in vitro* experiments, we observed a cytotoxicity of BCP ceramic although none was observed for HA and β-TCP ceramics (unpublished results).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 Biocetis (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

| | Formula | Ca/P ratio | Particle size (μm) | Specific surface ($m^2\,g^{-1}$) | Crystal structure |
|---|---|---|---|---|---|
| HA | $Ca_{10}(PO_4)_6(OH)_2$ | 1.667 | 1.05–5.98 | 3.29 | Hexagonal |
| β-TCP | $Ca_3(PO_4)_2$ | 1.50 | 0.80–4.47 | 3.0 | Rhombohedral |

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 Thermanox® (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 $10^5$ 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% $CO_2$, 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 (Thermanox®).

## 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 $2.1 \times 10^9$ micro-particles per ml. $2 \times 10^4$ cells (L929) per well with different concentrations of micro-particles (0, 10, 100, 1000 or 10 000 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 10 000 particles per cell for all samples (Figs. 1–3).

TABLE II Ceramic micro-particles influence upon cell viability

| Ceramics | Composition | Cell viability | |
|---|---|---|---|
| | | Without treatment (%) | After centrifugation (%) |
| BCP-1 | 40%β-TCP/60%HA | 57 | 82 |
| BCP-2 | 40%β-TCP/60%HA | 57 | 87 |
| BCP-3 | 30%β-TCP/70%HA | 52 | 80 |
| BCP-4 | 40%β-TCP/60%HA | 51 | 89 |

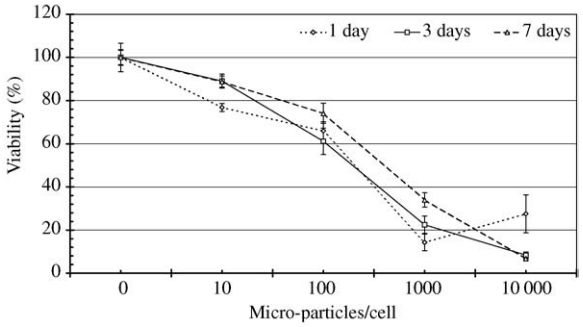


*Figure 1* Cell viability with number of BCP micro-particles.

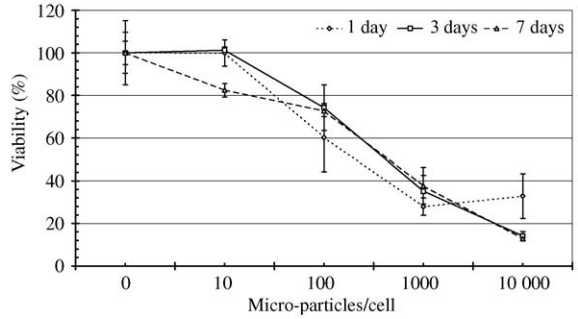


*Figure 2* Cell viability with number of HA micro-particles.

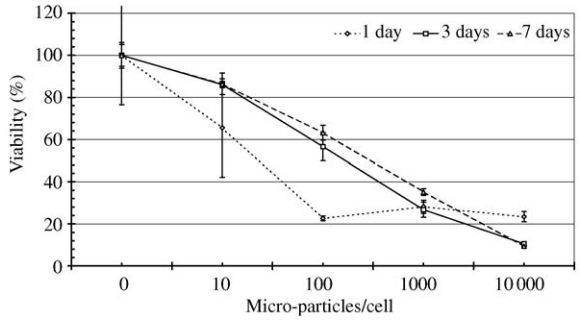


*Figure 3* Cell viability with number of β-TCP micro-particles.

### 3.3. Animal implantation

The ceramic granules of HA, β-TCP and BCP were implanted in the cavities of rabbit femoral condyle. After 12 weeks of implantation, new bone formation was considerable around implants and inside ceramic pores (Fig. 4), and was directly in contact with material for HA and BCP. On the contrary, for β-TCP there was a very thin space between implant and new bone, and the presence of micro-particles at the ceramic/bone interface was noted (Fig. 5). The macro-architecture of porous ceramics was scarcely changed in HA and BCP, but was visibly modified in β-TCP with a decrease of ceramic volume, an increase of the porosity, and a change of ceramic shape. Macrophages were more numerous in BCP than in β-TCP, but osteoclasts were rarely observed in all samples. Numerous micro-particles of identical size to the powder particles (0.5–5 μm) were found in medullar tissue and phagocytised by macrophages in BCP than in β-TCP and in HA (Fig. 6).

The histomorphometric results (Table III) showed that osteogenesis was significantly higher in β-TCP (21%) than in HA (18%), but was the weakest in BCP (12%). The material degradation was the highest in β-TCP (40%), the weakest in HA (0.7%), and intermediary in BCP (5%).

## 4. Discussion

Our *in vitro* findings confirm that ceramic micro-particles can induce inhibition and damage to cells but only when cells are in direct contact with micro-particles. Indeed the ceramics do not release toxic elements, since we observed the absence of cytotoxicity after centrifugation of the extraction medium. Thus, this inhibition could be relative to damages caused by the crystal shape of the micro-particles. The size and the concentration of particles could also play an important role. In the cytotoxicity study on four BCP samples formed by HA and β-TCP particles, the extracted medium without centrifugation showed an important cytotoxicity, although in our previous studies, pure HA or pure β-TCP ceramics never showed cytotoxicity. Why did we have different results with samples formed by a mixture of pure HA and β-TCP particles? The answer could be related to the different connections between powder particles after sintering of HA, β-TCP and BCP ceramics. The ceramic dissolution early breaks connections between particles before to degrade the particles

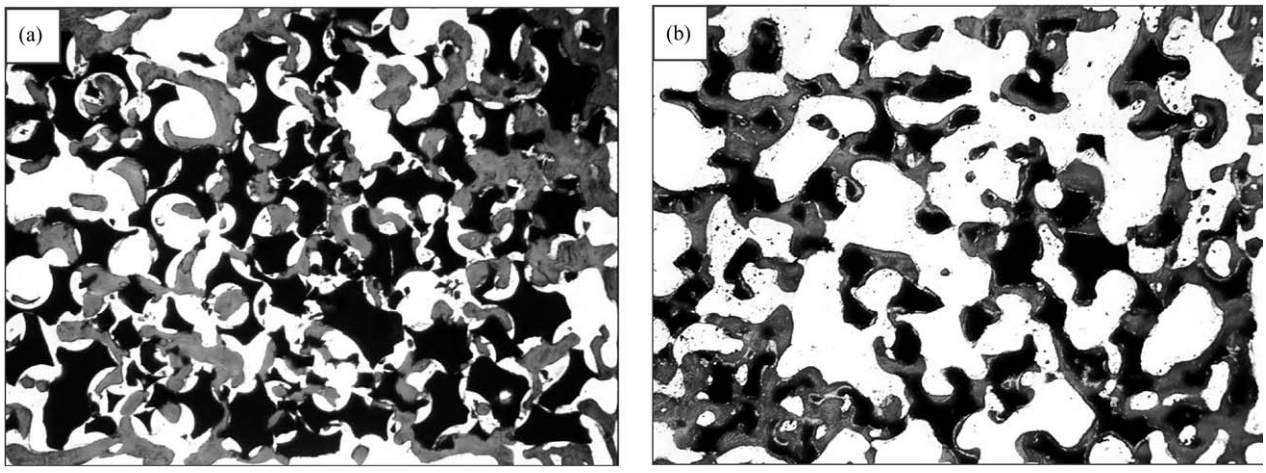


*Figure 4* After 12 weeks, new bone formed inside pores of the BCP (a) and β-TCP (b). The macro-architecture of porous ceramics was scarcely changed in BCP, but was visibly modified in β-TCP with a decrease of ceramic volume, an increase of the porosity, and a change of ceramic shape (b). Picro-Fuchsine van Gieson staining, original magnification × 25.

TABLE III Material degradation and osteogenesis in implants after 12 weeks

| Implant | Delay | Porosity (%) | Material (%) | Degradation (%) | Osteogenesis (%) |
|---|---|---|---|---|---|
| HA | 0 ws ($n=5$) | *59.17 ± 4.29* | *40.83 ± 4.29* | | — |
| | 12 ws ($n=5$) | 41.45 ± 4.22 | 40.53 ± 5.75 | 0.73 | 18.02 ± 3.53 |
| TCP | 0 ws ($n=5$) | *56.05 ± 7.10* | *43.95 ± 7.10* | | — |
| | 12 ws ($n=8$) | 52.41 ± 6.72 | 26.45 ± 7.76 | 39.82 | 21.14 ± 1.25 |
| BCP | 0 ws ($n=5$) | *55.56 ± 3.93* | *44.44 ± 3.93* | | — |
| | 12 ws ($n=8$) | 45.38 ± 5.63 | 42.24 ± 8.24 | 4.95 | 12.38 ± 6.55 |

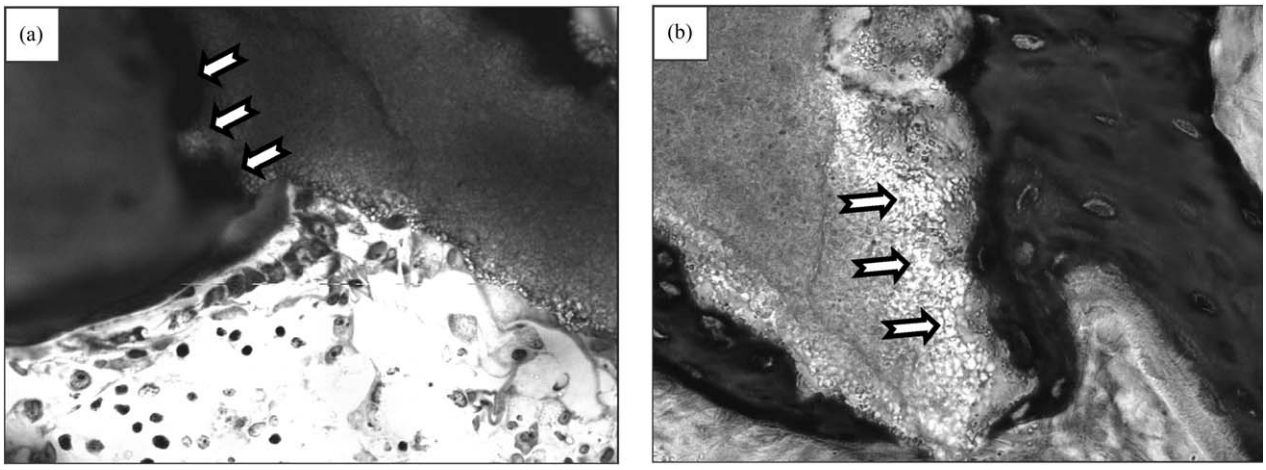


*Figure 5* After 12 weeks of implantation, ostegenesis was directly in contact with material (arrows) in BCP (a). On the contrary, for β-TCP there was a very thin space with presence of micro-particles (arrows) at the ceramic/new bone interface (b). Picro-Fuchsine van Gieson staining, original magnification × 100.

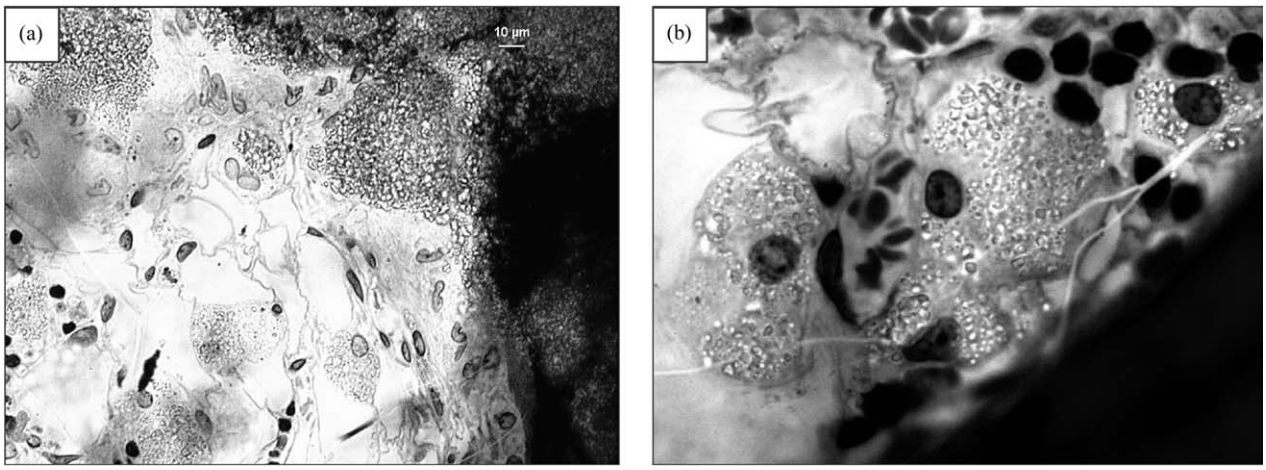


*Figure 6* After 12 weeks of implantation, numerous micro-particles were found in medullar tissue (a) and phagocytosed by macrophages (b) in BCP. Picro-Fuchsine van Gieson staining, original magnification (a) × 100 and (b) × 400.

themselves. If these connections were few and weak, this dissolution could provoke the release of a high quantity of micro-particles.

Each Ca–P ceramic is manufactured with a limited specific sintering temperature, since a misfit temperature could change physico-chemical and biological properties of ceramics. For example, the maximum sintering temperature for β-TCP ceramic is 1160 °C otherwise β-TCP could be transformed into α-TCP, and the temperature for HA is limited to 1300 °C otherwise HA could be transformed into oxyhydroxyapatite.

Sintering establishes the connections between particles since the high temperature melts partially particles and causes their fusion. Preparation of ceramics in pure HA or pure β-TCP requires only one temperature (1300 °C or 1160 °C) for sintering, which gives a good connection between particles. But biphasic ceramics are made of two different components (HA and β-TCP), therefore different sintering temperatures should be necessary. However, to produce these BCP ceramics the lowest temperature (1160 °C) is chosen to avoid transformation of β-TCP in α-TCP giving an incomplete sintering and weak connections between particles. Consequently, the BCP ceramics present a high percentage of micro-porosity which facilitates the dissolution by the biological fluids and the release of HA and β-TCP micro-particles after immersion or implantation [8, 9], the size of released micro-particles corresponding perfectly to the size of ceramic particles used for ceramic preparation (from 0.5 to 5 µm).

During degradation of BCP ceramics, released particles could be either HA or β-TCP particles. Although β-TCP particles can be easily degraded by cells, HA particles are phagocytosed by macrophages but are difficulty degraded and so, cause cell death and tissue inflammation. Thus, these particles *in vivo* could directly provoke the inhibition of cell proliferation or/and the cell damage in/around implantation site. Sometimes, the phagocytosed particles can be removed to the lymph nodes [10].

Our *in vivo* findings showed that released particles were more numerous in tissues around BCP implant than around β-TCP and HA implants, and that osteogenesis was significantly lower in BCP than in the two other ceramics demonstrating that the release of a too large number of undegradable particles can be harmful to tissue integration of BCP ceramics.

In conclusion, these findings highlight a risk of microparticles release from bioceramics which could be at the origin of local inflammation and cell damage and could perhaps modify osteogenesis. It must be paid attention to this problem especially with BCP ceramics because of the sintering difficulties of this bioceramic.

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