

Proliferation of human bone marrow stem cells cultured on alpha-tricalcium phosphate cement and titanium discs

Proliferação in vitro de células tronco da medula óssea humana sobre discos de cimento de alfa-trifosfato de cálcio e titânio

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Abstract

Objectives: to evaluate human bone marrow stem cells (HBMSC) cultured on α -tricalcium phosphate cement (α -TCP) and titanium (Ti) discs, as well as the osteoconductive properties of these biomaterials. **Methods:** discs of α -TCP and commercially pure Ti were manufactured and placed on polyethylene plates. Cells were cultured according to the following: group I – control (no discs); group II – α -TCP discs; group III – Ti discs. Propidium iodide staining was used to assess cell proliferation. Cells cultures were analyzed at 3, 7, 14 and 21 days. **Results:** Proliferation occurred in both biomaterials. At 3 days, greater cell proliferation was seen in group II than group III ($p=0.01$). Differences were not significant on the other time periods. Morphologic analysis showed circular morphology in group I, compatible with osteogenic cell lineage. **Conclusions:** both biomaterials were osteoconductive but not osteoinductive. This did not affect the initial stages of osteogenesis.

Keywords: Stem cells. Calcium phosphates. Titanium.

Resumo

Objetivos: avaliar culturas de células tronco da medula óssea humana (HBMSC) sobre discos de cimento de α -trifosfato de cálcio (α -TCP) e titânio (Ti), bem como a propriedade de osteocondução destes biomateriais. **Métodos:** discos de α -TCP e Ti comercialmente puro foram confeccionados e colocados em placas de polietileno. As culturas celulares foram distribuídas da seguinte forma: grupo I – controle (sem discos); grupo II – discos de α -TCP; grupo III – discos de Ti. Foi utilizado iodeto de propídio para possibilitar a constatação da proliferação celular. As culturas celulares foram avaliadas em 3, 7, 14 e 21 dias. **Resultados:** a proliferação das HBMSC ocorreu em ambos os biomateriais. No terceiro dia, uma maior proliferação celular foi constatada no grupo II em relação ao grupo III ($p=0,01$). Nos demais períodos não foram constatadas diferenças significativas. A análise morfológica demonstrou células com morfologia circular no grupo I, compatíveis com linhagem osteogênica. **Conclusões:** ambos biomateriais mostraram-se osteocondutores, mas não osteoindutores, não interferindo nos estágios iniciais da osteogênese.

Palavras-chave: Células-tronco. Fosfatos de cálcio. Titânio.

INTRODUCTION

Bone grafts and implants have been extensively used for the reconstruction of maxillary and alveolar bone defects, treatment of periodontal defects, maxillary sinus floor elevation, alveolar cleft management and other surgical procedures for oral rehabilitation (KENNEY et al., 1985; MANGANO et al., 2003; KOKEMUELLER et al., 2010; WEIJS et al., 2010).

The longevity of biomaterial implants depends on considerable understanding of cell behavior at the im-

plant-tissue interface. Whenever nonbiological materials are implanted in living tissues, there is an inevitable cell response. The form and characteristics of the implant surface may affect the events involved on this phenomena (SALTHOUSE and MATLAGA, 1983).

The α -tricalcium phosphate – α -Ca₃(PO₄)₂ – cement is a bioabsorbable biomaterial developed as bone substitute, described as bioactive and biocompatible. After setting, it is a substance similar to hydroxiapatite, a component of hard tissues in all vertebrates. It may be used for bone reconstruction or as scaffold (DOS SANTOS et al., 2002).

Titanium (Ti) and its alloys are some of the most important biomedical materials. They are used in dental

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implants because of high resistance to corrosion and biocompatibility, properties assigned to the passive layer of titanium dioxide that homogeneously coats its surface, formed immediately after the contact with aqueous solutions, air or other media that contain oxygen (PAN et al., 1997).

The cells of the bone marrow form a heterogeneous population containing osteogenic, fibroblastic and adipocytic cells. Osteoprogenitor cells differentiate into osteoblasts, which form an extracellular matrix that later become mineralized (SODEK and CHEIFETZ, 2000). Cell lineages from the bone marrow stroma may be used to regenerate different mesenchymal organs, such as bone tissue (THALMEIER et al., 2001).

This in vitro study aimed to evaluate the behavior of human bone marrow stem cells cultured on α -TCP and Ti substrates; to evaluate and compare the capacity of α -TCP and Ti to promote proliferation of human bone marrow stem cells; to evaluate α -TCP and Ti osteoconductive properties.

MATERIALS AND METHODS

Eight discs of α -TCP cement (Laboratory of Biomaterials, School of Engineering, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) and other eight identical discs of 99.9% commercially pure Ti (PROMM, Indústria e Comércio de Materiais Cirúrgicos, Porto Alegre, Brazil) were manufactured (15 mm diameter and 1 mm thickness, figure 1). For obtaining α -TCP, an appropriate mixture of γ - $\text{Ca}_2\text{P}_2\text{O}_7$ (prepared by dehydrating $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (IQUIMM) at 550 °C for 2 h) and CaCO_3 (Labsynth) was heated for 5 h at 1300 °C and quenched in air. After wet milling (anhydrous ethanol, ball mill) for 2 h and drying, was obtained the calcium phosphate cement (CFC) powder. The cement powder and the required amount (4.0 mL/g) of liquid solution (2.5%w/v Na_2HPO_4 ; accelerator) were mixed with a spatula. The paste thus obtained was packed into the holes of a silicone mold (15 mm diameter and 1 mm thickness). The samples in the mold were maintained for 24 hs at 100 % relative humidity (R. H.) and room temperature (27±1°C) for reaction of α -TCP into calcium deficient hydroxyapatite: $\alpha\text{-Ca}_3(\text{PO}_4)_2 + \text{H}_2\text{O} \rightarrow \text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5\text{OH}$. The discs were polished and sterilized by steam (autoclave, 121° C, 15 minutes) and placed on 24-well polyethylene plates.

Group I consisted of HBMSC cultured on polyethylene plate (control group); group II on α -TCP discs and group III on Ti discs.

The marrow sample was removed from the anterior iliac crest of an edentulous patient going under a reconstructive procedure. The patient had no other co-morbidities and signed an informed consent. The study was approved by the local ethics committee. The sample was immediately carried to the laboratory a sterile vacutainer. Immediately after, the cells were separated by density gradient centrifugation using Histopaque – 1077 (Dulbecco's Modified Eagle Media, Invitrogen, USA) and

placed in D-MEM medium supplemented with bovine fetal serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 50 $\mu\text{g}/\text{mL}$ gentamicin.

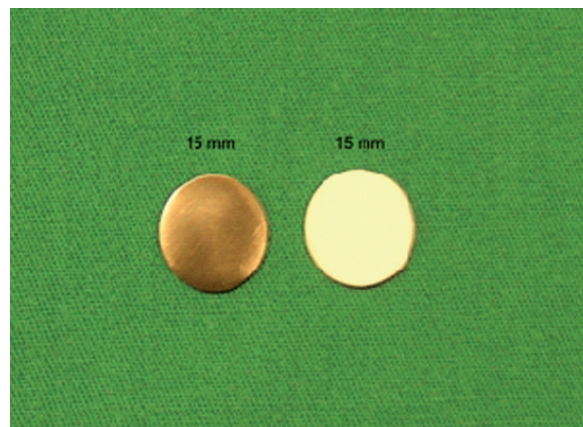


Figure 1 – Titanium (left) and α -TCP (right) discs measuring 15 mm diameter.

A 500 μl suspension of 7.2×10^4 cells per milliliter, determined by a hemocytometer, containing 40 ng of rhB-MP-4 per milliliter of D-MEM, was added in culture plates which had been previously prepared, receiving polyethylene, α -TCP and Ti discs (two units of each). One of the discs was used to evaluate cell proliferation by propidium iodide staining, and the other, to evaluate presence by RT-PCR for osteopontin and osteocalcin. Cultures were then incubated in a humidified incubator at 37°C with 5% CO_2 for 3, 7, 14 and 21 days.

Cell suspensions were added to the wells without discs, directly onto the polyethylene plate, to form the control group. Cell morphology and proliferation could be evaluated without the use of propidium iodide staining, for this group.

Morphology and proliferation of the HBMSC on α -TCP and Ti substrates were analysed by staining the nuclei with propidium iodide, a DNA-intercalating dye. They were removed from the wells for analysis and for micrographs of cell nuclei under the surfaces to be taken. A light microscopy was used under ultraviolet light (Axiolab, FT 400, Zeiss, Germany) at 50, 100 and 400x magnification.

For the evaluation and quantification of HBMSC, the biosystems were randomly microphotographed with a light microscope under ultraviolet light at a 100x magnification. After that, the images were digitalized with an image processing system using a scanner and a single measure standard (15x10 cm). A grid with standardized rectangles (2.5x3.25 cm) was traced over the images to simulate a hemocytometer and transferred to image software. The number per field of propidium iodide-stained nuclei HBMSC attached to the substrates was calculated. Cells in fourteen fields per group were counted at each time point, and their mean number was calculated.

The Student t test for independent samples was used for the statistical analysis of the comparison of cell

proliferation between groups. Analysis of variance (ANOVA) and the Tukey multiple comparisons test were used for the comparison between time periods.

RESULTS

Comparison at different time periods

Table 1 resume the mean numbers of HBMSC cultured on α -TCP and titanium (Ti) discs at the different time points.

Table 1 – Comparison of number per field of human bone marrow stem cells cultured on α -tricalcium phosphate and titanium discs at 3, 7 and 14 days.

Period	Group	N	Cells
3 days	α -TCP	14	17.9±8.4 ^A
	Ti	14	5.8±2.8 ^B
7 days	α -TCP	14	4.9±3.2 ^B
	Ti	14	6.1±4.3 ^B
14 days	α -TCP	14	8.4±2.7 ^B
	Ti	14	8.1±3.3 ^B
21 days	Media saturation		

*different index letters means significance ($P < 0.01$)

At 21 days, there was no attachment of HBMSC or any signs of cell proliferation in the α -TCP group. However, in the Ti group, there were bone marrow stem cells detaching from the substrate or in suspension, though without signs of cell proliferation on the surface. It was not possible to count cells in either group at this time period. This possibly occurred due to culture media saturation.

ANOVA revealed significant difference between results at 3 days and at the other time periods in the α -TCP group. There was greater proliferation of HBMSC at 3 days than at 7 and 14 days ($p=0.01$). At 7 days, there was a significant decrease in mean number of cells in comparison with results at 3 days. The results at 7 and 14 days did not differ significantly (Table 1).

According to the ANOVA results, there was no significant difference in the mean number of cells cultured on Ti discs ($p=0.18$) at the different time periods. However, there was a gradual increase in mean number of cells along time (Table 1).

Comparison between α -TCP and Ti

On day 3, the mean number of cells per field on the CFC substrate was 17.93. This was 3 times greater than the mean number on Ti ($P=0.01$). On day 7, there was a decrease in mean number of cells on α -TCP, but there was a trend to increased proliferation, although in a non significant difference, for cells on Ti. At 14 days, there was an increase in the number of cells cultured on α -TCP, and results between groups showed no significant differences.

The Student t test results did not reveal significant differences among groups at 7 and 14 days. However,

there was evident cell proliferation in both groups at all time periods (Table 1).

Morphology and cell proliferation

On day 3, the fields showed some HBMSC attached to the polyethylene plate, while others were in suspension. Morphologically, the cells were round, with small size variations and forming small colonies. HBMSC were attached to the α -TCP substrate and there was intense cell proliferation (Figure 2). HBMSC were detected inside the pores of the material. On the Ti substrate, there was moderate proliferation of attached HBMSC (Figure 3). At this time, there were cells attached to the edge of the Ti disc, and proliferation was observed.

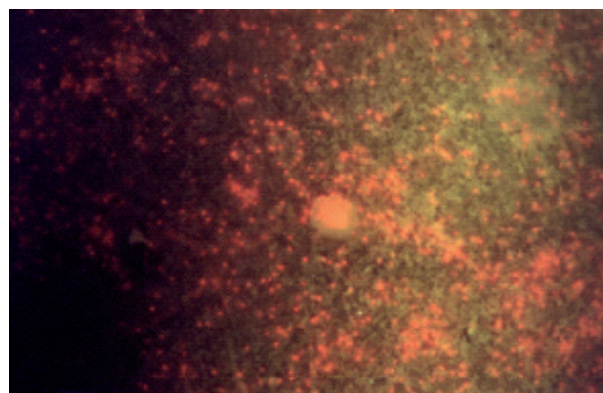


Figure 2 – HBMSC culture on α -TCP discs – 3 days. Cell nuclei stained with propidium iodide, in red. Cells attached to α -TCP substrate in intense proliferation; light microscopy, 100x magnification.

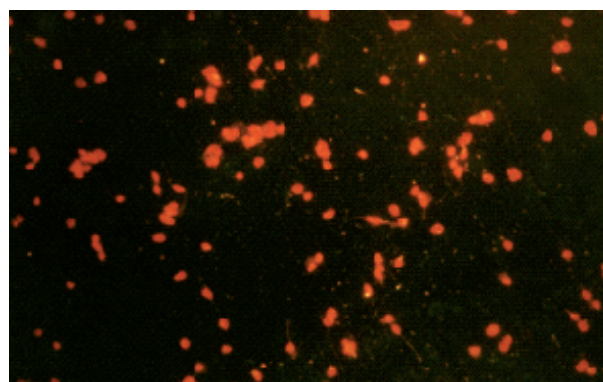


Figure 3 – HBMSC culture on Ti discs – 3 days. Cell nuclei stained with propidium iodide, in red. Cells attached to the Ti substrate in moderate proliferation; light microscopy, 100x magnification.

A growing number of HBMSC attached to the polyethylene plate and forming colonies was also found on days 7, 14 and 21. In the control group, round cells and cells with cytoplasmic elongations were found at all time points.

On day 7, morphologic analysis showed circular morphology compatible with osteogenic cell lineage.

HBMSC were attached to the α -TCP substrate in moderate proliferation. Cells were attached to the edge of the α -TCP disc; on the Ti group, HBMSC were attached to the substrate and in moderate proliferation.

On day 14, morphologic analysis showed a predominant circular morphology, which is compatible with osteogenic cells. A large number of HBMSC were attached to the α -TCP substrate in intense proliferation. Moreover, cells were detected inside the pores of the material. On the Ti disc, there was moderate proliferation.

At 21 days, morphologic analysis showed a predominant circular morphology, which is compatible with an osteogenic cell lineage, as well as round-shaped and fusiform cells and cells with cytoplasmic extensions (Figure 4). In the cultures on α -TCP and Ti discs, there were no attached cells or cells in proliferation anymore. On the Ti substrate, HBMSC previously attached to the disc were detaching.

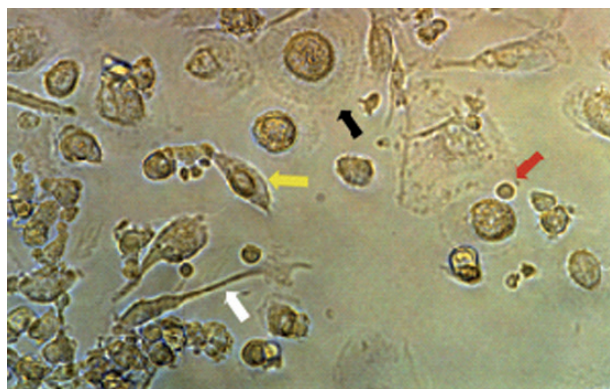


Figure 4 – HBMSC culture, control group – 21 days. Field shows circular morphology compatible with osteogenic cells (black arrow), round (red arrow) and fusiform (yellow arrow) cells, and cells with cytoplasmic extensions (white arrow); light microscopy, 400x magnification.

DISCUSSION

The use of models of in vitro cell cultures is useful to reach a better understanding of the characteristics of biomaterials, which may lead to better solutions and indications. Therefore, the results of in vitro studies spur the development of in vivo models and explore the associations between the properties of implant materials and their biological responses (KELLER et al., 2003). This study used an in vitro model to evaluate the behavior of HBMSC cultured on α -TCP and Ti. Other authors also evaluated the in vitro biological responses of cells on different materials (VROUWENVELDER et al., 1993; OZAWA and KASUGAI, 1996; HOTT et al., 1997; TOQUET et al., 1999; KNABE et al., 2002; HONG et al., 2003; KELLER et al., 2003; PARK et al., 2004; HARLE et al., 2006; SUN et al., 2006; GANDOLFI et al., 2011; PEREZ et al., 2013).

Morphologic analyses on day 3 showed the presence of round cells with small size variations forming small colonies. Similar morphologic findings were reported by Toquet et al (1999) up to the second day.

On day 7, there was formation of cell colonies in intense proliferation. Cells were elongated and had cytoplasmic extensions, in agreement with previous findings by Toquet et al (1999) and Park et al (2004).

As early as day 7, a circular morphology, compatible with osteogenic cells, was already found. At 14 and 21 days, however, circular morphology was predominant.

The proliferation of HBMSC on α -TCP substrates was greater on day 3 than on 7 and 14. On day 7, there was a decrease in the mean number of cultured cells on the surface of CFC. Similar findings were reported by Vrouwenvelder, Groot and De Groot, who observed high proliferation of osteoblasts on HA substrates on the third day of culture, but a decrease after 6 to 8 days. In our study, a trend towards increased proliferation was observed along time on Ti discs. At 3 days, there was greater proliferation in the α -TCP group than in the Ti group. There was no difference in cell proliferation between the α -TCP and Ti groups at 7 and 14 days, which is in agreement with findings by Ozawa and Kasugai (1996), although they used DNA quantification in their analysis. In our study, there was evident cell proliferation in both groups at 3, 7 and 14 days. Vrouwenvelder, Groot and De Groot (1993) also found similar cell proliferation on HA and Ti at 6 and 8 days using the same method as Ozawa and Kasugai (1996). Harle et al (2006) found similar results by day 4.

At 21 days, there were no HBMSC or signs of cell proliferation in the α -TCP group. In the Ti group, however, there were HBMSC detaching from the substrate or in suspension, but no signs of cell proliferation were found on the surface. In both groups, it was not possible to count cells at this time point. This finding may be explained by the saturation of the substrate at 21 days due to the large number of cells, which would block cell proliferation. Vrouwenvelder, Groot and De Groot found signs of cell death after 12 days. Those authors used substrates of 8 mm diameter, whereas in our study disc diameter was 15 mm. According to Hott et al (1997), in vitro cell proliferation and differentiation are limited by the size of the substrate.

In this study, HBMSC were found inside α -TCP pores. Toquet et al (TOQUET et al., 1999) also found HBMSC inside the pores of calcium phosphate ceramics. In a histologic study, Jarcho (1986) and Porter et al (2004) found bone tissue inside HA pores, as well as Meseguer-Olmo et al (2008). These findings reinforce the possibility of using α -TCP as matrix for tissue engineering.

Cell adhesion, attachment and surface spread are part of the first phase of the cell/material interaction, and the quality of this initial period is affected by cell proliferation and differentiation on implant interface (ANSELME, 2000). We found populations of attached HBMSC and HBMSC in suspension. There was evident proliferation of HBMSC on the α -TCP and Ti substrates at 3, 7 and 14 days, confirming their biocompatibility.

There was significant greater cell proliferation at day 3 on α -TCP than on Ti. Our findings suggest that there may be greater initial cell attachment on α -TCP than on Ti.

According to Hong et al (2003), there is a high concentration of apatite crystal ions on HA surface that may react with organic components and form an organic-inorganic interface that adsorbs onto ceramics and promotes cell adhesion. Therefore, HA provides adequate conditions for cell proliferation. Harle et al (2006) found similar results using HA sol-gel coatings. Turhani et al (2005) also found cell growth on different types of hydroxyapatite, using human osteoblasts.

Color changed from pinkish to yellowish, as indicated by the red color of the phenol in the culture medium, and confirmed acidity due to the presence of the α -TCP discs. Such phenomenon occurred more markedly up to day 3 and its residual effect was seen up to the day 7. After that time, pH was balanced, which was confirmed by the stabilization of the color of the medium. Knabe et al (2002) found that the change in pH of the culture medium affects cell behavior and inhibits proliferation. Dos Santos et al (2002) found in vitro cytotoxicity for CFC cement, which decreased along time. White and Shors (1986) reported that all types of hydroxyapatite and calcium phosphate undergo chemical dissolution at different degrees. It is our opinion that chemical dissolution of surface biomaterial in the medium may be responsible for the change in pH, which was also reported by Knabe et al (2002).

Between day 3 and day 7, there was a significant decrease in the number of bone marrow cells in the culture on α -TCP substrate. This may suggest a negative effect of the decrease of pH in the α -TCP culture, which may have caused cell death between the days 3 and 7. Such possibility is confirmed by the fact that cell proliferation resumed on day 7 to the 14.

Two requisites for biomaterials to be used as bone substitutes, or even as scaffolds, are their biocompatibility and non-toxicity (MEYER et al., 2004). This study confirms the biocompatibility of α -TCP and Ti, in agreement with previously reported in vitro and in vivo studies (WHITE and SHORS, 1986; PASSI et al., 1987; RICCI et al., 1992; OZAWA and KASUGAI, 1996; PAN et al., 1997; DOS SANTOS et al., 2002; PARK et al., 2004)

Human bone marrow stem cells could proliferate in vitro on α -TCP and Ti substrates under the conditions of this study. There was evident cell proliferation on both substrates at 3, 7 and 14 days. However, in vitro proliferation was limited by the size of the substrate, as observed at 21 days when no sign of cell proliferation was present. The effect of physical-chemical and microtopographic properties of biomaterial surfaces on cell behavior should be further investigated so that better materials are developed for high-quality osseointegration.

CONCLUSION

This in vitro study permitted the following conclusions: HBMSC followed a natural cycle of proliferation on α -TCP and Ti substrates; there was evident cell proliferation on α -TCP and Ti discs; at initial period, proliferation on

the α -TCP substrate was greater than on the Ti substrate; α -TCP and Ti substrates showed osteoconductive but not osteoinductive properties, without affecting the initial stages of osteogenesis.

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