Surface topography of hydroxyapatite affects ROS17/2.8 cells response

A topografia de superfície da hidroxiapatita afeta a resposta de células ROS17/2.8

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ABSTRACT: Hydroxyapatite (HA) has been used in orthopedic, dental, and maxillofacial surgery as a bone substitute. The aim of this investigation was to study the effect of surface topography produced by the presence of microporosity on cell response, evaluating: cell attachment, cell morphology, cell proliferation, total protein content, and alkaline phosphatase (ALP) activity. HA discs with different percentages of microporosity (< 5%, 15%, and 30%) were confected by means of the combination of uniaxial powder pressing and different sintering conditions. ROS17/2.8 cells were cultured on HA discs. For the evaluation of attachment, cells were cultured for two hours. Cell morphology was evaluated after seven days. After seven and fourteen days, cell proliferation, total protein content, and ALP activity were measured. Data were compared by means of ANOVA and Duncan's multiple range test, when appropriate. Cell attachment (p = 0.11) and total protein content (p = 0.31) were not affected by surface topography. Proliferation after 7 and 14 days (p = 0.0007 and p = 0.003, respectively), and ALP activity (p = 0.0007) were both significantly decreased by the most irregular surface (HA30). These results suggest that initial cell events were not affected by surface topography, while surfaces with more regular topography, as those present in HA with 15% or less of microporosity, favored intermediary and final events such as cell proliferation and ALP activity.
UNITERMS: Hydroxyapatite; Porosity; Surface topography; ROS17/2.8 cells.

RESUMO: A hidroxiapatita (HA) tem sido utilizada como revestimento de implantes e para substituição de tecido ósseo. O objetivo deste estudo foi avaliar o efeito da topografia de superfície da HA, resultante da presença de microporosidade, sobre a adesão, a morfologia e proliferação celulares, a medida de proteína total e a atividade de fosfatase alcalina. Discos de HA com diferentes porcentagens de microporosidade (< 5%, 15% e 30%) foram fabricados por uma combinação das técnicas de pressão uniaxial e sinterização. Células ROS17/2.8 foram cultivadas sobre os discos de HA. Para a adesão, as células foram cultivadas por duas horas. A morfologia foi avaliada após sete dias. A proliferação, medida de proteína total e atividade de ALP foram avaliadas após sete e quatorze dias. Os dados foram comparados por ANOVA e teste de Duncan quando apropriado. A adesão (p = 0,11) e a medida de proteína total (p = 0,31) não foram afetadas pela topografia de superfície. A proliferação após sete e quatorze dias (p = 0,0007 e p = 0,003, respectivamente), e a atividade de ALP (p = 0,0007) foram significantemente menores na superfície irregular (HA30). Esses resultados sugerem que eventos iniciais não são afetados pela topografia, enquanto superfícies com topografias mais regulares (microporosidade de 15% ou menos) favoreceram eventos intermediários e finais, como proliferação e atividade de ALP.

UNITERMOS: Hidroxiapatita; Porosidade; Topografia de superfície; Células ROS17/2.8.

INTRODUCTION

Hydroxyapatite (HA) has received considerable attention as a material for implants and bone augmentation procedures, since it bonds chemically directly to bone after implantation[6]. Synthetic HA for bone substitution can be produced by a combination of a powder pressing technique and sintering. Powder pressing techniques allow the obtainment of "green bodies", which are then sintered at high temperatures to produce the fusion of powder particles. Changes in the powder compaction and sintering conditions can result in a variety of chemical and porosity characteristics of HA[16,20]. Porosity can be classified, according to the diameter of pores, in macroporosity (> 10 mm) and microporosity (< 10 mm). It has been suggested that these structural modifications of HA influence cellular activity[16,20].

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Dziedzic *et al.*[8] (1996) reported that HA samples with different microporosities result in different bone-HA interfaces after implantation in rats. Preliminary results from *in vitro* studies using a primary culture of rat bone marrow cells and a rat osteosarcoma cell line have demonstrated that an increase in micropore percentage negatively influences HA biocompatibility[18,19].

Variations of micropore percentage result in different surface topographies[3,6] and authors have agreed that topography must be considered in terms of amplitude and organization[13]. Anselme *et al.*[1] (1997) observed a lower proliferation of human osteoblasts cultured on less organized Ti surfaces.

Considering that the surface characteristics of HA necessary for optimal osseointegration are still not fully understood, the aim of this study was to carry out an *in vitro* evaluation of the effect of varying the surface topography of HA on the response of osteoblast-like cells. For this, HA samples with less than 5%, 15%, and 30% of microporosity were evaluated using osteoblast-like cells derived from rat osteosarcoma (ROS17/2.8). These cells were used because they express numerous osteoblastic characteristics, including high levels of alkaline phosphatase (ALP) activity, synthesis of type I collagen, and osteocalcin[10]. The following parameters were evaluated: cell attachment, cell morphology, cell proliferation, total protein content, and ALP activity.

MATERIAL AND METHODS
Preparation of HA samples

HA discs (with the diameter of 12 mm and height of 2 mm) with different degrees of microporosity (< 10 mm), as shown in Table 1, were confected by means of a combination of uniaxial powder pressing and different sintering conditions. This procedure allows the confection of HA with identical chemical composition and crystallinity but with different porosities[20,21]. The discs were made of a high purity and crystalline HA powder

TABLE 1 - Axial load, sintering condition, and microporosity percentage of the HA samples.

Sample	Load (ton)	Sintering (ºC/hour)	Microporosity percentage
HA5	6	1,250/3	< 5
HA15	2	1,225/3	15
HA30	2	1,150/3	30

(Plasma Biotal, Tideswell, UK). The HA discs utilized in this study were divided in three groups: HA5, with less than 5% of microporosity, HA15, with 15% of microporosity, and HA30, with 30% of microporosity, as calculated from the actual density compared with the theoretical density of HA. The HA surface was evaluated by scanning electron microscopy (SEM - JSM-5410, Jeol, MA, USA). Surface roughness (Ra) was measured using a surface profilometer (Prazis Rug-03, São Paulo, Brazil), and an average was calculated for each disc, taking into account three measurements. The average Ra of each microporosity group was determined based on measurements of three discs (n = 3). All discs were cleaned in an ultrasonic bath and autoclaved before utilization in the cell culture experiments.

Cell culture

ROS17/2.8 cells were cultured in HAM'S F12 (Gibco - Life Technologies, Grand Island, NY, USA) supplemented with 8% fetal bovine serum, 50 mg/ml penicillin-streptomycin (Gibco), glutamine 5.4×10^{-7} M (Gibco), and 0.3 mg/ml amphotericin B (Gibco). Cells were cultured at a concentration of 0.825×10^4 cells/well on HA discs in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA). During the culture period, cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every forty-eight hours. In each plate, empty wells were only used as a control of culture conditions.

Cell attachment

For the evaluation of attachment, cells were cultured for two hours on HA discs (n = 5, for each microporosity). The culture medium was removed, and the wells were washed three times with PBS at 37ºC to eliminate unattached cells. The adherent cells were then enzymatically released (1 mM EDTA + 0.25% trypsin - Gibco) from the HA discs, and counted using a hemacytometer. Cell attachment was expressed as a percentage of the initial number of cells.

Cell morphology

For the evaluation of morphology, cells were cultured for seven days on HA discs and processed for SEM. First, cells were fixed with 1.5% glutaraldehyde (Sigma, St. Louis, MO, USA) buffered in 0.1 M sodium cacodylate (Sigma), and stained in 1% osmium tetroxide (Sigma). Then, cells were dehydrated through a graded series of alcohol, fol-

lowed by critical point drying at 32°C and 1,300 psi for one hour. After that, samples were sputter-coated with gold (10 nm) before examination under a SEM.

Cell proliferation

For the evaluation of proliferation, cells were cultured for seven and fourteen days on HA discs (n = 5 for each day and each microporosity). The cells were released and counted as described in cell attachment.

Total protein content

Total protein content after seven and fourteen days in culture on HA discs (n = 5 for each microporosity) was calculated according to the modified Lowry *et al.*[14] (1951) method. The culture medium was removed, the wells were washed three times with PBS at 37°C and were filled with 2 ml of 0.1% sodium lauryl sulfate (Sigma). After thirty minutes, 1 ml of the solution from each well was mixed with 1 ml of Lowry solution (Sigma) and left for twenty minutes at room temperature. After this period, it was added to 0.5 ml of the solution of phenol reagent of Folin and Ciocalteau (Sigma). It stood for thirty minutes at room temperature to allow color development, and absorbance was then spectrophotometrically measured (CE3021 - Cecil, Cambridge, UK) at 680 nm. The total protein content was calculated from a standard curve of bovine albumin and expressed as µg/ml. These data were normalized by the number of cells counted after seven and fourteen days, respectively.

ALP activity

ALP activity was assayed as the release of r-nitrophenol from r-nitrophenyl, using a commercial kit (Sigma), and the specific activity was calculated. From the same solutions used for calculating total protein content, 0.1 ml aliquots were assayed for measuring ALP activity. Absorbance was spectrophotometrically measured at 410 nm, and ALP activity was calculated from a standard curve obtained from different solutions of r-nitrophenol and sodium hydroxide 0.02 N. Results were calculated as µmol r-nitrophenol/hour, and data were expressed as ALP activity normalized by the total protein content and by the number of cells counted after seven and fourteen days, respectively.

Statistical analysis

Data were submitted to analysis of variance (ANOVA) and Duncan's multiple range test, when appropriate. Differences at $p \leq 0.05$ were considered statistically significant.

RESULTS

HA samples

Ra measurements presented the following results: HA5 = 0.38 µm, HA15 = 0.48 µm and HA30 = 0.49 µm. ANOVA revealed that Ra was not affected by the manufacturing conditions of HA (F = 2.79; gl = 2; p = 0.14). It was possible to observe, from SEM micrographs, that the amount of microporosity produced surfaces with different topographies (Figure 1). Thus, HA5 and HA15 presented more regular surfaces while HA30 presented a very irregular surface topography (Figure 1).

Cell attachment

Cell attachment was not affected by HA surface topography (ANOVA: gl = 2; F = 2.65; p = 0.11). Data are presented in Table 2.

Cell morphology

HA5 e HA15 presented more cells than HA30. Cells observed on HA5 and HA15 presented a polygonal morphology with dorsal ruffles and a large number of filaments connecting distant cells (Figures 2A and 2B). On HA30 discs, cells did not cover all surfaces. In addition, the formation of a monolayer was observed, with cells predominantly presenting a more flattened morphology, showing almost no dorsal ruffles and filaments (Figure 2C).

Cell proliferation

The number of cells was affected by surface topography after both seven and fourteen days (ANOVA: gl = 2; F = 14.31; p = 0.0007 e gl = 2; F = 9.61; p = 0.0032, respectively) in the following order: HA5 = HA15 > HA30. Data are presented in Figure 3.

Total protein content

Total protein content after seven and fourteen days was not affected by both surface topography (ANOVA: gl = 2; F = 1.21; p = 0.31) and period of culture (ANOVA: gl = 1; F = 1.58; p = 0.22). Data are presented in Table 3.
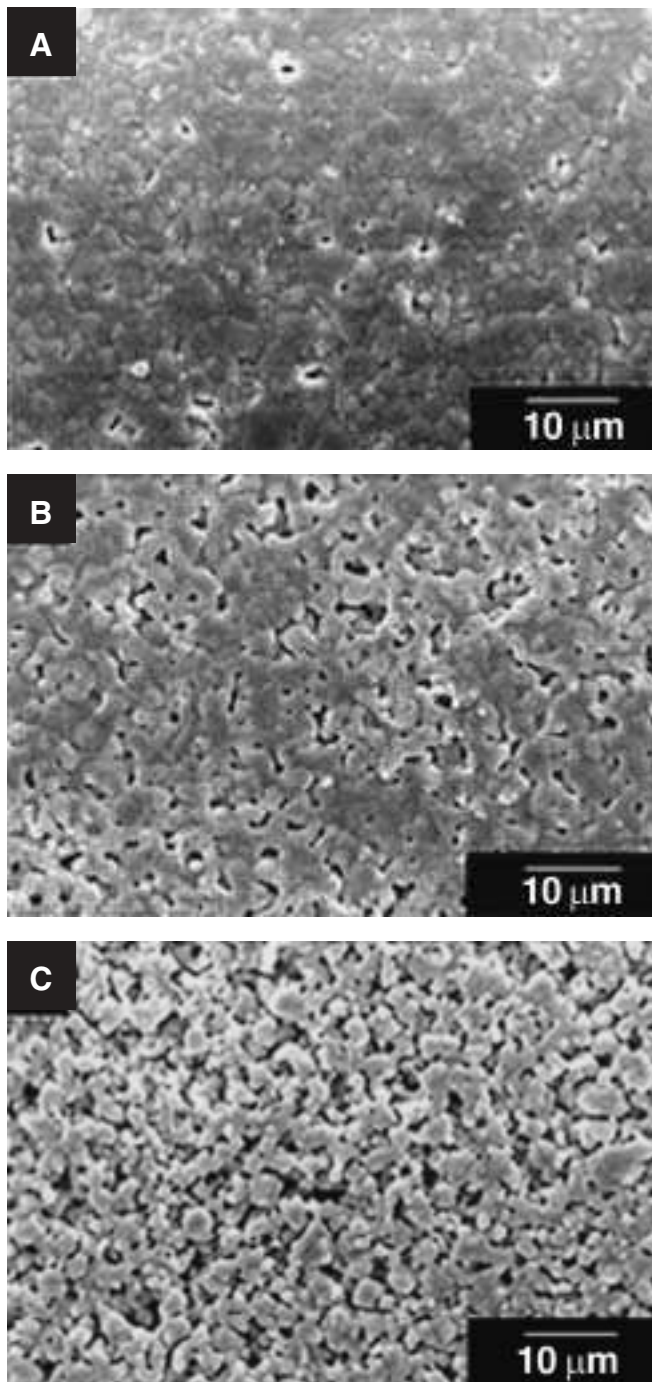
FIGURE 1 - Scanning electron microscopy (SEM) of the hydroxyapatite (HA) surfaces. (A) HA5 (5% of microporosity); (B) HA15 (15% of microporosity) and (C) HA30 (30% of microporosity).

ALP activity

ALP activity after seven and fourteen days was affected by surface topography (ANOVA: gl = 2; F = 9.69; p = 0.0007) in the following order:

TABLE 2 - Attachment of ROS17/2.8 cells, expressed as a percentage of the initial number of cells, on hydroxyapatite (HA) discs after two hours. Data are presented as mean ± standard deviation (n = 5).

Cell attachment	HA5	HA15	HA30
% of the initial number of cells	6.65 ± 4.15	7.4 ± 3.99	3.7 ± 1.38

HA5 = less than 5% of microporosity; HA15 = 15% of microporosity; HA30 = 30% of microporosity.

HA5 = HA15 > HA30. However, it was not affected by the period of culture (ANOVA: gl = 1; F = 0.21; p = 0.65). Data are presented in Table 4.

DISCUSSION

Early *in vitro* studies on the interaction between osteoblasts and biomaterials were essentially concerned with the effect of diverse range materials on cell response, without paying attention to the influence of surface characteristics[23]. However, it is now understood that the superficial properties of biomaterials play a critical role in the establishment of cell-biomaterial interfaces and that manufacturing conditions affect the superficial properties of HA[1,20].

The present results revealed that different combinations of powder pressing and sintering produced HA samples with different microporosities and, consequently, different superficial appearances. Despite these differences, Ra, a factor known to influence cell response, was not significantly affected by the manufacturing process of the HA samples. Hence, any differences in cell response observed in this study would be a consequence of differences in the microporosity of HA samples.

Osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events, involving cell attachment and cell proliferation, followed by the expression of osteoblastic phenotype[6]. This study evaluated the response of ROS 17/2.8 osteoblast-like cells, originated from rat osteosarcoma, cultured on HA discs with different surface topographies. The present results revealed that all HA samples, regardless of surface topography, are biocompatible, considering that they allowed cell attachment, proliferation, and expression of markers of osteoblastic differentiation.

The effect of surface characteristics on cells may be the result of the surface characteristics themselves or the result of the reaction that occurs as the surface is conditioned by the medium and
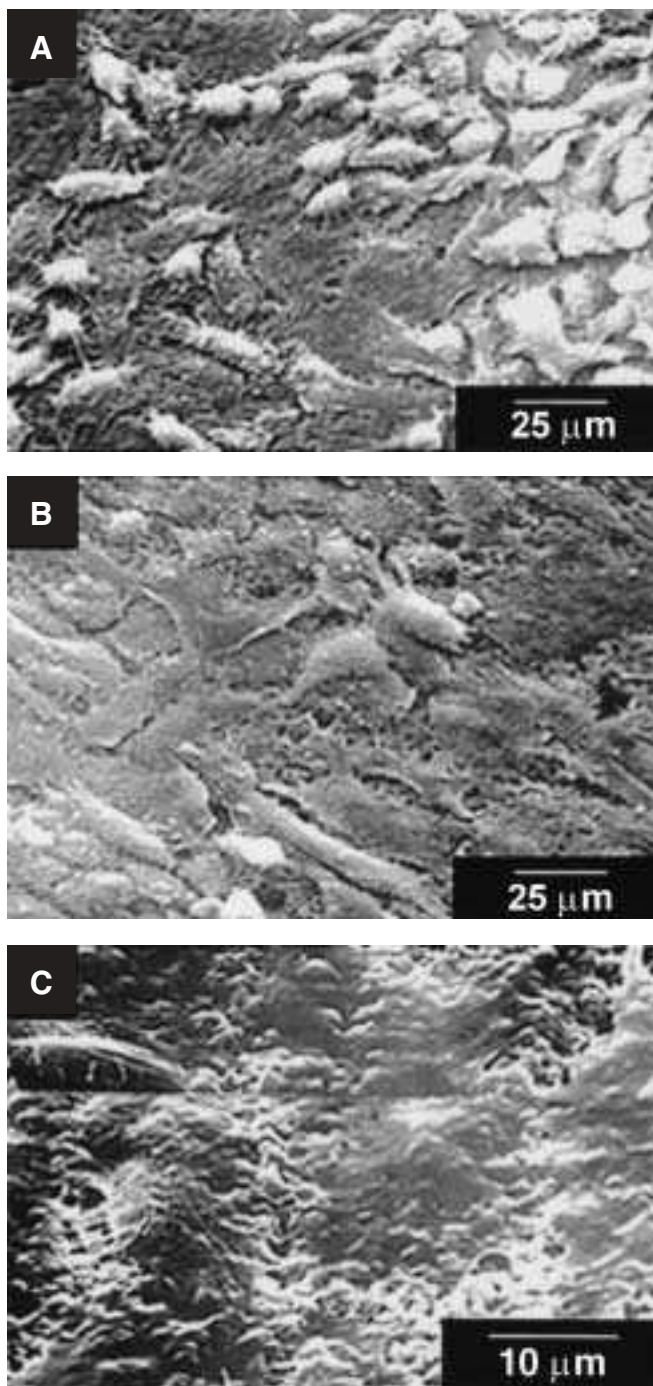
FIGURE 2 - Morphology of ROS 17/2.8 cells after seven days in culture on hydroxyapatite (HA) discs, evaluated under scanning electron microscopy (SEM). (A) HA with less than 5% of microporosity (HA5); (B) HA with 15% of microporosity (HA15), and (C) HA with 30% of microporosity (HA30).
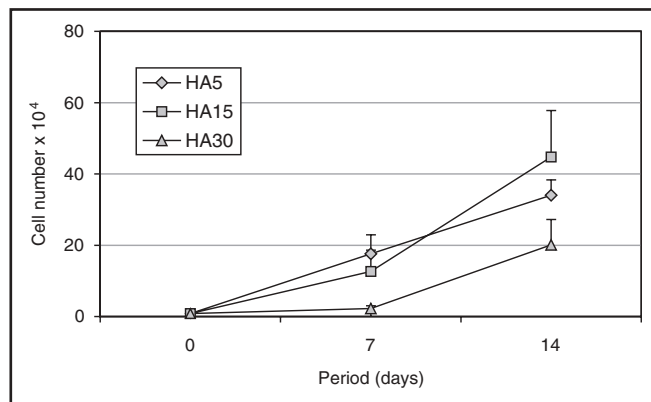

FIGURE 3 - Proliferation of ROS 17/2.8 cultured on hydroxyapatite (HA) discs. Data are presented as average values, and bars indicate standard deviation (n = 5). HA5 = less than 5% of microporosity; HA15 = 15% of microporosity; HA30 = 30% of microporosity.

TABLE 3 - Total protein content (μg/10^4 cells) produced by ROS17/2.8 cells cultured on hydroxyapatite (HA) discs. Data are presented as mean ± standard deviation (n = 5).

Period (days)	HA5	HA15	HA30
7	4.37 ± 0.65	7.06 ± 0.39	7.08 ± 7.08
14	4.44 ± 0.59	3.55 ± 0.12	6.13 ± 0.51

HA5 = less than 5% of microporosity; HA15 = 15% of microporosity; HA30 = 30% of microporosity.

TABLE 4 - Alkaline phosphatase (ALP) activity (μmol p-nitrophenol/hour) normalized by the total protein content and the number of ROS17/2.8 cells cultured on hydroxyapatite (HA) discs. Data are presented as mean ± standard deviation (n = 5).

Period (days)	HA5	HA15	HA30
7	2.84 ± 0.39	2.32 ± 1.01	0.58 ± 0.65
14	1.99 ± 0.92	2.44 ± 0.43	1.65 ± 0.13

HA5 = less than 5% of microporosity; HA15 = 15% of microporosity; HA30 = 30% of microporosity.

serum[15]. This initial interaction produces a layer of macromolecules that modify cell response. Fibronectin, a cell adhesion protein present in serum, has been shown to mediate cell attachment and spread on artificial substrates by interacting with glycosaminoglycans and with the cytoskeleton[7]. This study did not show any effect of surface topography on cell attachment. While it may occur, 2 hours – which was the period used in this evaluation – is a very short time to observe the development of a difference in protein adhesion that could affect cell attachment. Besides the period of incubation, it is also possible that cell attachment in

tissue culture is not seriously influenced by any characteristic of the material, since it has been shown that materials with different degrees of biocompatibility produce little or no differences in cell attachment[17].

In the present study, the amount of protein was normalized by the number of cells. Hence, these results evaluated the cell secretory activities that were not affected by surface topography. Similarly, Castellani *et al.*[4] (1999) did not find significant differences in the production of protein by rat bone marrow cells cultured on titanium with different surface topographies. Thus, it is possible that the total protein production, regardless of the chemical composition of the biomaterial, is not affected by surface topography.

Cell proliferation and ALP activity were affected by surface topography. HA samples with more regular surfaces such as HA5 e HA15 allowed greater cell proliferation and ALP activity than HA samples with irregular surface (HA30). These results suggest that more regular surfaces would facilitate cell growth and the expression of osteoblast phenotype markers. Cell morphology confirmed these findings, since cells cultured on more regular surfaces presented a polygonal morphology with dorsal ruffles and a large number of filaments. This morphology has been described as osteoblast feature[22]. On the contrary, cells cultured on irregular surfaces presented a flattened morphology showing almost no dorsal ruffles and filopodia. Other studies pointed out low cell proliferation and ALP activity on less organized surfaces[1,6], showing that the regularity and uniformity of the HA surface are important factors that affect cell proliferation and differentiation[2,12]. Therefore, differences in surface topography result in different degrees of HA biocompatibility, which confirms our previous results that revealed that HA30 presents, comparatively, low biocompatibility[18,19].

An explanation for these findings is that the physicochemical properties of the ceramic surface can affect the reorganization of proteins on ceramic specimens and change the profile of adsorbed proteins[8]. The type of substrate determines which integrins and extracellular matrix proteins are expressed by osteoblasts, providing information about how implant materials may affect osteoblastic differentiation[23]. The differences in protein adsorption can result in very different initial cell responses[23], and it is known that some proteins, such as fibronectin, have an important role in osteoblastic differentiation[11]. It is possible that organized surfaces favor the selective adsorption of proteins responsible for osteoblastic differentiation in serum. As a result, biomaterials that present more regular surfaces are more biocompatible.

CONCLUSIONS

- Varied manufacturing conditions produced HA samples with different microporosities. Surface roughness was not affected by the presence of micropores. However, surfaces with smaller percentages of microporosity presented more regular surface topography.
- All HA samples, regardless of surface topography, are biocompatible. However, samples with more regular surface topography were more biocompatible, which was evidenced by the greater cell proliferation and ALP activity observed on their surfaces, when compared with samples with irregular surface topography.

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