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Comparison of the osteogenic, adipogenic, chondrogenic and cementogenic differentiation potential of periodontal ligament cells cultured on different biomaterials

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ABSTRACT

It has been shown that the cellular responses such as adhesion, proliferation and differentiation are influenced by the surface properties, such as the topography or the surface energy. However, less is known about the effect of the chemical composition and type of material on the differentiation potential. The objective of the present paper is to compare the differentiation potential of periodontal ligament cells (HPLC) into adipocytes, osteoblasts, chondroblasts and cementoblasts of three type of materials (metals, ceramics and polymers) without using any biological induction media, but keeping the average roughness values within a limited range of 2.0–3.0 μm. The samples were produced as discs of 14 × 2 mm; ($n = 30$ for each type of material). Two samples of each type were chosen; stainless-steel 316L and commercially pure titanium for the metallic samples. The polymers were polymethyl methacrylate and high-density polyethylene, and finally for the ceramics; zirconia and dental porcelain were used. The surfaces properties of the samples (wettability, chemical composition and point of zero charge, PZC) were measured in order to correlate them with the biological response. To evaluate the potential of differentiation, human periodontal ligament cells obtained from extracted teeth were used since they are a promising source for periodontal tissue regeneration. Cell proliferation was initially tested to assure non-toxic effects using a viability colorimetric assay. Finally, the differentiation pattern was evaluated using real time reverse transcription quantitative polymerase chain reaction for 5, 10 and 15 days without adding any induction medium. The results indicated that the relative expression of genes related to a particular phenotype were different for each surface. However, not clear correlation between the type of material or their surface properties (morphology, chemical composition, wettability or point of zero charge) and the expression pattern could be identified. For example, bone markers were mainly expressed on cpTi and PMMA; one metallic hydrophobic and one polymeric hydrophilic sample which have similar R_a values but presented different topographical features, although both samples have in common a PZC below 7.

1. Introduction

Cell-materials interactions determine many of the cellular processes such as adhesion, spreading, proliferation and differentiation [1–6]. Therefore, understanding such interactions is critical for the development of implant materials and successful tissue engineering. The cell-surface interactions are not yet completely understood due to the complexity of the processes, but also due to the intermixing between the different surface physicochemical properties. A very well documented example is the effect of the matrix stiffness on the differentiation of stem cells. Initial experiments for tissue engineering using hydrogel scaffolds indicated that stem cells differentiate into neurogenic or adipogenic lineage when cultured on soft substrates, while stiff substrates promotes the differentiation into the osteoblastic lineage [7]. However, more recent experiments have shown that when the mechanical stiffness of some hydrogels was modified, the process did also altered the surface chemistry or surface functional groups and so it is not so obvious who is controlling the stem cell differentiation, surface composition or the mechanical stiffness [8,9]. Similar results have also been observed for titanium metallic implants, where different in-vitro studies have observed an increase in the osteogenic differentiation when micro-nano-roughened Ti surfaces are used [6,10–14]. However, the topographical modifications are usually obtained by chemical or

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physical processes that might also alter the surface composition or the surface energy, although this detail is not taken into account [2,15,16]. In general, it has not been possible to decouple the topographic modifications from variations in other surface properties, such as the chemical composition or the surface energy [17].

Although, there are still many unknowns, the clear message from the different reports is that the physicochemical properties of the biomaterials can modify the cellular microenvironment and deliver stem cell regulatory signals to induce differentiation into a specific lineage in a near physiological way. This is clearly an advantage and a challenge since the selection of the material should take into consideration both the functionality of the implant and an adequate cell response. Otherwise, undesired cell type attachment or differentiation might lead to the formation of a non-functional tissue around the implant [18].

Intuitively, and as a result of various studies, it is clear that the chemical composition of the surfaces has an impact on the short and long term cytotoxicity, which clearly limits the composition of the materials used for medical applications [18]. Furthermore, the selection of a material for a particular application is firstly determined by the bulk properties, such as rigidity or flexibility, transparence or opacity. The following requirement is the biocompatibility, which is mainly determined by the physicochemical surface properties of the biomaterial. Metals are used for replacing bone, polymers for soft tissues, etc. It is desirable that within each group, the surface properties could be further optimized to achieve the most appropriate biological response; micro-nano-rough surfaces for osteoblast differentiation in bone implants [19] or smooth surfaces to improve the adhesion of fibroblasts and connective tissue on polymers [20] or high surface energies to prevent the formation of clots in blood-contacting implants [21]. The difficulty lies in how to individually control the set of surface physicochemical properties, which themselves are strongly correlated, without altering also the functionality of the material for the desired application [22].

This is even more challenging when complex tissues as the periodontal tissue are considered. One current goal in dentistry is to induce tissue regeneration in the treatment of periodontal disease. Advanced periodontal disease shows extensive loss of the tooth-supporting periodontal tissues, which consist of bone, cementum and periodontal ligament [23]. The periodontal tissue regeneration involves the formation of all components of the periodontium; gingival, periodontal ligament, cementum and alveolar bone. Different approaches using bone grafts with or without isolating membranes have been proposed with limited success [24,25]. Human periodontal ligament (PDL) cells obtained from extracted teeth are a promising source for periodontal tissue regeneration [26–28]; it has been shown that the PDL cells can be either multipotent or composed of heterogeneous cell populations [29]. Moreover, they contain stem cells that have the capacity for proliferation, self-renewal and multilineage differentiation, such as osteoblasts, adipocytes, chondrocytes and neural cells in vitro [23,30,31]. Finally, they have also shown the ability to form cementum and periodontal ligament tissue in vivo [32–34]. However, there is very few information about the interaction between PDL cells and materials with different surface properties.

Herein, we examine the effect of the physicochemical properties of different biomaterials of common use in dentistry on the proliferation and potential of differentiation of human PDL cells. In order to perform this work, six different materials were selected; two metals, two ceramics and two polymers. The average roughness of all the samples was set to about 2–3 μm, in the range where osteoblastic differentiation has been reported to be promoted on metallic substrates [10,14,35]. We have used this roughness range since there is not information about the correlation between cell differentiation and roughness associated to polymers and ceramics. The chemical composition and other surface properties (composition, topography, wettability, and surface charge) were evaluated to find possible correlations with the gene expression as measured by real time reverse transcription quantitative polymerase chain reaction (RT-qPCR).

2. Materials and methods

2.1. Materials

The samples were produced as discs of 14 ϕ × 2 mm ($n = 30$ for each material). The selected materials were; commercially pure titanium (cp-Ti, Straumann™), stainless steel 316L (SS316L), Polymethyl methacrylate (PMMA, Lucitone 199™), high density polyethylene (HDPE, Poly-Maq Paloma 100™), dental porcelain (VITA VM13™) and Zirconia (Natura ZIR™). The surfaces were prepared to have average roughness between 2.0 and 3.0 μm, by roughening the surface using silicon carbide sandpaper (Fandeli™) of different grades. For metals, grade 600 was required, while for the polymers and porcelain sandpaper #360 was used. The ZrO_2 samples were prepared by sintering the ZrO_2 powder into molds of the proper size (14 ϕ × 2 mm) at 1500 °C. This process produced surface roughness in the adequate roughness range; therefore, no roughening process was required. Care was taken to follow exactly the same procedure for the preparation of the samples, so that the roughness was reproduced.

All the samples were ultrasonically cleaned for 20 min in acetone, followed by 20 min in 70% ethanol (in volume) before a final rinsing with distilled water.

2.2. Surface properties

The surface roughness was measured at two different scales; micro- and nanoscale. At the micro-scale, a contact Profilometry (Veeco Dektak 150™) was used. For every sample, three specimens were randomly selected for measurements. Three or four scans of 500 μm length each were done per specimens to obtain in total ten (10) surface profiles. The principal advantage of the profilometer is that the stylus is in contact with the surface and consequently the contact gives the amplitude level of the roughness with good accuracy and without depending on the optical properties of the surface. Nevertheless, the contact might be a disadvantage on surfaces of low rigidity, such as the polymeric samples, since the elastic deformation can disturb the profile [36]. Field emission electron microscopy images were also obtained to observe the topographical features at the micro-scale. Images at different amplifications were obtained using a scanning electron microscopy (JEOL JSM-7600F Field Emission Scanning Electron Microscope, SEM). The surface topography at lower scale was observed by atomic force microscopy using a Nano-surf Naio AFM. The images (SPIP software) were obtained using the contact mode with a silicon cantilever and areas of scanning of 30 μm × 30 μm.

2.2.1. Wettability

The contact angles were obtained using the sessile drop method in a Rame-Hart instrument after the cleaning process as described above. The drop image was stored and the image was later analyzed using the drop-analysis with Image J (Image Processing and Analysis in Java Software ®). Two liquids were used distilled water and fetal bovine serum (10%) Gibco® [37,38]. For each sample four drops were analyzed (three different samples per surface type) and the reported contact angles are the average of left and right angles.

2.2.2. Surface charge (point of zero charge)

The point of zero charge refers to the solution pH at which the surface is neutral within that medium. The surfaces in an aqueous solution are generally electrically charged as a consequence of the dissociation of the water in OH^- and H^+ groups, the acidity or basicity in the surface could be determine from the point of zero charge (PZC). The PZC values were obtained from potentiometric titration curves following the procedure employed in previous works [39,40]. The PZC values of each material provide important information about the surfaces properties that can be related with the wettability measurements for understanding

the biological behavior of these materials in the differentiation capabilities.

2.3. Biological tests

The use of human tissue from the oral cavity for the generation and culturing of human fibroblasts was reviewed and approved by the Ethics Committee at the National University of Mexico School of Dentistry (UNAM). Tissue samples were obtained from the donors that underwent routine oral surgery procedures.

2.3.1. Culture of human periodontal ligament cells (HPLC)

Human periodontal ligament cells (HPLCs) were isolated and grown as described previously [41]. Cells between the 1st and 2nd passages were used for the experiment. The cells were grown in DMEM media supplemented with 10% fetal bovine serum purchased from Gibco-BRL (Gaithersburg, MD, USA) and antibiotics (streptomycin 100 μg/ml, penicillin 100 units/ml). Cell cultures were maintained in an atmosphere of 95% air, 5% CO_2 at 37 °C with 100% humidity.

2.3.2. Cell proliferation

Cytotoxic effect and effect on proliferation were evaluated at 0, 24, 48 and 96 h on the surfaces to be tested using the colorimetric MTT (tetrazolium) [3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay [42]. The MTT analysis is dependent on the reduction of the tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. HPLCs were plated at 1×10^4 density in a 1 ml volume in 24-well plates (Costar, Cambridge, MA, USA™). HPLCs were cultured during 4 days, replacing the medium with fresh medium daily. At the end of the treatment intervals, 100 μl of MTT solution (5 mg/ml: Boehringer Mannheim, Indianapolis, IN, USA®) in phosphate-buffered saline (PBS) was added to the wells and incubated at 37 °C for 4 h. After the MTT incubation, 1 ml lysing buffer Dimethyl sulfoxide (DMSO) was added to each well and incubated 1 h at 37 °C. The absorbance of 100 μl of the resultant solution was read in a microplate reader at 570 nm. The absorbance or optical density is directly proportional to the number of living-viable cells present in the culture.

2.4. mRNA isolation and real-time reverse-transcriptase polymerase chain reaction (RT-qPCR)

HPLCs were cultured during 5, 10 and 15 days in DMEM medium supplemented with 10% FBS and antibiotics as described. At term, cells were collected and analyzed for mRNA expression of cartilage, bone, cementum and adipogenic markers by real-time RT-qPCR.

The evaluation of the differentiation of cells in vitro is commonly done by the measurement of the expression of multiple relevant biochemical markers. For osteoblasts differentiation, the most used ones are the phosphatase alkaline (ALP), bone Sialoprotein (BSP), osteocalcin (OCN) and RUNX2 [43,44]. Cementogenesis is a critical process for regeneration of the periodontium and although it has not been possible to identify specific cemetoblastic markers [34], some results suggest that HACD1/CAP (3-Hydroxyacyl-CoA Dehydratase1/cementum attachment protein) [45] and cementum protein 1 (CEMP-1) [33,46] can be used as markers since they have been expressed during the formation of cemetoblasts. For the chondroblastic differentiation, typically SOX9, Collagen type II (Col II), Collagen type X (Col X), Collagen type XI (Col XI) and Aggrecan (Aggrecan or ACAN) are used [43]. Finally, for the adipocytes, the differentiation process follows the sequential expression of three key transcription factors (C/EBPbeta, C/EBPalpha and PPARgamma) [47,48]. In this work, we have measured the expression of peroxisome proliferator-activated receptor gamma (PPARgamma or PPAR-γ) and the Lipoprotein lipase (LPL), which is one of the first genes expressed during the adipocyte differentiation process [49].

Table 1
Primer sequences used for real-time RT-qPCR analysis of gene expression.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	CAACGGATTTGGTCGTATTGG	GCAACAATATCCACTTTACCAGAGTTAA
BSP	AACGAAGAAAGCGAAGCAGAA	TCTGCCTCTGTGCTGTTGGT
OCN	GTTGCAGGCTCAATCCATTT	CCATCCTCATACCTGCACCT
ALP	AGCACTCCCACTTCATCTGGAA	GAGACCCAATAGGTAGTCCACATTG
HACD1/CAP	TCCAGACATTTGCCTTGCTT	TTACAGCAATAGAAAAACAGCATGA
CEMP1	TGAGAACCTCACCTGCCTCT	ACCCCTTAGGAAGTGGCTGT
SOX	GTAATCCGGGTGGTCCTTCT	GACGCTGGGCAAGCTCT
Collagen II	CGGCTTCCACACATCCTTAT	CTGTCCTTCGGTGTCAGGG
Collagen X	GTGGACCAGGAGTACCTTGC	CATAAAAGGCCCACTACCCA
Collagen XI	GTCATATGCTGCCTTGGGAT	AATGGAATCACGGTTTTTGG
Aggrecan	ACAGCTGCAGTGATGACCCT	TTCTTGGAGAAGGGAGTCCA
PPARγ	AGGCCATTTTGTCAAACGA	GAGAGATCCACGGAGCTGA
LPL	TCAGCTGTGTCTTCAGGGG	CTCCAGAGTCTGACCGCCT

Primer sequences for human genes encoding cartilage, bone, cementum and adipogenic markers and GAPDH are listed in Table 1 and were acquired from Invitrogen Carlsbad, USA®.

Total RNAs were extracted according to the manufacturer's recommended protocols with Trizol Reagent (Invitrogen, Carlsbad, USA®). Ten nanograms were used per reaction and the level of mRNA expression was quantified by the one-step real-time RT-qPCR method using SuperScript® III Platinum® SYBR® Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA). A reaction (25 μl) was set up with the following qPCR conditions: (cDNA synthesis) 50 °C for 3 min, denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and finally 40 °C, 1 min. Amplifications were performed in a Corbett Rotor-Gene 6000 (Qiagen, Valencia, CA, USA®). All experiments were performed in triplicate and expression levels of the above mentioned molecules were obtained using delta-delta Ct method normalizing for GAPDH [50].

2.5. Statistical analysis

For profilometry, 10 scans were done on each type of surface, but using 3 specimens of each group. The roughness values were not so different among the 3 specimens and the parametric roughness data for the 10 scans presented a normal distribution. Since profilometry indicated that the roughness was similar for samples prepared using the same methodology, for AFM and SEM only one sample was analyzed on different zones and at different amplifications. For contact angle, four drops were measured in each sample and the left and right angles were calculated, leading to 560 angles for each liquid. The results were analyzed using the IBM SPSS® Statistics 20 software where ANOVA and post-hoc test (Tukey) were performed to compare between the two liquids. The cell cultures were done by triplicate. Finally, the RT-qPCR analysis was done using the GraphPad Software Prism 6.0 ® and the statistical significance was determined by Two Way ANOVA and multiple comparisons with Tukey. ?? values <0.05 were considered to be significant.

3. Results

3.1. Surface characterization

Fig. 1 shows the topographic features of the six samples at the different length scales and amplifications. A representative profile for each sample is shown in the first column of Fig. 1, indicating also the average roughness values, R_a (2D profile). It can be seen that the R_a values ranged between 2.0 and 2.6 μm. Both metallic surfaces (cp-Ti and SS316L) showed similar profiles with acute and noisy peaks regularly spaced in agreement with the SEM (second column Fig. 1) and AFM

(third column Fig. 1) images that showed the longitudinal scratches produced by the one-directional roughening process. Such scratches are also observed for the PMMA (1D) sample, but not so clear for the HDPE (1C); the roughness profiles of both polymers are not so different between them, but the peak-valley distances are definitely smaller in comparison to the metallic surfaces. Nevertheless, the average micro-

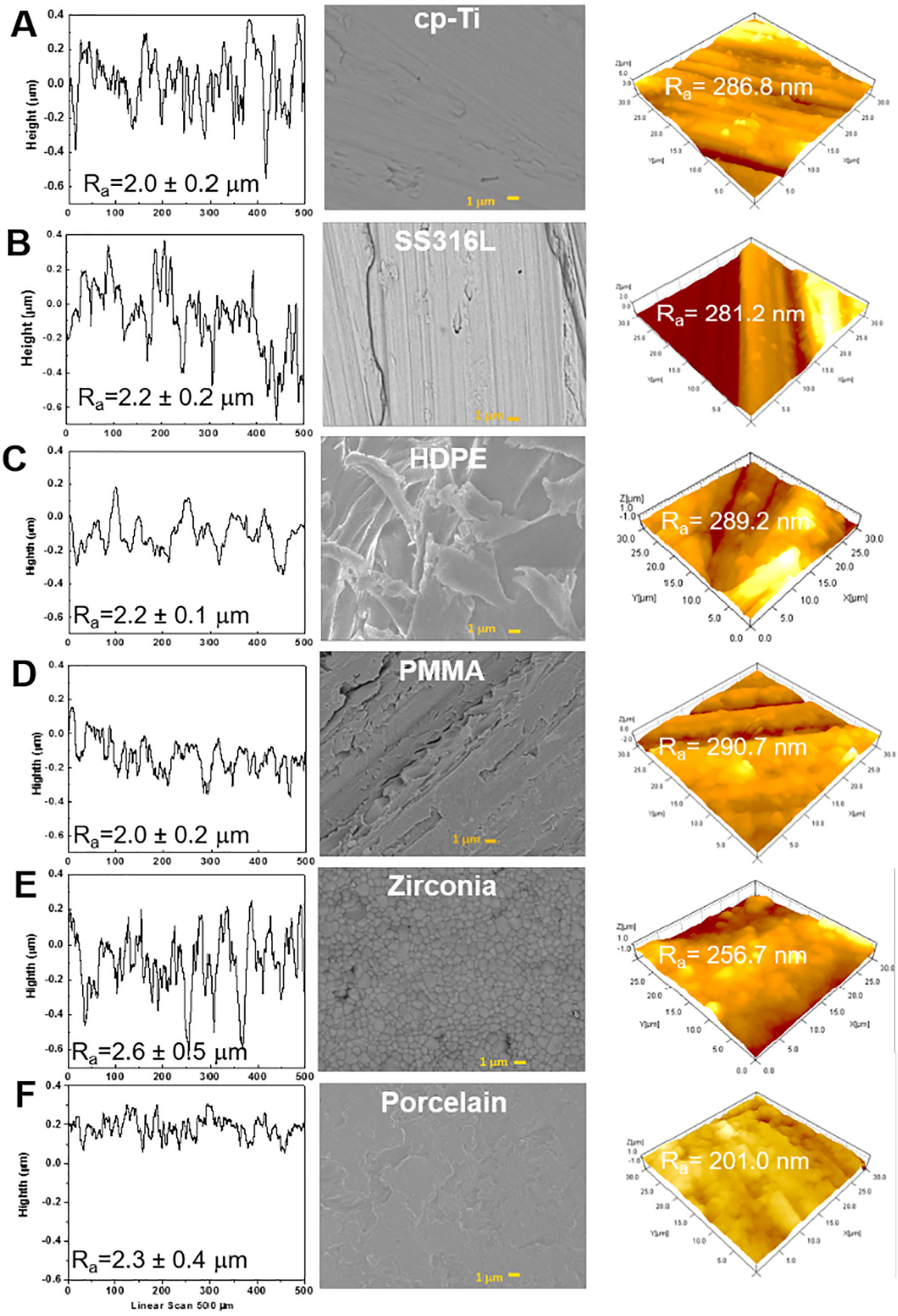

Fig. 1. 1A) cp-Ti, 1B) SS316L, 1C) HDPE, 1D) PMMA, 1E) zirconia and 1F) porcelain. Profilometry, random line scan 500 µm (axis x) and axis y: −0.6 to 0.4, Scanning electron microscopy images (SEM 5000 X) and atomic force microscopy (measured at 30 µm × 30 µm × 3 (axis z) amplification).

roughness values are 2.0 and 2.2 μm for the PMMA and the HDPE, respectively. Finally, the ceramic samples showed slightly larger average roughness values; 2.6 μm for zirconia (1E) and 2.3 μm for the porcelain (1F). The profile is smoother in the porcelain with shallower peak-valley distances.

The results presented in Fig. 1 indicated that although the average roughness (R_a) values were similar, the micro-scale topography of the samples is not properly represented by this parameter, i.e. the R_a values are not so different but the features and their organization looks very different for each sample. In order to fully and properly characterize the surfaces, it might be necessary to measure other profile parameters or perform analysis of the fractal dimension of the surfaces, so that the value reported for the surface roughness really represent the visual aspect observed by SEM [51].

At the nano-scale range observed by the AFM measurements (third column), we can see that again the S_a surface average roughness (3D) is not so different among the samples; it ranges from 201.0 nm for porcelain (1F) to 290.7 nm for the PMMA (1D), i.e. a variation of 90 nm, but again the features appeared different among the samples.

3.2. Wettability

The contact angles (CA) measured for both liquids (deionized water and fetal bovine serum 10%) are summarized in Fig. 2. One important result is that the hydrophilic-hydrophobic character of the samples is the same when water or FBS are used; no significant statistical differences were observed. On the other hand, following the criteria of Vogler [37,38], three surfaces showed a more hydrophilic character, while the other three were clearly hydrophobic. Stainless steel and the two polymers showed contact angles slightly above 65°, which is the lower limit defined by Vogler [38] for hydrophilic surfaces. The ceramics and the titanium surfaces showed a clear hydrophobic character, being the porcelain the most hydrophobic of the six materials with CAs around 20°. For smooth samples, one could say that larger CAs are an indication of larger surfaces energies and therefore stronger electrostatic and polar interactions between the surface and the biomolecules. However, for rough surfaces, the CAs are also determined by the topography and not only by the surface energy.

3.3. Surface charge (point of zero charge PZC)

The pH value corresponding to the PZC was obtained from the inflection point in potentiometric titration curves (the maximum value in the differential curve). The PZC values were compiled in Table 2. Most of the PZC values (SS316L, zirconia, Porcelain and HDPE) are close to 7.0, indicating that under the culture conditions, where the pH was fixed at 7.0, these surfaces will be neutrally charged. However, PMMA and cp-Ti surfaces showed PZC values of 4.8 and 4.6, respectively. Accordingly, at the pH of 7.0 used for the cell cultures, these two materials will present negatively charged surfaces that could create electrostatic interactions between the cell-membrane and the surface, not possible on the other samples.

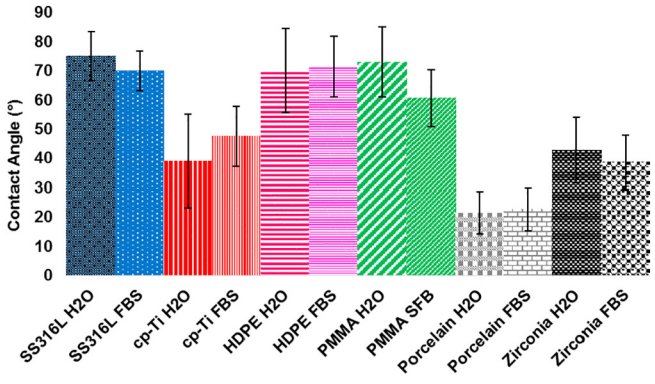

Fig. 2. Contact angle for both water and fetal bovine serum (10%) solution. The wettability was significantly different among the samples ANOVA $p \leq 0.05$. No statistical difference was observed between both liquids for the same sample.

3.4. Cell proliferation

Our results show that the six surfaces exhibited high proliferation rates after 4 days in culture, with the exception of PMMA surface that shows a decrease of about 50% in the proliferation in comparison to the control. The cp-Ti exhibited 85% cell proliferation compared to control at 96 h. The HDPE surface also permitted cell proliferation at high rate since after 96 h there was an 87% cell proliferation compared to control. The zirconia, SS316L and porcelain showed above 70% when compared to control (Fig. 3). These results confirm that the materials tested were not cytotoxic.

3.5. Real-time reverse-transcriptase polymerase chain reaction quantitative (RT-qPCR)

Gene expression profiles of isolated cell fractions from HPLCs are shown in Fig. 4. Quantitative PCR demonstrated that bone markers such as BSP, which is a hydroxyapatite crystal nucleator, was highly expressed by HPLCs at 10 days when cultured on cp-Ti and PMMA surfaces (Fig. 4A). Osteocalcin, a molecule that promotes hydroxyapatite crystal growth, was observed to be highly expressed by most of the materials at 7 days, except by the HDPE surface (Fig. 4B). ALP, a protein which is closely related to the mineralization process was highly expressed at 15 days by all the surfaces tested. Nevertheless, on cp-Ti and HPDE, the ALP expression was larger than the other surfaces (Fig. 4C).

Cementum-related proteins such as HACD1/CAP and CEMP-1 are shown in Fig. 5. The HACD1/CAP was mainly expressed at the initial stages of mineralization by all the materials, except Zirconia. The surfaces of cp-Ti, SS316L and porcelain presented slightly but significantly larger expression than the others (Fig. 5A). CEMP-1 which promotes octacalcium phosphate crystal nucleation and participates during the biomineralization process was only observed in large quantities for cp-Ti at 15 days and on the HDPE after 5 days (Fig. 5B).

Three cartilage markers were tested on these surfaces (Fig. 6). SOX9 a transcription factor that mediates chondrocyte differentiation, was expressed by cells plated on all the surfaces. Nevertheless, cp-Ti was the most suitable surface for the expression of this molecule during the whole culture period, showing the higher levels at 10 and 15 days. At the initial stages of cartilage differentiation SS316L, zirconia and porcelain also showed values of expression similar to cp-Ti. Col II, a marker for the cartilage phenotype, showed the higher expression values on PMMA, HDPE and porcelain. Col X, a marker for fully differentiated chondrocytes, was highly expressed at 10 days by cp-Ti, followed by porcelain and zirconia surfaces, the mRNA values decreased at 15 days (Fig. 6C). Col XI, a marker for hypertrophic chondrocytes, was mainly expressed at 5 days for all the surfaces tested. However, cp-Ti showed similar values at all periods (Fig. 6D). Aggrecan, a marker for pre-hypertrophic chondrocyte, was elevated at 5 days in porcelain and zirconia surfaces and at 10 days by cp-Ti surface (Fig. 6E).

The adipogenic markers are shown in Fig. 7. PPAR-γ is an early marker for the adipogenic phenotype and was expressed by all the surfaces at all times tested. The levels of this marker on the cp-Ti increased

Table 2
The point of zero charge of the surfaces measured using the potentiometric titration method.

Material	SS316L	cp-Ti	Zirconia	Porcelain	PMMA	HDPE
pH	7.3 ± 0.1	4.6 ± 0.3	7.4 ± 0.1	6.7 ± 0.3	4.8 ± 0.2	7.2 ± 0.1

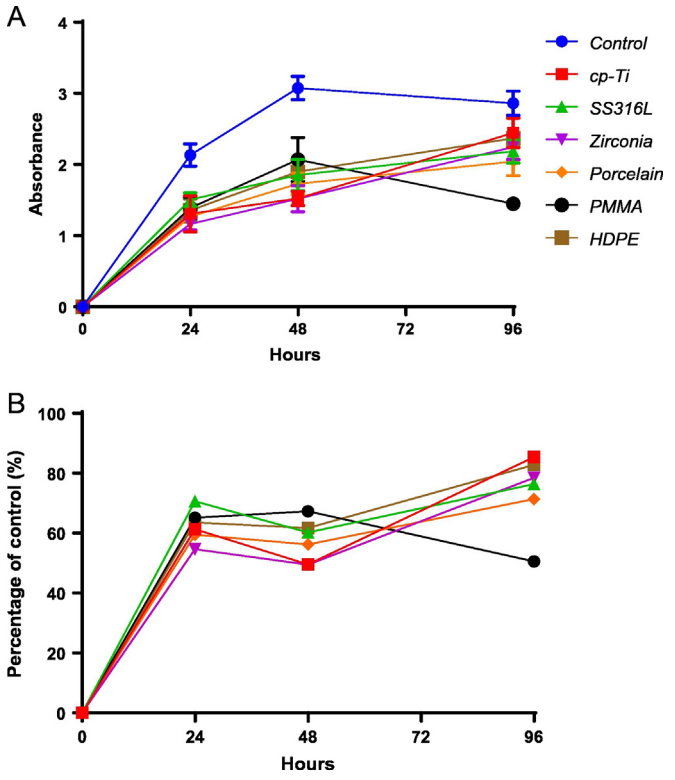

Fig. 3. Proliferation assay after 24, 48 y 96 h with periodontal ligament cells using the MTT assay. A. Absolute value of absorbance measured at 570 nm as indicated by the MTT assay. B. Percentage of proliferation estimated considering the control as the 100%.

as a function of time attaining the maximum values for 10 and 15 days in comparison to the other surfaces.

Meanwhile, the opposite trend was observed for the SS316L, being higher at 5 days (Fig. 7A). Finally, LPL, another marker for the adipogenic phenotype, was expressed at initial stages by the six surfaces tested. The values increased at 10 days on the cp-Ti surface but at later stages, it decreased for all surfaces and even no expression was observed on some surfaces (Fig. 7B).

4. Discussion

The aim of the current paper is to evaluate the possible correlations between the physicochemical properties, including the composition, of different biomaterials of common use in dentistry on the proliferation and potential of differentiation of human PDL cells. Therein, six different materials were selected; two metals, two ceramics and two polymers and we prepared all of them to present similar numerical average roughness. The roughness values were selected according to the information regarding the effect of the topography to improve the osseointegration [14,15]. However, the aim of the paper was to evaluate if the differentiation of periodontal ligament cells was influenced or regulated by the physico-chemical properties of surfaces belonging to three common types of materials; polymers, ceramics and metals. For such purpose, it was necessary to select one surface finish and since there is very few information about the correlation between topography and cell behavior for polymers and ceramics, we used the extensive data relating the osteoblastic differentation on metallic surfaces, which define the ideal roughness ranging between 2 and 4 μm [6,12,19]. The average roughness, R_a was chosen as a standardization parameter for the six surfaces trying to minimize the effect of the surface topography on cell response. Nevertheless, our first finding was that despite the average roughness value, R_a, were within the desired range of 2–3 μm, for which the literature have shown that the expression of bone markers such as ALP or OCN are not strongly modified [12], R_a was clearly not able to characterize the topography of the six different samples and significant variations in the topography were observed. The results are in agreement with Ponche et al. [36] argument that R_a is unable to characterize lateral roughness and that in general, more than one surface roughness parameter are required to fully characterize the surface topography. Therefore, we cannot discard that the differentiation patterns measured by RT-qPCR might be also affected by the diverse morphology of the samples observed qualitatively by the SEM images, since cell adhesion, morphology and proliferation have been shown to be dependent on the surface topography or architecture of the samples [6].

Periodontal Ligament Cells were used since their potential to differentiate into a wide range of cell lineages has been shown by different authors [23,27,28,30,52,53]. Moreover, the HPLCs are a potential source for periodontal regeneration, subject of interest for dental applications. However, few is recognized about the interaction between these cells and biomaterials [53,54], particularly it is not known if the lineage of the culture cells can be preserved and/or if the surfaces induce differentiation into specific phenotype. In this study, we cultured HPLCs on six different surfaces; 2 polymers, 2 metals and 2 ceramics for periods up to 15 days to evaluate the expression profile of osteoblasts, adipocytes, chondroblasts and cementum related genes. Lineage specific genes were evaluated after culturing the cells (5, 10 and 15 days) without adding any differentiation media by quantitative real time PCR. The surface properties of the six materials were characterized aiming to identify possible correlations between the gene expression profile of the HPLCs cells and the material's properties, such as surface composition, wettability and surface charge.

The proliferation results indicated that human periodontal ligament cells were able to attach and growth on all surfaces at a slightly lower rate than on the tissue culture plastic. Similar lower proliferation on rough samples has also been observed for titanium surfaces. Since 2002, Boyan et al. [10] showed that the cell (fetal rat calvaria cells) number decreased as the surface roughness increased from 0.6 to 3.97 and 5.21 μm in comparison to the control tissue culture plastic. Such a decrement has also been confirmed when using human osteoblasts or human mesenchymal stem cells culture on Ti alloys with roughness in a similar roughness scale [35]. In those cases, only differentiation into osteoblast lineage was investigated and the reduction in the cell number was associated to the maturation of the osteoblasts that stops cell division while producing the proteins associated to bone growth [55] and such production was enhanced by the micro-nano-roughness. We observed a similar reduction in the proliferation rate in comparison to the control for all the surfaces, which actually have similar roughness values, suggesting that the roughness induced reduction in the cell number is not unique for Ti-based surfaces, although nothing can be said about the effect of the specific topographic features (peaks, valleys, skewedness, etc.…).

The hypothesis proposed for this research was that the physicochemical properties of the materials were able to induce different microenvironments which in turns regulate the cell signaling and the expression of different genes or proteins related to different cell lineages. In accordance, we expected that the chemical composition and intrinsic properties of the family of materials (metals, ceramics or polymers) presented patterns that could be family-associated. However, after analyzing the RT-qPCR, we concluded that the expression and/or up-regulation of certain markers is not associated to any type of material, but as discussed below maybe associated to specific surface properties. An overall outcome was that we observed markers from the four lineages (osteoblasts, chondrocytes, adipocytes and cementoblasts) expressed on all surfaces. A plausible explanation might be related to the heterogeneity of the HPLCs, which contain mesenchymal stem cells with the potential to differentiate to osteogenic, adipogenic, cementogenic and chondrogenic cell lineages and to express the genes related to the above mentioned phenotypes. To some extent, the different cell lineages were able to attach and proliferate on the surfaces preserving their lineages, which is indeed a good result

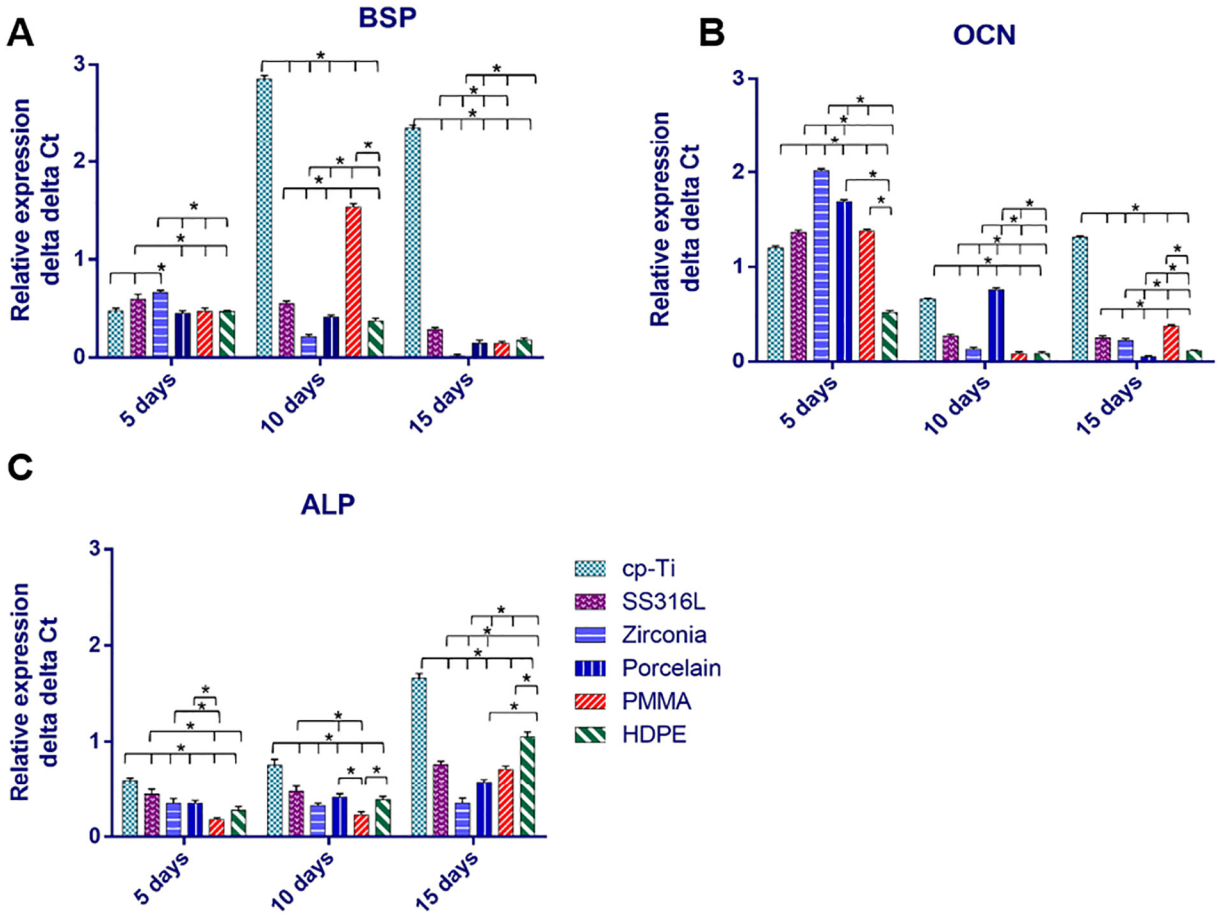

Fig. 4. Gene expressions of osteoblast markers including BSP (A), OCN (B) and ALP (C) after 5, 10 and 15 days culture with HPLCs without induction medium. Tukey's multiple comparison tests. Statistical significance * $p \leq 0.05$ ($n = 3$).

for the treatment of periodontal disease using cell therapies. Alternatively, the result could also indicate that the proposed methodology using heterogeneous cells and not induction media is not enough to evaluate the effect of the surface properties on the potential of differentiation.

However, if we concentrate on the markers that were up-regulated above the other surfaces and for more than one evaluation period, some interesting correlations can be observed. Osteoblasts markers were largely expressed on the cp-Ti surface; BSP was larger than on all the other surfaces at 10 and 15 days, OCN at 15 days and ALP was larger for the three evaluation periods and actually increased with time. Such results are clearly in agreement with the current knowledge about the extraordinary properties of the titanium to induce osteoblasts differentiation in vitro. However, cp-Ti showed up-regulation of SOX-9, Col X, Aggrecan, PPARγ and LPL markers too. Interestingly, cementum marker (HACD1/CAP) was up-regulated at the initial stages of mineralization (5 days) and markedly, cementum protein 1 (CEMP1) at the late stages of mineralization (15 days), indicating that cp-Ti surface promoted HPLCs differentiation toward a cementoblastic phenotype. Therefore, we could say that Ti has higher ability to preserve the cell lineages in

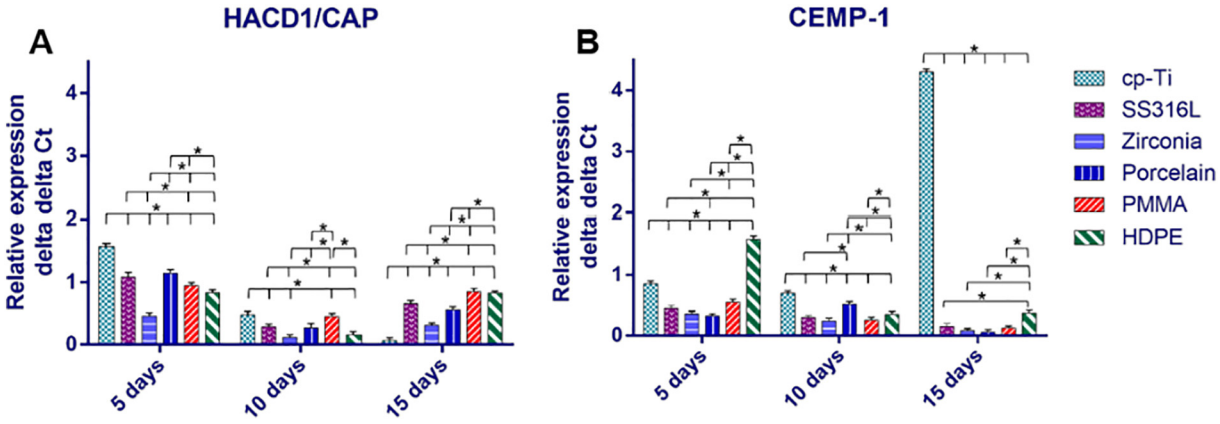

Fig. 5. Gene expression of cementoblast markers including HACD1/CAP (A) and CEMP-1 (B) after 5, 10 and 15 days culture with HPLCs without induction medium. Tukey's multiple comparison tests. Statistical significance * $p \leq 0.05$ ($n = 3$).

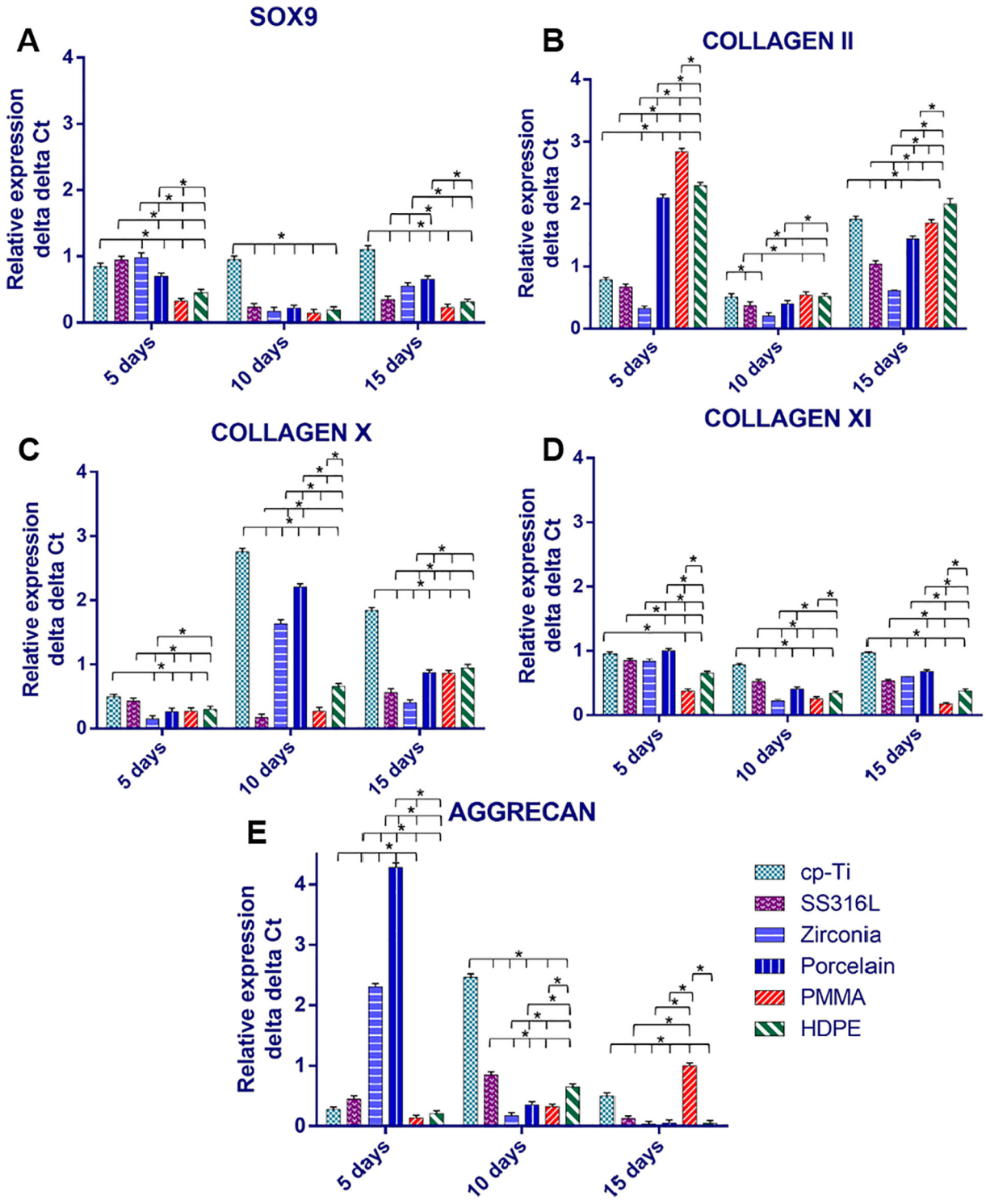

Fig. 6. Gene expression of chondrogenic markers including SOX9 (A), COL II (B), COL X (C), COL XI (D) and Aggrecan (E) after 5, 10 and 15 days culture with HPLCs without induction medium. Tukey's multiple comparison tests. Statistical significance * $p \leq 0.05$ ($n = 3$).

comparison to the other surfaces. The following material that presents up-regulation of more than one osteoblast-marker was the PMMA: BSP (10 days) and OCN (5 days). PMMA also showed up-regulation of Col-II and Aggrecan (15 days). These two surfaces; cp-Ti and PMMA have in common a lower pzc value (4.6 and 4.8, respectively) in comparison to the other 4 surfaces that showed pzc values close to the neutral pH. The pzc values lower than 7 indicate that under the culture conditions, these surfaces will be negatively charged while the others remain basically neutral. On the other hand, cp-Ti showed an hydrophobic behavior while PMMA was more hydrophilic. The combination of hydrophobicity and low pzc values makes cp-Ti completely different to the other surfaces. Hydrophobic interactions between the surfaces and the biomolecules in the media, including cells, are attractive and of a longer-range nature than the hydrophilic surfaces [56] and the presence of an electrostatic charge can significantly increase the distance and type of interactions.

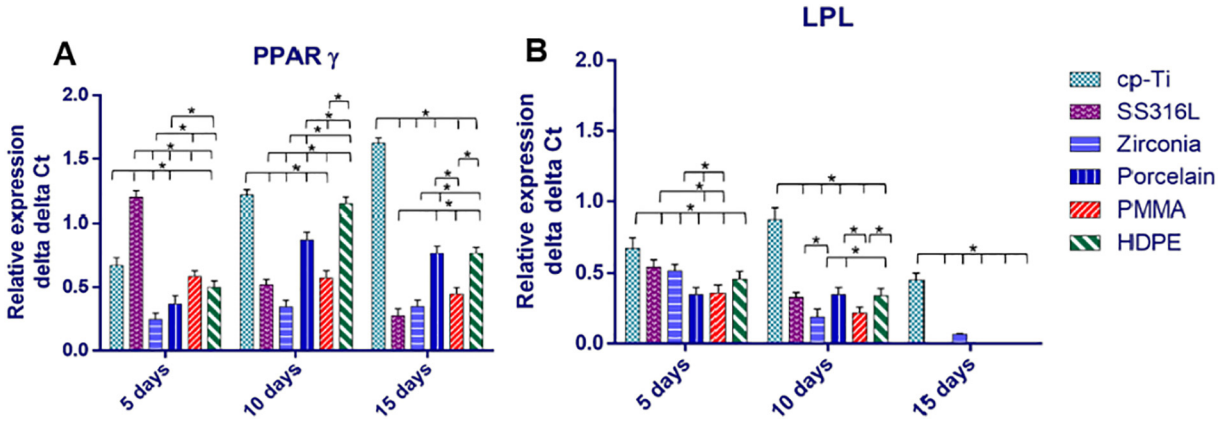

Fig. 7. Gene expressions of adipogenic markers including PPARg (A) and LPL (B) after 5, 10 and 15 days culture with HPLCs without induction medium. Tukey's multiple comparison tests. Statistical significance * $p \leq 0.05$ ($n = 3$).

The other two surfaces presenting hydrophobic character but not low pzc values were the ceramics. However, there was not a clear pattern of expression of markers; only Aggrecan (5 days) and Col-X (10 days) were up-regulated in both surfaces and Col-I on the porcelain at 5 and 15 days. These markers are all associated to chondrogenic lineages, where also cp-Ti showed up-regulation. Could this be an indicative that hydrophobic surfaces favored in some way the chondrogenic differentiation or preservation?. At this point, there is not enough evidence to support this as a conclusion, but it is certainly an interesting hypothesis to explore considering the current difficulties on the treatment of degenerative cartilage diseases including osteoarthritis [57].

5. Conclusions

The objective of the work was to explore if the physicochemical properties of different biomaterials could modify the proliferation and potential of differentiation of human periodontal ligament cells. The results indicated that the proliferation of HPLCs was similar on all surfaces and above 50%, suggesting that non cytotoxicity was observed. The reduction in comparison to the control is probably associated to the larger roughness of the samples. The analysis of gene expressions obtained by the RT-qPCR data was not very conclusive as to identify a particular pattern of differentiation associated to metals, ceramics or polymers, maybe due to the differences in the topography that could not be properly controlled or that the method used without adding differentiation induction media complicates the analysis leaving too many free parameters.

Nevertheless, the RT-qPCR data clearly showed that cp-Ti surfaces allow HPLCs to differentiate toward different cell phenotypes including osteoblastic, cementoblastic, adipogenic and chondrogenic, in addition to promote cell proliferation. Moreover, the combination of hydrophobicity and low PZC values makes Ti completely different to the other surfaces, probably explaining the up-regulation of a large number of genes on this surface. Therefore from our results we can conclude that cp-Ti showed the higher ability to differentiate the heterogeneous HPLCs population, including mesenchymal stem cells, when compared to the other surfaces.

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