

EFFECT OF DIFFERENT TREATMENTS OF TITANIUM SURFACE ON THE BIOLOGICAL PROPERTIES OF MESENCHYMAL STEM CELLS

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Abstract

The surface topography of titanium (Ti) influences the biological characteristics of associated cells. This study analyzed the behavior of murine mesenchymal stem cells (MSC) cultivated on Ti surfaces subjected to different treatments, including calcium phosphate deposition (CaP), pitting corrosion (PC) and plasma anodization (PA). The results showed that viability, morphology, immunophenotype and differentiation were not modified by cultivation on Ti surfaces, and that PA and CaP were the treatments giving the best conditions for cell proliferation.

Key words: mesenchymal stem cells, titanium surface treatment, anodization, adherence, proliferation, differentiation

1. INTRODUCTION

Titanium and its alloys are highly biocompatible, and have been used successfully for over thirty years in implants. Titanium is not considered an osteoinductive material, but has the ability of osseointegration, which contributes to its success in clinical applications (Li & Kawashita, 2011). However, there are unresolved problems associated with its use as a biomaterial. Its protective oxidized surface does not allow the rapid formation of a solid interface with the surrounding tissue. Moreover, the relatively high hardness of titanium, when compared to living bone, can lead to stress shielding problems and subsequent loss of the implant. Although these and other problems related to the use of titanium-based implants (reviewed by Jemat et al. 2015) have not yet been thoroughly resolved, the evidences indicate that Ti and its alloys are superior to other metals, so that its use in dentistry should increase in the near future. New alternatives to mitigate these disadvantages are thus of great importance.

The early integration of host cells into the biomaterial is one of the most relevant features of an efficient bioengineering system. The surface topography of an implant influences the adhesion of cells, modifying other biological factors such as differentiation, proliferation and migration (Hu et al. 2012). The surface of titanium biomaterials may thus have role in bone formation in implants, allowing better osseointegration.

Titanium surfaces can be modified by chemical or physical processes, but the ideal topography is not yet established, since it is difficult to associate the surface properties with clinical outcome. Implant stability studies are normally assessed in vivo by resonance frequency analysis (RFA) or through post-mortem investigations using mainly the histological examination of the contact between host tissue and the implant. In vitro studies provide an excellent research tool, since the interactions between cells (Mozumder et al. 2011) or extracellular matrix (Pittrof et al. 2012) with the surfaces of materials can be evaluated in detail. Models in culture are interesting by allowing more controlled conditions, especially on the cellular level, and collaborate with the reduction of animal experiments.

Mesenchymal stem cells (MSC) are one of the most interesting cell types to be analyzed in this experimental approach. These cells can be defined by their ability to self-renew, to differentiate into specialized cell types, and by a specific panel of surface markers (Dominici et al. 2006). Besides their

high potential to form osteoblasts, MSC are also able to secrete bioactive molecules with immunomodulatory, angiogenic, chemoattractant and anti-apoptotic properties, which explain their potential as tools in tissue engineering (da Silva Meirelles et al. 2006).

In the present study, we investigated the biological response of murine MSC on different treatments of titanium surfaces. To better understand this interaction, multiple parameters were simultaneously analyzed: cell adhesion, viability, proliferation, morphology, immunophenotype and differentiation.

2. MATERIAL AND METHODS

2.1. Reagents, culture media and solutions

Complete culture medium (HDMEM) was prepared by complementing Dulbecco's modified Eagle's Medium (DMEM) with HEPES (free acid, 2.5–3.7 g/l) and 10% fetal bovine serum (Cultilab, Sao Paulo, Brazil). Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution containing 10 mM sodium HEPES (HB-CMF-HBSS) combined with DMEM (1:1) was used for washing and resuspending the cells. All reagents used in this study were from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. Plasticware was from TPP (Trasadingen, Switzerland).

2.2. Sample preparation

Coupons with the dimensions 1 x 1 x 0.8 cm were cut from commercially pure titanium from CAMACAM (Ti Cp) ASTM F67 grade 2, polished up to 1 μm using diamond paste and rinsed with alcohol in an ultrasound bath. The following surface treatments and their combinations were applied to the polished Ti surfaces after properly masking the samples with epoxy:

Pitting corrosion (PC) for the production of micro pores: Homogenous pitting of the polished Ti substrates was produced at a potential of 1.8 V, by keeping the samples for 5 min in 1 M NaBr at 80°C in a three electrodes cell. The samples were immediately thereafter cleaned with acetone in an ultrasound bath.

Plasma anodization for the production of nanopores: Plasma anodization of polished Ti samples was carried out at room temperature (RT) in 1 M H_3PO_4 , in a two-electrode cell using a laboratory potential source. The counter and reference electrode was a Pt grid. A constant current density of 40 mA/cm^2 was applied between the electrodes up to a potential of circa 200 V, at which the strong sparking of the Ti electrode started and was maintained for further 100 seconds. The samples were then cleaned with acetone in ultrasound. The treatment was applied to samples without any previous treatment (PA) or to samples previously pitted (PCPA).

Chemical deposition of calcium phosphate (CaP) for pores closure: The anodized Ti samples were dipped in two consecutive baths [0.1 M $\text{Ca}(\text{OH})_2$ saturated with 1 M H_3PO_4 (suspension) and 0.02 M $\text{Ca}(\text{OH})_2$ filtered, respectively], at room temperature, and immediately dried in hot air, in cycles repeated up to five times to improve the coverage.

The samples were autoclaved before used as substrates in the cell culture procedures.

2.3. Surface analyses

The Ti samples with different surface treatments, produced by pitting, anodization, chemical deposition of Ca-phosphate and the combination of these processes were analyzed with a JEOL JSM-5800 scanning electron microscope (SEM) provided with an energy dispersive x-ray spectrometer (EDS). For the statistical analysis of pores distribution, before and after the chemical closure of pores, randomly acquired SEM images were quantified with the Image-J v. 1.62 software.

2.4. Mesenchymal stem cell culture

MSC were isolated and cultured from the liver of adult C57Bl/6 mice, as previously described (da Silva Meirelles et al. 2006). Briefly, livers were disaggregated in HB-CMF-HBSS, digested with collagenase I, suspended in HDMEM, plated on 6-well plates and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2 . Media was changed and non-adherent cells were removed every

three days, and after the establishment of the permanent culture the cells were trypsinized and passaged every three or four days.

Cultures were observed with an inverted phase-contrast microscope (Axiovert 25; Zeiss, Hallbergmoos, Germany). Photomicrographs were taken with a digital camera (AxioCam MRc, Zeiss), using the AxioVision 3.1 software (Zeiss). Cultures in passages 5 to 8 were used for all experiments. Unless otherwise stated, all experiments were performed in duplicate.

All animal procedures in this study were conducted in accordance with humane animal care standards, and experimental procedures were performed according to institutional guidelines, after approval of the project by the Research Ethics Committee.

2.5. MSC adhesion to Ti surfaces

The Ti samples were placed in wells of a 24-well tissue culture plate and seeded with 40 µl of HDMEM with 1×10^6 MSC. After 4 hours incubation at 37°C, the samples were washed with HB-CMF-HBSS, transferred to another well of a 24-well plate and incubated with 1.5 ml 0.25% trypsin and 0.01% EDTA in HB-CMF-HBSS for 10 min. After washing in HDMEM, viable cells were counted with trypan blue in a Neubauer chamber.

2.6. Characterization of MSC on Ti surfaces

MSC were cultivated on surface-treated Ti samples for different periods of time and analyzed for the biological parameters described below. Experiments were performed in triplicate.

Cultures were routinely observed with an inverted phase-contrast microscope (Axiovert 25; Zeiss, Hallbergmoos, Germany). For detailed observation, after 7-day culture the samples were rinsed with HB-CMF-HBSS, fixed with ethanol for 5 minutes at RT and stained for 2.5 minutes with Giemsa. Photomicrographs were taken with a digital camera (AxioCam MRc, Zeiss), using AxioVision 3.1 software (Zeiss). MSC cultivated during 7 days on Ti were also analyzed on a scanning electron microscope (Zeiss EVO MA10). The samples were fixed by immersion in a mixture of 2.5% glutaraldehyde buffered with 0.1 M phosphate (pH 7.3) under refrigeration at 4°C for 24 hours. After fixation, the samples were washed in three changes of phosphate-buffered solution 0.1 M and, subsequently, dehydrated in ascending series of alcohol, followed by acetone. The samples were dried using Critical Point Dryer (Balzers CPD-030) and coated with Au, using a Sputter Coater (Balzers SCD-050).

For analysis of proliferation, Ti samples were seeded as described above with 1×10^6 MSC, incubated for 4 h at 37°C, washed with HB-CMF-HBSS, transferred to another well of a 24-well plate and incubated with 1.5 ml HDMEM during 48 h. In a second experimental group, 1×10^5 cells were seeded and the samples were incubated for 7 days, with a change of medium on days 3 and 5. After 48 h or 7 days, cells were trypsinized and counted as above. A cell number index was calculated by dividing the number of recovered cells for the number of cells originally present in the Ti samples.

The osteoblastic differentiation of MSC on Ti surfaces was analyzed by seeding 1×10^5 MSC on Ti samples as above, and incubated for two weeks with osteogenic-inducing medium (HDMEM supplemented with 10^{-8} M dexamethasone, 5 µg/ml ascorbic acid 2-phosphate and 10 mM β-glycerophosphate). Calcium deposition, which indicates osteogenic differentiation, was observed by washing the samples with HB-CMF-HBSS, fixing with 4% paraformaldehyde for 15-30 minutes at RT, and staining for 5 minutes at RT with Alizarin Red S stain at pH 4.2. The intensity of staining was analyzed with the ImageJ software (NIH ImageJ, Version 1.42-l).

For immunophenotyping, MSC cultivated during 7 days on Ti as described above were trypsinized, washed in HB-CMF-HBSS and incubated for 30 minutes at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine CD29, CD44 and CD45. The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected.

2.7. Statistical analysis

All experiments were triplicated. Data were analyzed using the Prism 5 software (GraphPad Software Inc., San Diego, CA). The significance of differences in the results was evaluated by a one-way analysis of variance (ANOVA). A difference of $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Incubation of 10^6 MSC with Ti samples for 4 hours resulted in adherence of 3.5 to 5.2×10^5 cells in three of the treatment protocols tested (Fig. 1). One of the treatments (plasma anodization, PA) resulted in adherence of a smaller number of cells, but the difference was not significant. This is an important result when Ti is considered for bioengineering using MSC, since one of the main features of these cells is their adherence to the substrate. The level of adherence observed after incubation, about 50% of the MSC originally seeded, is less than normally observed in conventional plating (close to 100% adherence), but similar to that observed in other studies (Giordano et al. 2006; Fiedler et al. 2013).

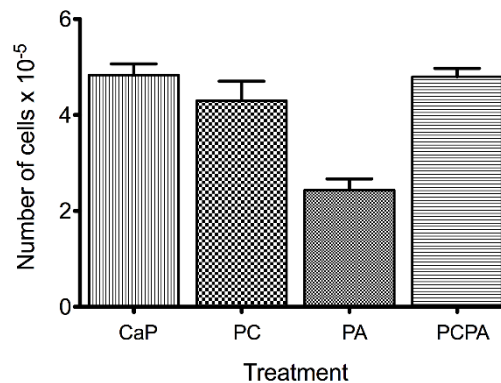


Fig. 1. MSC adhesion on Ti samples after seeding of 10^6 cells. Results are shown as number of adherent cells.

After trypsinization and collection, the viability of adherent cells was evaluated with Trypan blue staining. Similar to other studies, cell viability was $>90\%$ in all cases, irrespective of the type of treatment of titanium surfaces and the period of incubation (Giordano et al. 2006; Chan et al. 2014). No differences were found when the 4 types of treatments were compared. These results indicate low cytotoxicity of metal, as expected due to its biocompatibility properties. The characteristically adherent morphology of MSC could be observed by Giemsa staining of cultures maintained for 7 days on Ti samples, and was not modified by the different treatments (Fig. 2). The cells tended to concentrate on the corroded titanium surface produced by pitting, when compared with plain areas. Similar results were described by Eisenbarth et al. (2002) for other cell types, but contrasting results have also been reported (Martin et al. 1995).

The adherent cells could also be observed by scanning electron microscopy (Fig. 3), showing the mesenchymal stem cells with their characteristic fibroblastoid morphology in contact with Ti submitted to all treatments. Cells with similar characteristics were observed by Castellani et al. (1999) and Giordano et al. (2006), whereas Eisenbarth et al. (2002) described the presence of a large amount of cytoskeleton in cells firmly attached on titanium surfaces. More recently, Santander et al. (2015) reported improved adhesion capacity and higher proliferation rate of human MSC cultured on Ti surfaces treated with a combination of a shot blasting and anodizing procedure.

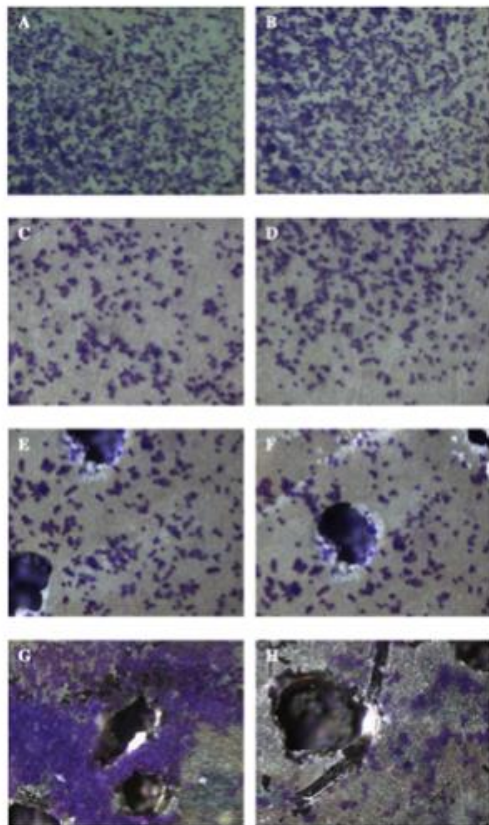


Fig. 2. Giemsa-stained MSC cultivated on titanium surfaces with different treatments. A and B, calcium phosphate deposition; C and D, plasma anodization; E and F, plasma anodization and pitting corrosion; G and H, pitting corrosion. Original magnification x100.

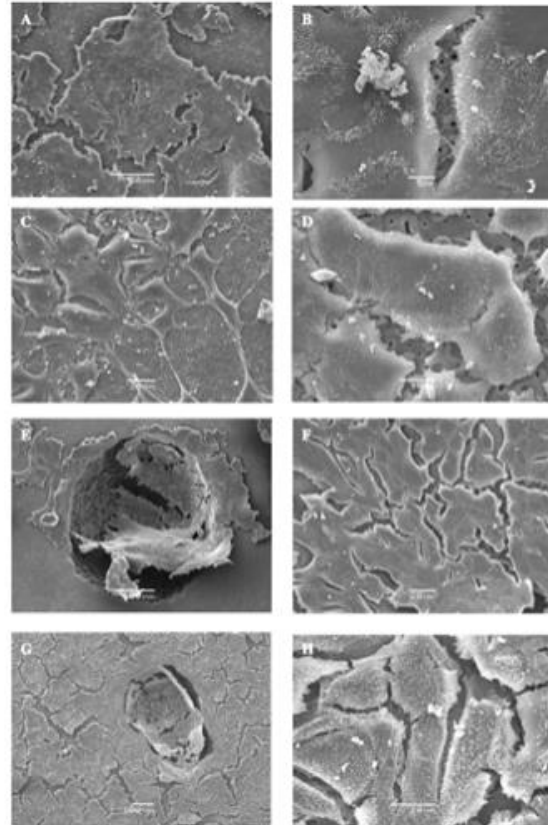


Fig. 3. Scanning electron microscopy image of MSC cultivated on titanium surfaces that received different treatments. A and B, calcium phosphate deposition; C and D, plasma anodization; E and F, plasma anodization and pitting corrosion; G and H, pitting corrosion.

For analysis of the proliferation rate of MSC on Ti surfaces, the cells were seeded in two concentrations (10^6 and 10^5 cells/sample), and cultured during 2 or 7 days, respectively. After trypsination, viable cells were counted to evaluate the cell number index. After 48 h the initial cell population showed a similarly slight increase in all cases (Fig. 4A). After 7 days, the cell number index was not modified in Ti samples treated with CaP, but showed a large increase in the remaining samples, particularly when treated with plasma anodization ($p < 0.05$, Figure 4B).

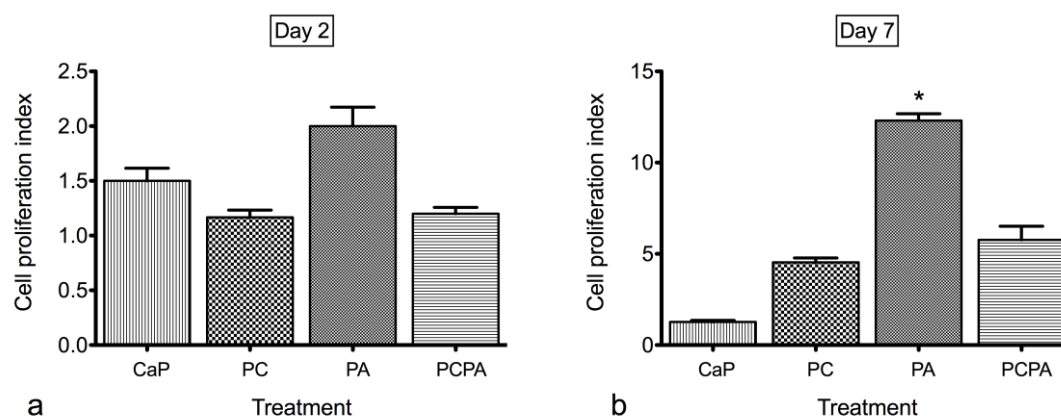


Fig. 4. Cell proliferation of MSC incubated on Ti samples for 2 or 7 days at 37°C in 5% CO₂.
*Significantly different from all other conditions, $p < 0.05$.

The only significant difference observed in MSC proliferation was for PA-treated surfaces, which induced higher proliferation rates of MSC on day 7. Interestingly, treatment with calcium phosphate showed a higher number of live cells on day 2, but a much smaller number in long-term (7 days). This may have happened for a faster proliferation and occupation of the available surface by cells that, due to lack of space for further expansion, began to die. Alternatively, cells might have detached from the Ti surface to the culture plate, a possibility that has not been tested in this work. A similar process could happen with other treatments later. Results showing intensive cell proliferation on Ti surfaces were also reported by Sader et al. (2005) and de Peppo et al. (2014).

To investigate the osteogenic potential of treated Ti samples, MSC were cultivated with treated Ti samples for two weeks with osteogenic-inducing medium. Staining with Alizarin Red showed that cells cultured on Ti surfaces are capable of osteogenic differentiation, with similar intensity in all cases (results shown for CaP and PA, Fig. 5).

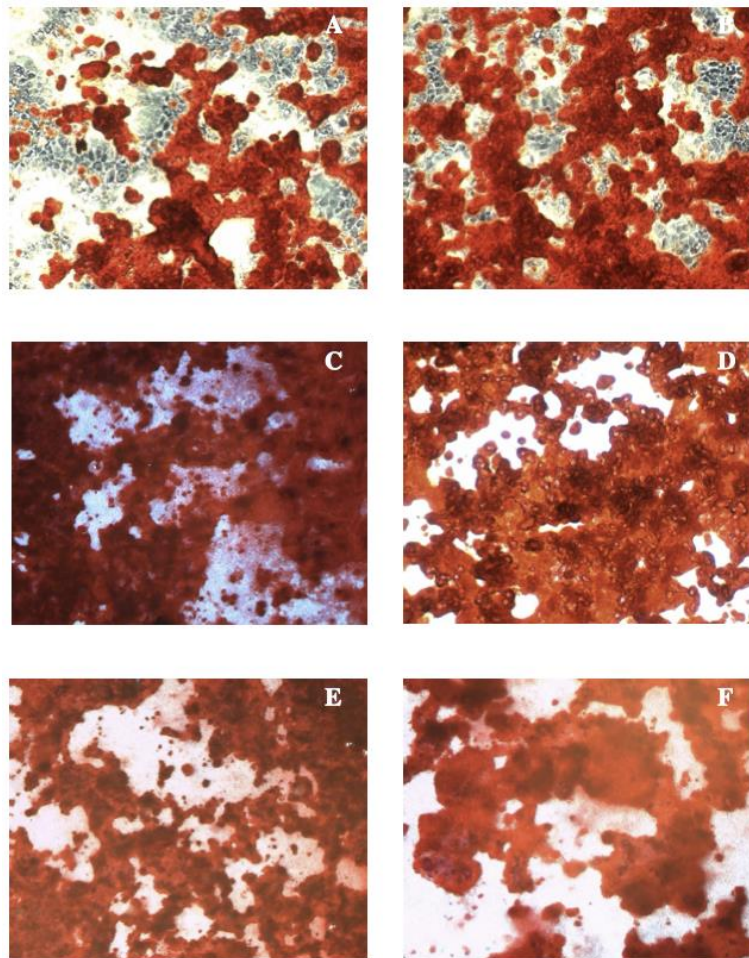


Fig. 5. Photomicrographs representative of the osteogenic differentiation of MSC after culture with inducing medium on different Ti surfaces. A and B, control cultures (plastic surface); C and D, titanium surfaces treated with calcium phosphate; E and F, titanium surfaces treated with plasma anodization. Original magnification x 100.

Although the analyses did not allow the quantification and comparison between treatments, the results show that MSC maintained the osteogenic potential when cultivated on all types of treated Ti surfaces, which is important for this type of bioengineering process. Varying results have been reported in other studies, showing for example greater osteogenic potential in cells cultivated on rough surfaces (Martin et al. 1995; Giordano et al. 2006), or the absence of difference among treatments (Sader et al. 2005). Hempel et al. (2013), on the other hand, observed higher osteogenic potential in human MSC cultivated on polished than sandblasted/hot acid etched titanium surfaces. Martin et al. (1995) have proposed that the surface differences affect more the differentiation potential of MSC than adhesion and proliferation. Size, besides the surface structure, may also affect the osteogenic potential of biomaterials. de Peppo et al. (2014) investigated the osteogenic differentiation of human MSC cultivated on titanium-coated nanostructures, showing that osteogenesis was influenced by the size of the underlying structures.

The cultivation on Ti surfaces for 7 days did not significantly modify the immunophenotype of MSC. As shown in Table 1, a low frequency of cells positive for CD45, and a high frequency of cells positive for CD29 and CD44 were observed in all cases. The cells cultured on Ti samples treated with calcium phosphate showed a slightly lower frequency of cells positive for CD44 and CD29, but the differences were not significant.

Some studies (Rosa & Beloti 2003; Hackenberg et al. 2014) have shown that the contact of stem/stromal cells with titanium surfaces can induce differences in the expression of membrane markers, with involvement of molecules that promote adherence of these cells. Other reports however, agree with the results observed in the present work, with no modification in the immunophenotype of cells cultivated on Ti surfaces (Blanco et al. 2011).

Table 1. Immunophenotyping of MSC cultured on Ti samples for 7 days at 37°C in 5% CO₂. Results shown as frequency (%) of positive cells for each surface marker.

Ti treatment	Surface marker		
	CD45	CD29	CD44
Control (cultivated on plastic surface)	5.9	96.6	90.4
Calcium phosphate deposition	4.3	72.8	39.1
Plasma anodization	2.4	86.9	75.8
Pitting corrosion	4.9	88.6	73.6
Plasma anodization and pitting corrosion	5.2	88.7	75.9

4. CONCLUSION

Our results showed that titanium substrates treated by chemical deposition of calcium phosphate, pitting corrosion, plasma anodization or a combination of these last two procedures, were capable to maintain the viability, immunophenotype and proliferation and differentiation potential of murine mesenchymal stem cells after variable periods of cultivation. The chemical deposition of calcium phosphate on anodized Ti samples constitute to be the best surface for cell proliferation. There is not yet a consensus on the most adequate surface treatments for titanium implants to be used in osteogenesis. A combination of factors must be considered, including the types of surface, the different cell types used and the different methodologies employed. The present study adds information to the search for a standardization of techniques, treatments and cellular types employed in bone tissue engineering.

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