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Surface Reactivity of Calcium Phosphate Based Ceramics in a Cell Culture System

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ABSTRACT: Surface reactivity of Calcium Phosphate materials – Hydroxyapatite (HA), Tricalcium Phosphate (β -TCP), Hydroxyapatite-Tricalcium Phosphate (HA-TCP) were elucidated in a cell culture system. MG-63 osteoblast-like cells were seeded onto the ceramic discs to evaluate changes in the cell morphology and functionality with respect to the different substrates.

The dissolution and re-precipitation of calcium phosphate phases on the surface of the discs in the culture medium was found to be prominent on β -TCP when compared with HA. Low calcium (Ca), magnesium (Mg) and alkaline phosphatase (ALP) levels and high phosphorous (P) levels in the medium of β -TCP were observed. This indicated that P must have leached out into the medium from β -TCP and Ca in turn deposited from the medium onto β -TCP resulting in the apatite phase transformation. The low ALP activity in β -TCP medium is however an indication of low osteoblastic activity.

Under the phase contrast microscope, the osteoblast cells around HA material were found to be confluent and viable, while in the vicinity of β -TCP only cellular debris was observed. In the case of HA-TCP, only a few viable cells surrounded the material amidst the debris. Scanning electron microscopy revealed numerous cells on the surface of HA showing different cell behaviour like anchorage, attachment, adhesion and spreading in the early time period as the surface was only slightly disturbed with re-crystallisation. But with time the entire surface of HA had changed due to precipitation and re-crystallization which did not support cell behaviour while the cells surrounding the material showed normal growth. On the contrary, cells were scarcely observed on the entirely changed surface of β -TCP and HA-TCP even from the earlier days of the

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culture and the morphology of cells surrounding the material too started changing.

These results establish that HA promoted the activity of osteoblast cells. HA surface remained unaltered for some time, while the surface of β -TCP underwent dissolution of surface ions and resulted in the re-crystallization of apatite over the surface. The resulting changes in the surrounding milieu of β -TCP with high phosphate and low Ca levels probably was responsible for the death of the cells.

KEY WORDS: *in vitro*, HA, β -TCP, HA-TCP, dissolution, re-crystallization, osteoblast cells.

INTRODUCTION

Calcium phosphate based hydroxyapatite (HA) and Tricalcium Phosphate (β -TCP) are major components of materials used as bone substitutes for almost the past three decades. The differences in the chemistry [1,2], the crystallographic structure [3], and the porosity [4,5], possibly govern the bioactivity of the materials, which in turn favoured the formation of new bone [6].

Cell culture models are extensively investigated in the elucidation of cellular attachment and adhesion to Calcium Phosphate materials [7–12] for orthopaedic and dental applications. The cell density, the cell morphology and the viability differ according to the surface reactivity and physico-chemical nature of the substrate. Knowledge of the behaviour (anchorage, attachment, adhesion, spreading) and growth of specific bone forming cells on a material in a well controlled environment as in cell culture could help in predicting *in vivo* cell behaviour. The formation and deposition of bone directly onto the implant requires a surface that is not only non-toxic but also one that promotes the activity of osteoblasts. In short, the adhesion and growth of cells on ceramics are considered to be regulated by the time dependant variation of the surface structure.

Here, we have adopted a cell culture model to study the surface behaviour of the in-house synthesized ceramic discs seeded with MG-63 osteoblast-like cells under physiological conditions and to evaluate their morphology and functionality.

MATERIALS AND METHODS

Materials

The materials used were in-house synthesized sintered disc shaped calcium phosphate based ceramics – *viz* HA, β -TCP and a

composite of HA and β -Tricalcium Phosphate (HA-TCP) seeded with osteoblast-like cells (MG-63).

Preparation of Materials

Hydroxyapatite and β -TCP were prepared by the wet chemical method followed by the freeze drying technique [13] starting from calcium nitrate and ammonium dihydrogen phosphate solution. The powder was pressed in the form of a 12 mm diameter and 1.5 mm thick disc and sintered at 1200°C for 2 h. The sintered density for HA was approximately 98%, while the β -TCP was only 70% dense. The phase analysis was carried out by XRD and the Ca/P ratio was determined by chemical analysis (data not shown). The HA-TCP composite was prepared by mixing equal weights of HA and β -TCP and pressed as in the case of HA and β -TCP samples. The sintering temperature was 1200°C for 2 h. All materials were cleaned with soap solution and acetone in an ultrasonicator, air dried and steam sterilised.

Cell-culture

MG-63 osteoblast-like cells (supplied by National Centre for Cell Sciences – NCCS, Pune, India is a well characterised cell line, originally isolated from osteosarcoma) were used for these experiments.

The cells (plating density of 1×10^3 cells/cm²) were seeded onto HA, β -TCP and HA-TCP discs which were placed in 24-well tissue culture plates (Nunc, USA) in Minimum Essential Media (Hi-Media, India) containing 10% heat inactivated calf serum and 1% penicillin-streptomycin. Culture was maintained in 5% CO₂ atmosphere at 37°C and 100% humidity for 144 h, without any change of media. The experiment was stopped at 144 h, since it was observed that the cell growth would be affected, if fresh nutrients are not supplied further.

As control – (1) HA and β -TCP discs were placed in cell free medium for 2 h only to detect the changes, if any, on the surface of the discs and (2) cells alone in the medium was maintained for 144 h for biochemical analysis.

Cell Morphology

To evaluate changes in cell morphology, with respect to different substrates, cells around the material were examined by Phase Contrast Microscopy (Leica, MPS-32-PCM) and (2) cells on materials were evaluated by Scanning Electron Microscope (SEM). For SEM, the samples were rinsed three times in phosphate buffered saline (PBS) and fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide in

phosphate buffer. Thereafter, the discs were rinsed with phosphate buffer, sequentially dehydrated in 50, 70, 90 and 100 % methanol and dried in a Critical Point Dryer (Hitachi- HCP-2). A thin layer of gold was sputter coated onto samples prior to examination in SEM (Hitachi-S2400).

Biochemical Analysis

The media alone were removed from the test and control wells (triplicates) after 144 h of incubation and stored in aliquots at 4°C for biochemical analysis. The media were tested for Calcium (Ca) and Magnesium (Mg) concentration using atomic absorption spectrophotometry (Hitachi-220). Phosphorous (P) concentration was measured spectrophotometrically using the Modified Method Kit for Phosphorous (Qualigens-Glaxo) and absorbance was measured at 680 nm and expressed as ppm (parts per million). Alkaline Phosphatase activity was determined using a kit based on Kind and Kings' method (Qualigens-Glaxo) and absorbance was measured at 510 nm and expressed as KAU (King Angstrom Unit).

RESULTS

Morphological Evaluation

Phase Contrast Microscopic Evaluation

Morphologically the osteoblast-like cells around HA discs were spindle shaped and showed no toxicity by 144 h and the cells around HA materials were observed to be of the same morphology as cells alone (Figure 1A). But cells in the vicinity of β -TCP were gradually showing changed morphology and finally by 144 h cell degeneration was observed (Figure 1B). In the case of HA-TCP, only a few cells in the medium showed normal morphology similar to cells alone, while the rest were degenerated in the culture by 144 h (Figure 1C).

Scanning Electron Microscope Evaluation

(a) HA Sample

Isolated areas over the surface of the dense HA material underwent gradual microstructural changes in the medium alone by 24 h (Figure 2A). In areas where there were no surface changes, normal adhesion, spreading and proliferation were noted (Figure 2B). But in the cell culture medium prolonged for 144 h the entire surface re-precipitated. So intact grains and grain boundaries in these areas were disrupted completely exhibiting blanket coverage of precipitates of

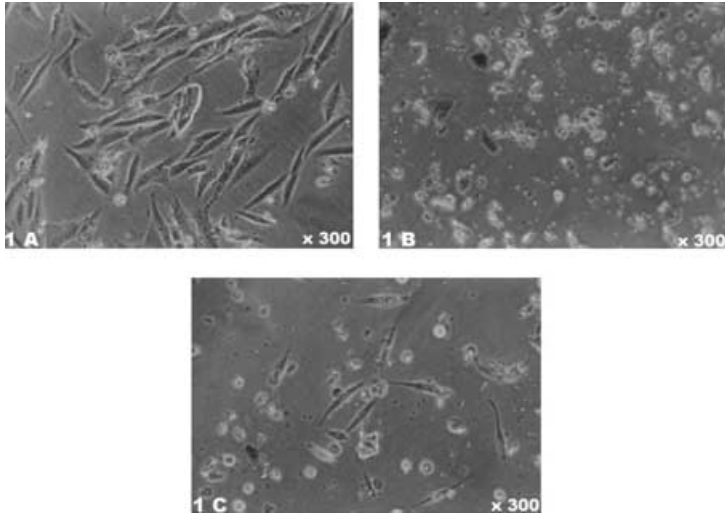


Figure 1. Phase Contrast Micrographs of MG-63 osteoblast-like cells in the culture medium surrounding – (A) HA as normal cells; (B) β -TCP as degenerated cells and (C) HA-TCP as normal cells with degenerated cells . Magnification $\times 300$.

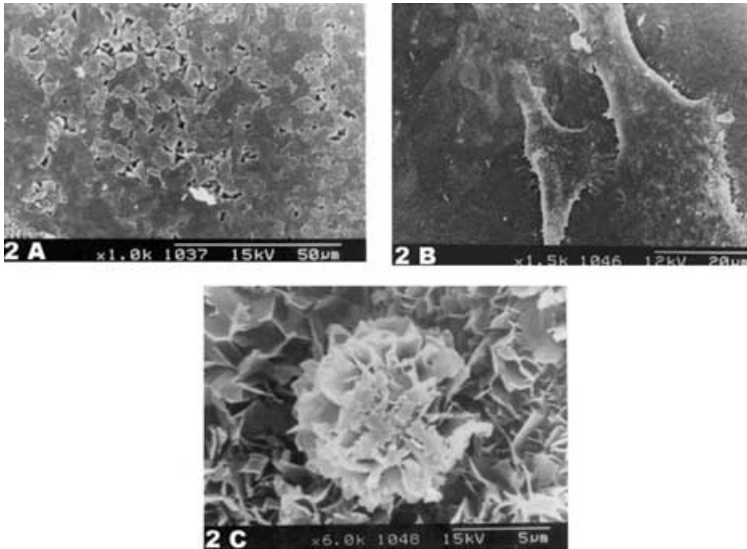


Figure 2. Scanning Electron Micrographs of the surface topography of HA disc – (A) in the cell free culture medium for 24 h, showing patches of slightly disrupted areas; (B) in the cell (MG-63) culture medium for 24 h with adhered cells and (C) in the cell (MG-63) culture medium for 144 h showing the re-precipitated surface, magnified as calcium phosphate plate-like crystals with hardly any normal cells.

calcium phosphate plate-like crystals (Figure 2C), which did not support any cell attachment due to the changed topography of HA.

(b) β -TCP

In the case of β -TCP, the microstructure is different from HA as the density was lower. Here, the surface was affected to a greater extent in the medium when compared to HA. The creation of new needle-like crystallites from in between the pores of the grains of β -TCP exhibited a flower-like pattern on the sample surface (Figure 3A, B). The new phase may be due to the re-crystallised apatite and very scarce cells were found adhered on the surface. The attached cell failed to spread on the re-crystallised topography of β -TCP (Figure 3C, D).

(c) HA-TCP

The re-crystallised surface topography of HA-TCP in the medium was intermediate between that seen on HA and β -TCP. The portion of the cells on re-crystallised free areas of HA-TCP adhered and spread flat

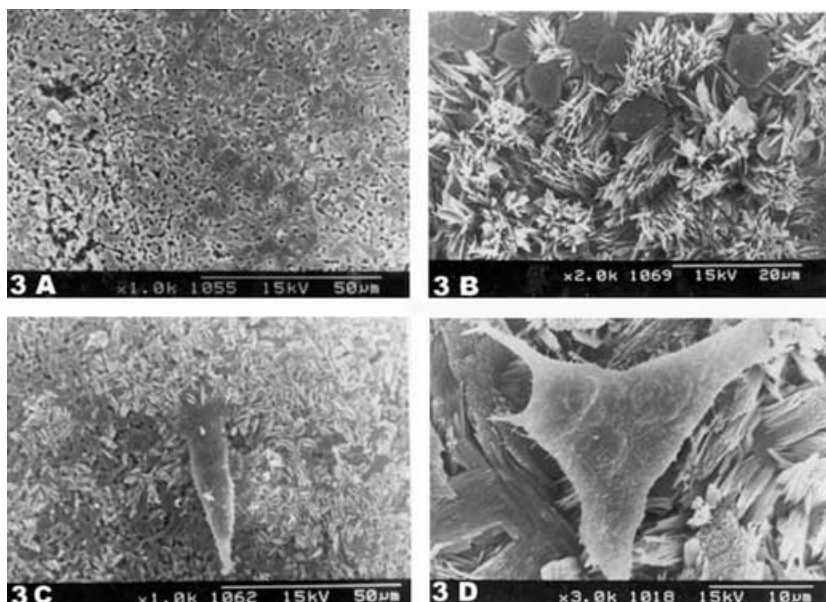


Figure 3. Scanning Electron Micrographs of the surface topography of β -TCP disc in the culture medium – (A) alone without cells for 24 h, showing the shaken grain arrangement and (B) with MG-63 cells for 24 h, exhibiting a cell spotted amidst the needle-like crystallites in between the shaken grains; (C) for 144 h showing a magnified image of the new topography with sprouts of needle-like crystallites and (D) with a magnified image of a cell lying embedded in between the sprouts of needle-like crystallites.

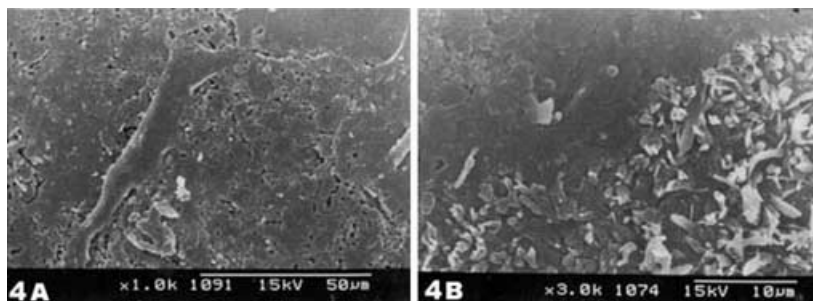


Figure 4. Scanning Electron Micrographs of the surface topography of hydroxyapatite-Tricalcium phosphate (HA-TCP) disc in the cell culture medium (A) after 24 h, showing a partially re-crystallised disturbed surface with cells adhered on the surface and (B) after 144 h, showing a magnified image of the re-crystallised area of plate-like and needle-like crystallites.

while those on the re-crystallised areas failed to spread out (Figure 4A). Grains and grain boundaries were clearly disrupted with re-crystallization areas of plate-like and needle-like intermediate crystallites of different calcium phosphate phases (Figure 4B).

(d) Cells Seeded on the Coverslip (Control)

Confluent normal morphology of cells were observed on the coverslip until 144 h.

Biochemical Analysis

The levels of P, Ca, and Mg in the medium of HA were comparable with control and ALP concentration indicated functional activity of the cells. Low Ca and Mg levels and high P concentrations in the medium surrounding β -TCP, showed that Ca has been precipitated as apatite phase resulting in the phase transformation of the surface of β -TCP. Low ALP concentration indicated less functional activity of the cells in the medium containing β -TCP when compared with the other materials. Ca, P, Mg and ALP levels observed in the medium of HA-TCP were intermediate when compared with the media of HA and β -TCP (Table 1).

DISCUSSION

Surface properties of biomaterials play a critical role in the establishment of cell-material interface. With respect to bioactive calcium phosphate ceramics, it is observed that the chemical composition,

Table 1. The biochemical analysis of Calcium (Ca), Phosphorous (P), Magnesium (Mg) and Alkaline Phosphatase (ALP) in the cell culture medium of hydroxyapatite (HA), Tricalcium phosphate (β -TCP) and HA-TCP composite, after 144 h of incubation.

	P (ppm)	Ca (ppm)	Mg (ppm)	AIP (KAU)
HAP and cells in medium	16.2 \pm 1.5	59.1 \pm 3.3	15.3 \pm 2.4	0.77 \pm 0.27
β -TCP and cells in medium	22.1 \pm 3.1	32.6 \pm 4.9	9.7 \pm 1.5	0.33 \pm 0.09
HA-TCP and cells in medium	15 \pm 2.6	55.8 \pm 7.2	14.7 \pm 1.8	0.62 \pm 0.08
Cells in the medium alone	17.5 \pm 2.0	60.9 \pm 6.4	14.2 \pm 25	0.64 \pm 0.10

physical characteristics and crystal structure play an important role in the cell response [14,15]. *In vitro* cytocompatibility studies are increasingly concerned with the influence of surface topography, surface change and consecutive adsorption of proteins on cell attachment and its proliferation [16]. In this study, we have investigated the morphological and functional response of osteoblast-like cells to HA, β -TCP and a composition of HA-TCP mixture in the disc form. Calcium Phosphate ceramics with Ca/P ratios of either 1.67 or 1.5 have been reported to be biocompatible [1,17]. However, the factors concerning the bioresorption/biodegradation of calcium phosphate ceramics have not been completely elucidated [18].

Dense HA (Ca/P 1.67) being a bioactive ceramic, the solubility product is low, implying a low rate of dissolution. So there was only a gradual microstructural change of the HA surface by 24 h in the medium alone (Figure 2A). A favourable surface for cell attachment, adhesion and spreading (Figure 2B) was evident by 24 h, promoting ALP activity and cell proliferation, thereby displaying the cytocompatibility of the ceramic to osteoblasts. On HA after 24 h in cell culture, flattened morphology of osteoblast cells with extended long cytoplasmic processes over the surface and into micropores, were reported by Hing et al. [19] and cell proliferation is considered to be related to ALP activity [20]. High surface free energy and presence of hydroxyl groups on HA substrate has also promoted cell adhesion on its surface as described by others [21–23]. But by 144 h in the cell culture medium, the entire surface of HA re-precipitated, which did not support cell attachment and adhesion (Figure 2C). The substances leached from HA can cause dramatic changes in local pH which in turn affects protein adsorption on HA and this may affect subsequent cellular responses towards the material [24]. Even though we did not find any cytotoxic response of osteoblasts on our HA discs, it is reported that fine particles of HA can cause cell damage *in vitro*. This toxicity evidenced by membrane damage is due to direct

contact between cells and particles and is largely independent of the chemical nature of the particle [25]. However the cells around the material showed normal cell growth and proliferation (Figure 1A) where P, Ca, Mg levels were low with high ALP levels which indicated osteoblastic activity (Table 2). Costa and Fernandes [26] has reported a decrease in the levels of calcium and phosphorous with HA samples which represent the ongoing process in the mineralisation process – a calcium phosphate deposition in culture. Anselme et al. [27] related this modification of calcium and phosphorous concentration in cell culture medium to a dissolution phenomenon of materials as no such variation is seen in control wells. This fluctuating chemical (Ca, P, Mg, ALP) environment in the culture medium has in turn favoured the growth of the osteoblasts around the material for biomineralization. The extracellular pools of Ca, P, and Mg are in equilibrium with much larger intracellular pools. The ion levels of the culture medium are adjusted in accordance with the solubility kinetics of different materials via removal of eluted ions by metabolism or circulatory processes. The role of ion levels in cell adhesion and cellular metabolic activity should be clarified, since particularly divalent cations including Mg^{2+} , are known to be active in cell adhesion mechanisms [28,29]. So besides Ca and Phosphate contents, we have measured the concentration of Mg in the culture medium. ALP appears to play a crucial role in the initiation of matrix mineralisation providing localised enrichment of phosphorous and after that the expression of the enzyme is down regulated [30–32]. The surface roughness, porosity and particle size of biomaterials, indeed influence the activity of osteoblast cells [25,33,34] and the stability of HA is less if the surrounding media is sufficiently acidic [35].

Porous β -TCP with a Ca/P ratio of 1.5 in porous form was used in this study. It is an unstable ceramic where the solubility product is high, leading to high rate of dissolution. The higher porosity of β -TCP enhances the rate of dissolution [36,37]. As β -TCP dissolved, the solubility of β -TCP surface approached the solubility of HA and decreased the pH of the solution which further increased the solubility of β -TCP and enhanced the dissolution. The presence of micropores in the sintered material increased the surface area and ensured contact with the medium and increased the rate of dissolution. The above possibilities were confirmed by the surface morphology of β -TCP as observed with SEM (Figure 3A–D) which showed that most of the surface of β -TCP has undergone dissolution and re-precipitation, leading to needle-like apatite crystallites.

The variation in solubility of calcium phosphates could in turn be a cause in changes of cell adhesion. This is because when the surface of

β -TCP changes, the altered surface characteristics of the material affect protein adsorption and subsequent protein mediated cell function [38–40]. Cells were scarcely distributed on the re-precipitated surface. This could be the reason why cell contact and adhesion was poor on β -TCP, very apparent by the round appearance of the cell (Figure 3C, D) that failed to attach well and spread on the new surface.

The change in morphology of osteoblast-like cells surrounding β -TCP in the cell culture medium was evident by 96 h of incubation and by 144 h resulted in death of the cells (Figure 1B). In cells, calcium ions has the primary function of signal transduction and acts as a secondary messenger and phosphorous ions helps in energy production and supply to the cell while magnesium ions are known to inhibit the formation of apatites and stabilise the structure of whitlockite. The dissolution/re-crystallisation at the ceramic surface and the exchange of ions between the cell culture medium resulted in high phosphorous and low calcium, magnesium and ALP levels which inhibited the growth of cells that lead to cell death [41–45]. So it is quite obvious that cell death has resulted in low ALP (Table 1).

Thus, the more soluble the material, the greater the inhibition of ALP activity, which involves the calcium ions through an increase in cytosolic concentration and low magnesium levels promotes its transformation to an apatite phase. A rise in calcium concentration which indicates that dissolution occurs, until supersaturation is achieved followed by calcium uptake indicating re-precipitation reaction in early hours was interpreted as a phenomenon of a cell-mediated mechanism, which could mimic one of the steps *in vivo bone* growth [46]. But Ansleme et al. [27] confirmed this modification of calcium and phosphorous concentration in the culture medium as rather a dissolution phenomenon which is a physicochemical reaction rather than a biochemical process, because it occurs even without cells.

However in our study, physicochemical transformations of the β -TCP surface appeared to prevent osteoblast-like cells to attach and grow *in vitro*. This inhibition is provoked either by a direct effect on cells or by a local modification of pH [47,48]. Low surface free energy of β -TCP and lack of hydroxyl ions [49] could also contribute to non-adherence of cells in the acidic pH medium of our experiments. The acid environment can cause partial dissolution and precipitation of macrocrystals of calcium phosphate materials and bring a change in the level of phosphorous and calcium ions in the microenvironment. Continuous leaching of ions – phosphorous and calcium from bioactive surface and the formation of a fresh apatite layer can prevent the availability of fibronectin or other adhesion proteins to cells [50]. Intensive solubility and high reactivity of

TCP may damage adherent cells and lower cell adhesiveness. Completion of the dissolution-precipitation phenomenon gradually may lead to a stable phase to support cell growth with time. Thus the stability of the surface crystal structure is considered to be the dominant factor for control of cell adhesion [37].

However a combination of HA and TCP (50% by weight) gave intermediate results with respect to morphological (Figure 6A) and biochemical results (Table 1). It was found that the dissolved calcium and phosphorous in the medium were removed from solution by re-deposition on the immersed HA-TCP ceramics, which suggested that the stability of the surface was closely related to both the reactions of association and dissociation of calcium and phosphorous in tissue culture medium [37]. Change in morphology of osteoblast cells in medium surrounding HA-TCP was evident by 96 h and after 144 h resulted in cellular debris supported with very few viable cells (Figure 1C) indicated by the ALP activity levels. The HA phase of the HA-TCP supported by the phosphorous, calcium and magnesium levels in the medium (Table 1) is intermediate between HA and TCP medium levels and favoured the growth of few cells among the cellular debris subjected to the effect of the TCP phase. Cell anchorage and attachment seen on HA-TCP must be due to the combination effect of TCP and HA (Figure 4A, B).

It is conceivable that immersion of ceramics in culture medium initiates the surface phase transformation [51,52]. It has been observed that the apatite phase on surface of ceramic can be formed by a chemical reaction of the ceramic with the ions in the biological fluid resulting in dissolution and re-precipitation [15,35,36,44,46–48,53–56] and the modified cell culture media can in turn influence the growth and behaviour of cells.

Differences in osteoblast material interaction seen in different ceramic materials may be due to the surface aspects of the materials which depend on topography, chemistry or surface energy [16]. Furthermore, differences in morphological and functional responses observed during osteoblast substrate interaction may be due to the surface reactivity of the material [9]. Stability of surface crystal structure is a dominant feature for the control of cell adhesion [37]. Topography as well as surface roughness also influences cellular response [50]. The surface reaction include dissolution, precipitation and ion exchange accompanied by absorption and incorporation of biological molecule [57]. Biodegradation or dissolution is mainly governed by the crystal structure, neck geometry and crystallinity of the material [58]. The dissolution of material may be responsible for the

inhibition of cell attachment and growth of cells [27,19]. Cell material interaction studies between anchorage dependent cells and their substrate can also affect the functional responses of the cells as substrate characteristics can influence attachment and spreading through development of focal contacts and extracellular contacts.

CONCLUSION

The difference in cell behaviour observed on calcium phosphate ceramics can be correlated with the surface chemistry of materials. As HA has relatively a stable phase, the cells spread well in 24 h. But with increased time (144 h), the change in surface morphology led to non-adherence of cells. But the culture medium surrounding HA was not affected much and hence there was normal cell growth. In the case of TCP surface, cell growth and spreading was less than HA. Microenvironment was affected implying cell death, which may be due to leaching of ions from the substratum changing the pH of the medium. Inability of cells to attain a foothold on the re-crystallised surface, resulted in an unstable interface and prevented proper anchorage; attachment and adhesion in an acidic medium with high P and low Ca and Mg levels leading to cell death. HA-TCP behaved in an intermediate fashion.

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