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THE INFLUENCE OF SURFACE CHEMISTRY ON OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS.

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ABSTRACT – Calcium phosphate (CaP) based ceramics are used as bone graft substitutes in the treatment of bone defects. The physico-chemical properties of these materials determine their bioactivity and osteoinductive potential: macrostructure, microstructure and chemical composition. In a previous study, two tricalcium phosphate (TCP) ceramics were compared, TCP-S (small microstructure dimension) and TCP-B (large microstructure dimension), but several physico-chemical properties were varied. So, it is not known which of them was essential for osteogenic differentiation. Therefore, in the present study, all the conditions were maintained constant for all samples, except one: chemical composition of the surfaces. Hereby, three types of TCP-S based discs were compared. All the samples have similar microstructure but different coatings: TCP-S, TCP-S with carbon and gold coatings. Firstly it was confirmed that surface topography, the ion release and protein adsorption properties were the same regardless the surface chemistry. *In vitro*, both cell proliferation and osteogenic differentiation were enhanced over time. Nevertheless, no significant difference was found among the human bone marrow stromal cells (hBMSCs) cultured on different types of TCP-S samples. Regarding cell shape, there were no differences between the three groups of samples. The expression of osteogenic markers, collagen-I, alkaline phosphatase, osteopontin and osteocalcin, was also assessed and the results showed some significant differences among the three groups of samples. The current *in vitro* data reveal that these surface chemistries do not affect osteogenic differentiation of hBMSCs.

INTRODUCTION

Considering the increased incidence of osteodegenerative diseases in the rapidly aging populations of developed countries, there is considerable demand for bone substitutes. Although autologous transplant was the gold standard procedure, its clinical use is limited. Another important drawback is the need for a second operation that may lead to donor site morbidity, chronic post-operative pain, hypersensitivity and infection. However a large number of biological and synthetic substitute bone materials have been reported. They do not differ significantly in their clinical application and can be easily, cost-effectively, and efficiently used with minimum extra expense (1) (2) (3).

Calcium phosphate based (CaP) biomaterials aroused as a good example of substitute bone material due to their similarity in composition to the bone mineral (a calcium phosphate in the form of carbonate apatite) and similarities in some properties of bone that include biodegradability, bioactivity, and osteoconductivity (4). Another very important property of bone is the osteoinductivity that allows bone to repair and regenerate itself (up to a point). Materials, as CaP, that can induce bone formation are called osteoinductive materials. The material properties which so far have been suggested to play a role in osteoinduction are chemical composition, overall geometry of the implant and porosity. Microstructural surface properties, including grain size, microporosity, surface roughness and specific surface area have been suggested as critical factors in osteoinduction as well (5). The goal of the current study was to try to find out if surface chemistry itself could influence osteogenic differentiation. Here all the conditions were maintained constant for all samples, except chemical composition of the surfaces. Hereby, three TCP-S discs were compared. All with equivalent macrostructure and similar microporosity but different coatings: gold and carbon coatings comparing with TCP-S without coating.

MATERIALS AND METHODS

PREPARATION OF TCP-S, TCP-S WITH C AND TCP-S WITH AU DISCS

TCP-S discs (diameter of 9 mm, thickness of 1 mm) were kindly provided by Xiaoman Luo (Xpand Biotechnology BV). Prior to the coating process, TCP-S discs were ultrasonically cleaned in water. For carbon coated TCP-S was used a carbon coater (Edwards 306) at a voltage/current of 36V/60mA with vacuum 10x10-5 mbar for 4-5 seconds. Gold coated TCP-S (i.e. TCP-S with Au) was prepared using a sputter coater (Cressington Sputter Coater 108 Auto) by treating discs with two cycles of 90 seconds each with a current of the 30mA for coating the ceramic discs.

PHYSICOCHEMICAL PROPERTIES OF TCP-S, TCP-S WITH C AND TCP-S WITH AU DISCS

Chemical compositions of TCP-S, TCP-S with C and TCP-S with Au discs were determined using a scanning electron microscope (SEM, Philips XL30) coupled with an energy dispersive x-ray spectrometer (EDS, EDAX, AMETEK

Materials Analysis Division). Microstructure was studied with SEM in the secondary electron mode. The surface roughness was analyzed with an atomic force microscopy (AFM; PicoScan Controller 2500 – Quadrexed Multimode; Molecular Imaging). A super sharp TESP cantilever: 42N/m, 320kHz, 2-5nm ROC, No Coatings (Bruker AFM Probes.) was used in tapping mode. Images were taken at a scan rate of 1 Hz on 100 µm2 area was done at 3 different surface points for each sample (n=3) with Nanoscope (612r1®,Digital Instrument Veeco). Obtained images were analyzed with Scanning Probe Image Processor (SPIPTM, version 4.2.2.0) to obtain roughness measurements (Ra, Rq, and Rmax) and high quality 3D images.

Ion release measurements

To evaluate the influence of surface chemistry on the ion release, TCP-S, TCP-S with C and TCP-S with Au discs were immersed in 1 ml of basal medium (BM) and incubated at 37°C in humid atmosphere with 5% CO_2 for 1, 4, 7 and 14 days. Basal medium consists of Minimal Essential Medium-alpha (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Lonza Group Ltd), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), 2 mM L-Glutamine (Gibco) and 0.2 mM L-ascorbic acid 2-phosphate (ASAP, Sigma-Aldrich Co., A8960). Calcium and phosphate ion concentrations in culture medium containing samples and controls (i.e. BM without ceramics) were measured with Quantichrom™ Calcium Assay Kit DICA-500 (Bioassay systems) and Quantichrom™ Phosphate Assay Kit DIPI-500 (Bioassay systems) respectively, following the manufacturer's guidelines. Absorbance measurements of each standard solution and samples were performed with spectrophotometer (Thermo Scientific Multiskan GO UV/Vis Spectrophotometer) at 620 nm. Calcium and phosphate ion concentrations were calculated through standard calibration curves and expressed as mean ± SD. Three samples were used per material (n=3).

Protein adsorption

To calculate the amount of protein adsorbed by discs, TCP-S, TCP-S with C and TCP-S with Au discs were incubated for 1, 4, 7 and 14 days in 1 mL of basal medium at 37 °C in humid atmosphere with 5% CO2. The protein amounts remaining in the medium and adsorbed on the discs were measured with the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Absorbance measurements were performed with spectrophotometer at 562 nm. A calibration curve was prepared using standard BSA solutions.

In order to measure the amount of protein adsorbed by ceramics, 100 µl of RIPA buffer (Thermo scientific) was added to each sample after washing three times with PBS and kept at 4°C for 10 minutes. Thereafter the amount of protein detached from the ceramics into RIPA buffer was measured. The RIPA buffer consists of 10% RIPA buffer (10X) supplemented with protease phosphate and 89% demi water. Three samples were used per material (n=3) and the protein content was expressed as mean ± SD.

Expansion of hBMSC

The used hBMSC were obtained from three different donors at passage 2. The cells were stored in a liquid nitrogen vessel (Custom Biogenic Systems).

The cells were thawed directly to the medium and cultured in T-flaks (T175, ThermoScientific). For expansion of hBMSCs proliferation medium was used that contained basal medium and 1% of basic fibroblast growth factor (bFGF, Instruchemie).

Cells were cultured at 37°C in a humid atmosphere with 5% CO2, medium was refreshed twice a week and cells were subcultured until 90% confluence.

Cells distribution

Cells were fixed with 10% neutral formalin and stained with 1% methylene blue solution (Sigma-Aldrich) in order to observe the adherent cells on the discs, after washing with phosphate-buffered saline (PBS) solution three times.

Cell distribution on the discs was observed with a light microscope (LM; E600, Nikon SMZ-10A, Japan) equipped with KL 1500 LCD camera (SCHOTT).

Cell shape and morphology

Specimens for SEM were prepared for the high-vacuum imaging environment. Firstly, samples must be fixed to preserve their native structure. In this study, it was used specimens from methylene blue experiment. So, they were already fixed. Following fixation, samples were then dehydrated with graded ethanol (70, 80, 90, 95 and 100%) to ensure that all water was removed. Then they were dried using critical point drier (CPD 030. Balzers), mounted on appropriate stubs and sputter-coated with gold-palladium to prevent charging effects that hinder suitable image formation. Images were collected using SEM (Phillips). Then, in order to confirm the results obtained with SEM, cells were cultured under basic conditions for 4 days, fixed and immunohistochemically stained for actin with Alexa 568-Phalloidin (1:60 in 1%BSA, Invitrogen) and cell nuclei with DAPI (1:1000, Invitrogen). Fluorescence images of isolated cells were taken with the fluorescence microscope (NIKON ECLIPSE E600).

Metabolic activity

Metabolic activity of the cells was monitored using Presto Blue assay. A fresh presto blue working solution was made in a concentration of 10% presto blue solution (Invitrogen) in basal medium, avoiding the light. After aspiring medium from the wells, 1mL presto blue solution was added to each sample and the well plate was placed in the incubator at 37°C for 10 minutes. Thereafter, 200µl solution was taken from each well and placed in a white 96-well plate in triplicates, avoiding the light. The fluorescence was measured using a spectrophotometer (VICTOR3 1420 Multilabel counter Perkin

Elmer). The wavelengths of excitation and emission were 540-570nm and 580-610nm, respectively.

OSTEOGENIC DIFFERENTIATION – ALP STAINING

Sigma-Aldrich Alkaline Phosphatase kit was used to study osteogenic differentiation staining. The experiment was conducted following the manufacturer's protocol. After the 7 and 14 days of cell culture, the BM was removed and the samples were washed three times with PBS and fixed for 30 seconds. The fix solution was previously prepared mixing by citrate working solution (2 ml citrate concentrated solution with 100 ml deionized water) and acetone. Then these samples were rinsed with deionized water and then incubated with alkaline dye mixture for 30 minutes, avoiding the light. This alkaline dye mixture was constituted of 2 ml Naphtol AS MX-PO4 and 48 mL prepared diazonium salt solution, which was prepared by dissolving one fast blue salt capsule in 48 ml distilled water, using magnetic stirrer at room temperature. Finally, the samples were washed 3 times with deionized water and observed under the stereo microscope (LM; E600, Nikon SMZ-10A, Japan).

OSTEOGENIC DIFFERENTIATION ASSAY (ALP ACTIVITY)

Cell osteogenic differentiation was measured with an alkaline phosphatase (ALP) assay (CDP-S*tar* assay kit, Roche). Before the analyses, the samples were rinsed three times with PBS and stored at -20°C until further use. 500 µl of DNA cell lysis buffer (prepared according to manufacturer's instructions of CyQuant Cell Proliferation Assay kit instructions) was added onto each sample, followed by two cycles of freezing and thawing at -20°C and room temperature, respectively. After these two cycles, an ALP assay was done immediately according to the manufacturer's instructions. Briefly, 10 µl of cell lysate and 40 µl of CDP S*tar* substrate were incubated in white 96-well plate for 20 minutes avoid light. In the end the luminescence was measured using a spectrophotometer (VICTOR3 1420 Multilabel counter Perkin Elmer). ALP expression was normalized to DNA content. Three samples were used per material (n=3).

BONE-RELATED GENE-EXPRESSION

Bone-related gene expression was evaluated with quantitative real-time polymerase chain reaction (PCR) assay. RNA isolation was performed using Trizol reagent (Invitrogen) and Nucleospin RNA isolation kit (Macherey-NagelGmbh& Co.) according to the manufacturer's instructions. Total RNA was measured using a NanoDrop spectrophotometer (Nanodrop technologies, USA). The RNA was used to synthesize complementary DNA (cDNA) with iScriptcDNA Synthesis kit (BioRad) according to the manufacturer's instructions. PCR analysis was performed with the Bio-Rad real-time PCR system (Bio-Rad, Hercules, CA, USA) on markers of alkaline phosphate (ALP), collagen type I (Col I), osteocalcin (OC), and osteopontin (OPN), with beta-2 microglobulin (B2M) as the house-keeping gene for normalization. Primer sequences for ALP, Col I, OC, OPN, and B2M are listed in Table 1. Finally, the real-time PCR reaction was run at 95 °C for 10 min followed by 95 °C for 30 s; 60 °C for 1min and 72 °C for 1 min for 40 cycles; the dissociation curve at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15s. Data was analyzed using Bio-Rad iQ5 software. The relative amounts of target genes normalized by B2M were calculated by 2-ΔCT method where $\Delta CT = CT_{,Target} - CT_{,B2M}$. All experiments were done in triplicate (n=3).

Table 1 - Primer sequences of the studied bone-related genes.

Gene	Forward primer	Reverse primer
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
COL-I	AGGGCCAAGACGAAGACATC	AGATCACGTCATCGCACAACA
OCN	GGCAGCGAGGTAGTGAAGAG	GATGTGGTCAGCCAACTCGT
OPN	CCAAGTAAGTCCAACGAAAG	GGTGATGTCCTCGTCTGTA
B2M	GACTTGTCTTTCAGCAAGGA	ACAAAGTCACATGGTTCACA

RESULTS AND DISCUSSION

PHYSICOCHEMICAL CHARACTERIZATION OF DIFFERENT TCP-S DISCS

The EDX results of TCP-S, TCP-S with C and TCP-S with Au discs showed all the discs had Ca, P, O elements (Figure 1A). TCP-S with C discs showed an extra peak of carbon and TCP-S with Au discs had an extra peak of gold. Surface microstructure of the discs was observed with SEM (Figure 1B), showing no differences of surface topography among TCP-S, TCP-S with C and TCP-S with Au discs. Therefore, the surface coatings of carbon and gold did not change the surface microstructure. Similar results were confirmed by AFM results (Figure 1C): no differences in surface topography were observed with AFM images (Figure 1C).

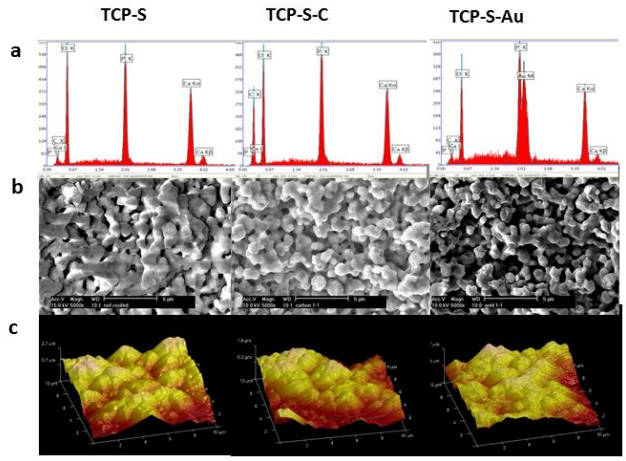

Figure 1 - Chemical compositions (A), SEM pictures of surface (B), AFM topography (C).

ION RELEASE

Figure 2A depicts the results obtained on calcium concentration: in all solutions, the calcium concentration decreased from day 1 to day 14 as compared to BM indicating that calcium was not released from the ceramics, but was rather adsorbed on the discs. There was no significant difference between the three samples, which means that calcium ion release is the same whatever the coating. It is also important to note that the concentration of calcium ion in the medium is always lower than the controls. On the other hand, the analysis of phosphate concentration (Figure 2B) shows that the concentration of phosphate ion accumulated in the medium decrease until day 4 as compared to basic medium. From this day there is an increase of concentration but it is always lower than the control. It seems that phosphate ions precipitate until day 4 and after that day, they are released. The simplest possible explanation is that phosphate ions start to dissolve again.

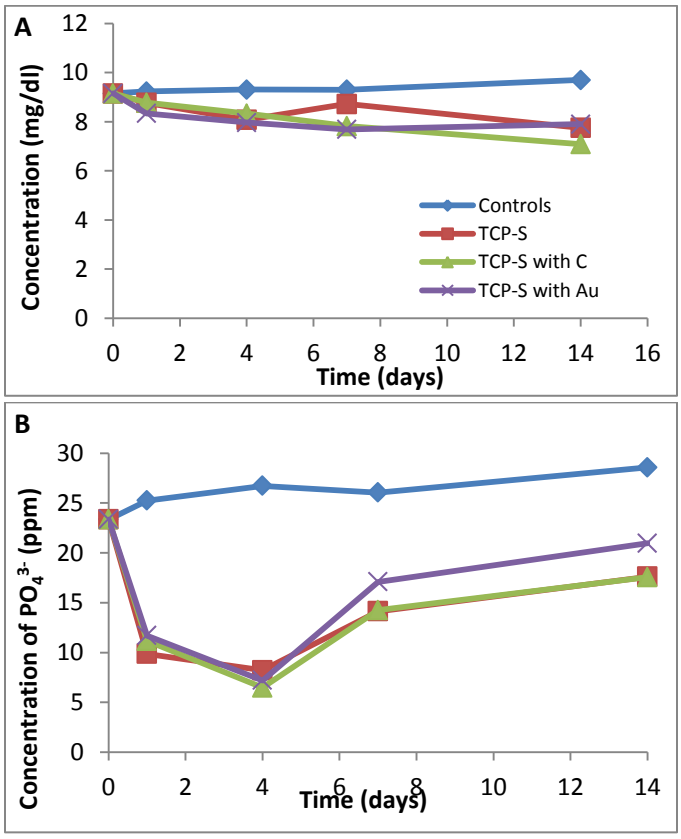

Figure 2 – Calcium (A) and phosphate (B) ions exchange profile.

Neither in calcium profile nor in phosphate profile significant difference was found among the different samples. The results showed that all the ceramic discs have similar calcium and phosphate ions exchange profiles respectively. This is an important statement because in the case of CaP containing materials, ion exchange between the material and the medium may significantly modify the composition of the latter, and hence the environment for cells to grow and differentiate. Furthermore, changes that take place in the medium will vary depending on the properties of the material, resulting in a study where same cells are cultured in different environments, making comparisons between them difficult and less reliable, but that is not the case here.

PROTEIN ADSORPTION

To measure the quantity of proteins, two methods were used: In the first method it was quantified proteins on the medium while in the other method it was measured the amount of proteins adsorbed on the discs. From Figure 3A, the amount of proteins in the medium did not change over time. No significant differences in the amount of proteins in the medium were found among the samples and also compare to the controls at all the time points. From the results showed in Figure 3A it is not evident if there was adsorption of proteins or not. In order to try to find out the amount of protein adsorbed on the discs has been calculated. RIPA buffer was used to separate the proteins from the discs. Once these proteins were separated it is possible to quantify their concentration. In this way it is clear that the discs adsorbed proteins from the medium, however no differences were found between the discs (Figure 3B). So, taken together, all the samples showed the similar calcium and phosphate ions exchange and protein adsorption potential. Therefore, in this study, it can be assumed that the effect of ions exchange and protein adsorption as a constant parameter in study of cellular behavior of hBMSCs on the TCP-S, TCP-S with C and TCP-S with Au discs.

HBMSCS ATTACHMENT AND PROLIFERATION

To evaluate the distribution of hBMSCs on TCP-S, TCP-S with carbon and TCP-S with golden coatings, cells were cultured for 1, 7, 14 and 21 days and stained with methylene blue. As observed with stereo microscopy, hBMSCs were homogeneously distributed on all types of TCP-S discs 1 day after cell seeding and

at later time points the staining was more intense (Figure 4), which means that TCP-S discs, regardless the type of coating, supported hBMSCs attachment, growth and proliferation, whatever the type of coating. There was no significant difference in cell adhesion regardless of the different chemical composition of the coatings. It means these surface chemistries do not affect cells attachment. There should be other parameters playing a dominant role, for example microporosity.

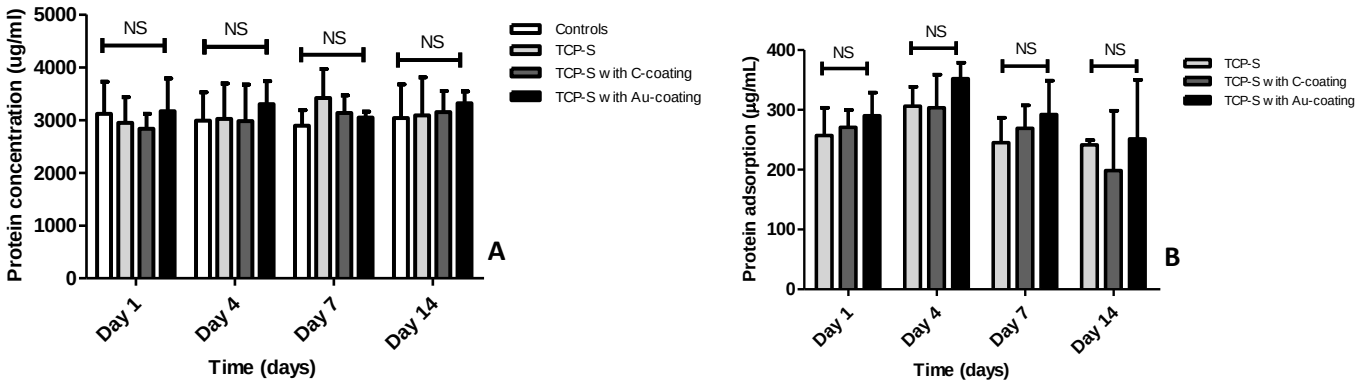

Figure 3 - Concentration of protein in the culture medium (A) and adsorbed on the discs (B).

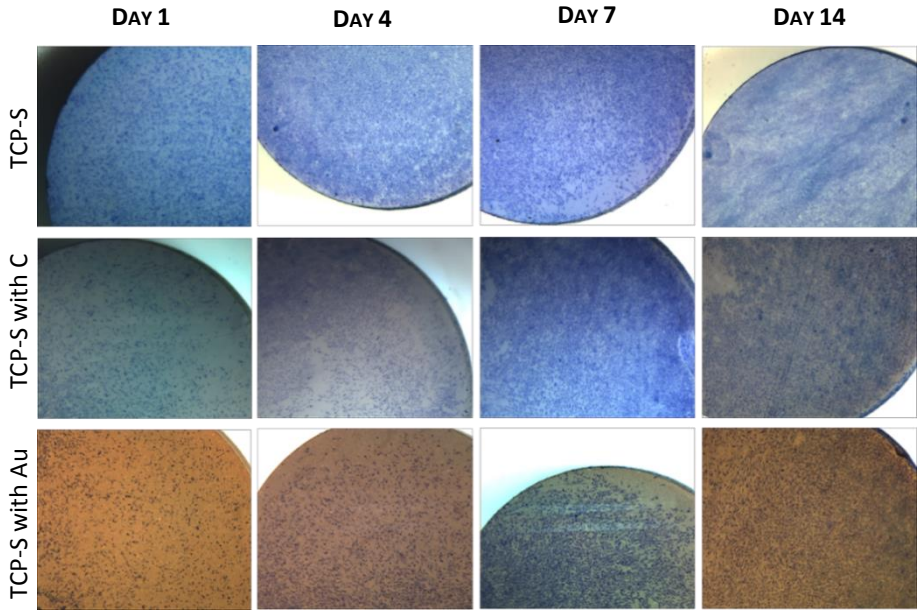

Figure 4 - hBMSC distribution on TCP-S discs with different coatings.

CELL SHAPE AND MORPHOLOGY

At day 1 and 4 of culture (Figure 5), the cell shape of hBMSCs cultured on the various types of TCP-S was very similar. The morphology of the cells growing on the ceramics studied initially showed a spreading habit with long and thin extents. After day 7 hBMSCs show a flattened appearance and presented a close contact with the material. Furthermore cells increased in number and formed aggregates, partially coating the surface of the ceramics. The cells from this day also presented abundant filopodia and intercellular connections.

Although the images from Figure 5 allow an analysis of the cell shape, they were obtained to study cell attachment to these different coatings. So, to confirm that morphologies achieved with SEM are characteristic of this type of cell and of these time-points, an immunofluorescence protocol was used. After 4 days of incubation in basal medium. hBMSCs were fixed and

stained for actin (Figure 7). Cells exhibited a characteristic spindle-shape phenotype with actin fibers extending across the cytoplasm arranged in several directions, as revealed by Alexa Fluor 594 phalloidin staining (Figure 7, A-B). During culture time of hBMSCs on different coatings, there were no differences in actin cytoskeleton organisation which then resulted in similar morphologies of cells cultured in basic medium. Cells grown in different discs appeared always with spindle-shape morphology (Figure 7).

Several authors (6) (7) (8) proposed that the hBMSCs shape change from fibroblast-like morphology to a more flattened and polygonal morphology when they are moved from basic medium to an osteogenic medium culture. In the present study, as hBMSCs were cultured under basic medium and their cell shape was not altered, it can be therefore assumed that this surface chemistry alone is not responsible for osteogenic differentiation.

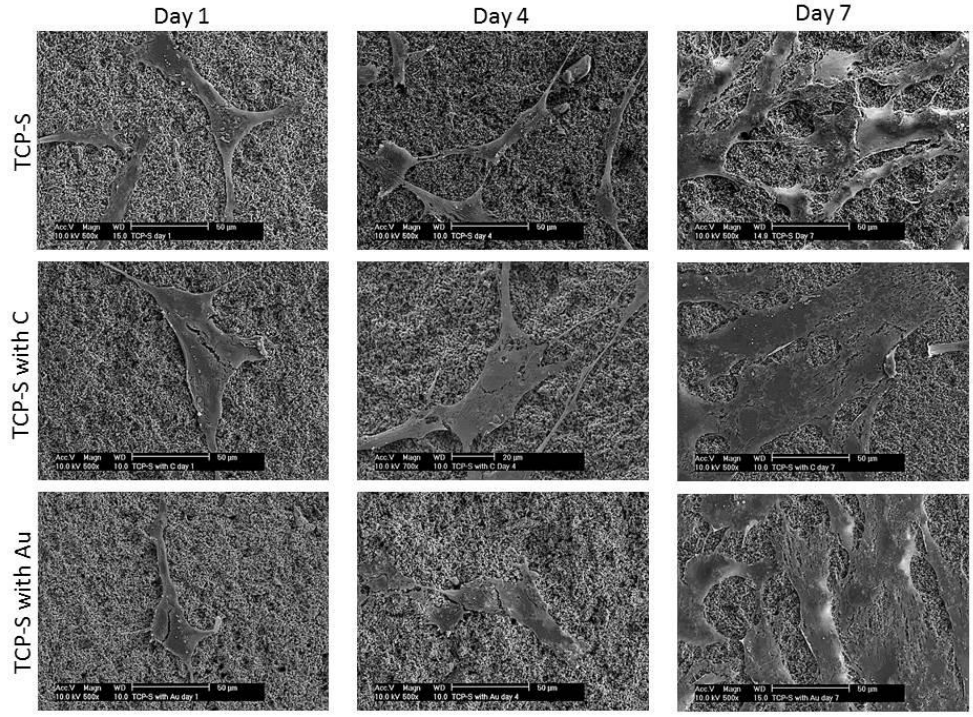

Figure 5 – SEM images of the hBMSCs growing on the TCP-S discs with different coatings after 1, 4 and 7 days.

METABOLIC ACTIVITY

Metabolic activity of the cells cultured on TCP-S discs without coating increased along the culture days, as expected, since at least the number of cells increases over time (Figure 6). hBMSC on TCP-S with C or Au coatings had an increase of their metabolic activity only from day 4 to day 7, as between days 7 and 14 there was no increase. At both day 4 and 7, no significant difference in metabolic activity of hBMSCs was found among the three types of coating. However, at day 14 the metabolic activity of hBMSCs cultured on TCP-S discs were significantly higher than the cells cultured on TCP-S with C and TCP-S with Au. A possible explanation for this could be that in the early days hBMSCs are proliferating and as it shown in Figure 4 cells seem to grow equally on all samples. Hence there were no significant differences in the metabolic activity at days 4 and 7. At day 14, on the other hand perhaps some cells begin to differentiate, being this differentiation more evident in TCP-S discs without coating.

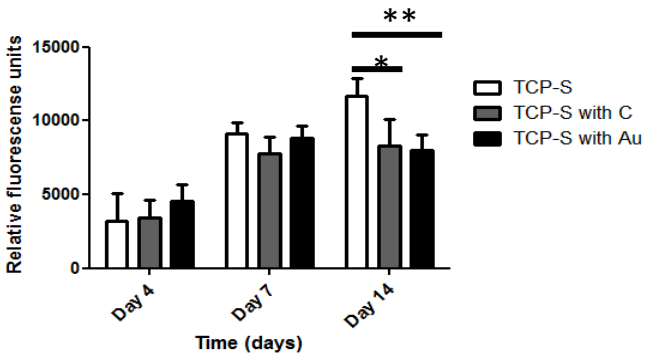

Figure 6 - Metabolic activity of hBMSC on different TCP-S discs over culture time.

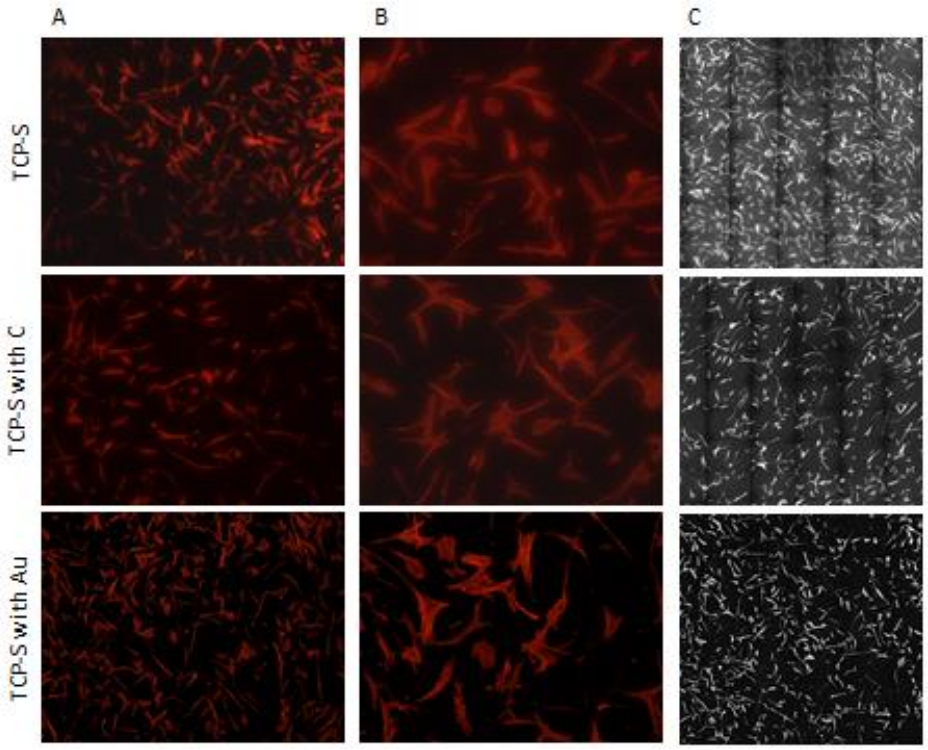

Figure 7 - Immunohistochemical staining of hBMSCs cultivated in BM against actin after 4 days of cultivation. Representative images are presented here with different magnifications 4X (A) and 10x (B). Cells were stained with fluorescent phalloidin and images were converted to grayscale where actin is white (C)

It is known that the surface properties of a biomaterial affect metabolic activity as well as affect the cell adhesion and many other biological responses of cultured cells (9). In this study, the difference in surface properties was adjusted by varying the chemical composition of the coating. hBMSCs could not recognize the difference of the surface characteristics on various coatings, and still revealed a similar behavior in the performance of metabolic activity at days 4 and 7. hBMSCs probably could not distinguish those differences and only revealed slightly different levels of metabolic activities, excluding on day 14 where differences were significant.

The non-existence of differences may be due to the fact of roughness is a constant parameter (9). Besides, the metabolic activities of hBMSCs on the TCP-S with various coatings were always lower than those on the TCP-S without coating. However, this method tells us nothing about the type of metabolic activity. It is required to study osteogenic differentiation itself.

OSTEOGENIC DIFFERENTIATION

The osteogenic differentiation was detected by alkaline phosphatase (ALP) staining at a 7 and 14-day time point. ALP is a generally used marker for early osteogenic differentiation. Osteogenic differentiation of hBMSCs was confirmed by a positive ALP staining at day 7 and 14 for all discs (Figure 8). From the images is evident that all showed ALP staining. At day 7, ALP expression is similar for all type of discs. In contrast, at day 14 there seems to be more ALP expression on discs without coating than the others, which may represents an increase on osteogenic differentiation.

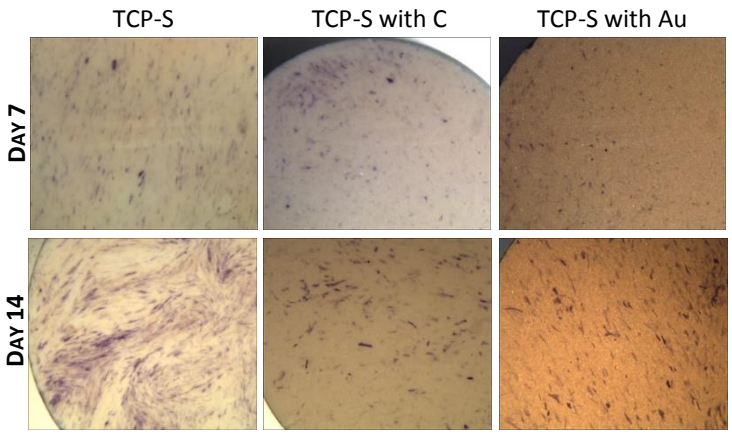

Figure 8 - ALP staining for the osteogenic differentiation.

Although ALP staining is mainly used as a qualitative method, if a quantitative study is desirable, it is certainly

necessary to employ another assay, such as ALP activity measurement.

ALP activity of hBMSCs was then detected and quantified via an enzymatic assay (CDP *Star*) at day 7 and 14 of cell culture.The ALP activity of hBMSCs normalized by presto blue content over time, cultured on TCP-S, TCP-S with C and TCP-S with Au is shown in Figure 9. Here, no differences in ALP activity of hBMSCs were observed at day 7. However, the hBMSCs on TCP-S with Au discs had significantly higher ALP activity at day 14 time point than TCP-S with C and TCP-S without coating indicating an enhanced osteoblastic phenotype of hBMSCs on TCP-S with Au. It should be noted that ALP activity of hBMSC was enhanced from day 7 to day 14 without osteogenic reagents (L-ascorbic acid, glycerolphosphate and dexamethasone).

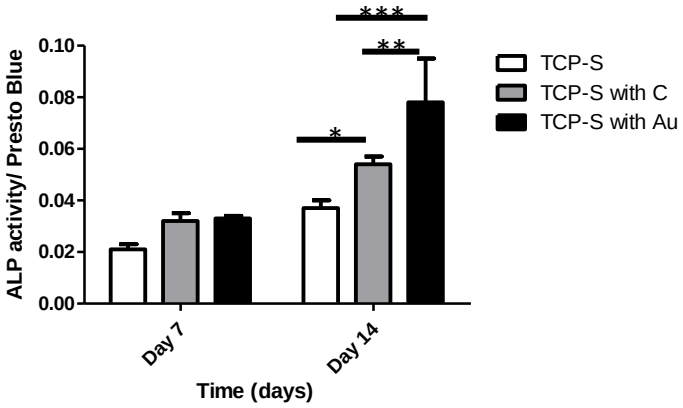

Figure 9 – ALP activity normalized by metabolic activity.

This may indicate that these cells are moving towards an osteogenic lineage only due to the TCP's properties. Nevertheless, in order to confirm this trend, the analysis of another marker of bone formation like osteocalcin could be relevant.

BONE-RELATED GENE-EXPRESSION

In this study, it was developed quantitative RT-PCR assays (Figure 10) for genes encoding the most typical osteoblast-related membrane and extracellular matrix molecules (i.e. ALP, Col-I, osteocalcin (OC), osteopontin (OPN)). Gene expression of Col-I, ALP and OC were higher at day 7 compared to day 4 and 14; OPN expression decreased from day 4 to day 7 and then slightly increased after day 7 until day 14 on all the samples. No significant differences in gene expression of Col-I were found among TCP-S, TCP-S with C and TCP-S with Au at any time points tested. As for OC and ALP gene expressions it was noted as being significantly higher on TCP-S on day 14 when compared with TCP-S with Au which indicates that surface chemistry had indeed some influence on this expression pattern. As ALP is an early marker and OC a late one, it would be easier to justify if the differences were found in ALP and OC expressions on day 7 and on day 14, respectively. Still regarding the OC, its expression was significantly higher on TCP-S discs when compared to TCP-S with C at day 14. On the other hand, OPN gene expression decreased from day 4 to 7 and increased from day 7 to day 14 on both coating materials and it was significantly lower on TCP-S at day 4 as compared on TCP-S with Au. In this experiment there seemed to be a small effect of surface chemistry on expression of genes evolved in osteogenic differentiation as suggested by ALP activity assay (Figure 9). However, the results found in these two experiments were different. Actually, contradictory results between gene expression and proteins had been presented in some studies (7) (10). With the intent to getting more solid results the experimental procedure could have been repeated with more donors in order to analyse whether the results are consistent or not. Due to the fact that not all the mRNA originates protein, it is usually to rely more in the results at the protein level. So, to study the expression of OC and OPN at the protein level could also be of interest.

In this case it is difficult to affirm with certainty that surface chemistry has influence on the expression of these osteogenic markers since the experimental outcomes obtained were different between gene and protein levels.

Another important aspect to this discussion is related with the lineage-specificity of certain markers. Some recent studies have shown that some of these markers are shared between osteogenic and adipogenic differentiated hBMSCs (11). For this reason, many studies still have to be conducted in order to understand how these proteins and signaling pathways are involved in the differentiation process. Nevertheless, it is still important to search for new markers that function as tools for evaluating the lineage commitment of hBMSCs.

CONCLUSIONS

The aim of this master thesis was to study if different surface chemistry affected cellular behaviour of hBMSCs. Several experiments were performed concerning cell proliferative capacity, metabolic activity, morphological differences and osteogenic differentiation.

To study the discs properties, measurements on the amount of calcium and phosphate ions in the culture medium and also on the protein adsorption were carried out. In none of these assays significant differences were found between each type of coating, allowing us to conclude that the study was performed under the same culture environments, regardless the type of coating.

Regarding to *in vitro* studies, no differences in cell proliferation nor in cell shape were found. However, metabolic activity was enhanced in TCP-S discs without coating at day 14. Different trends were obtained among

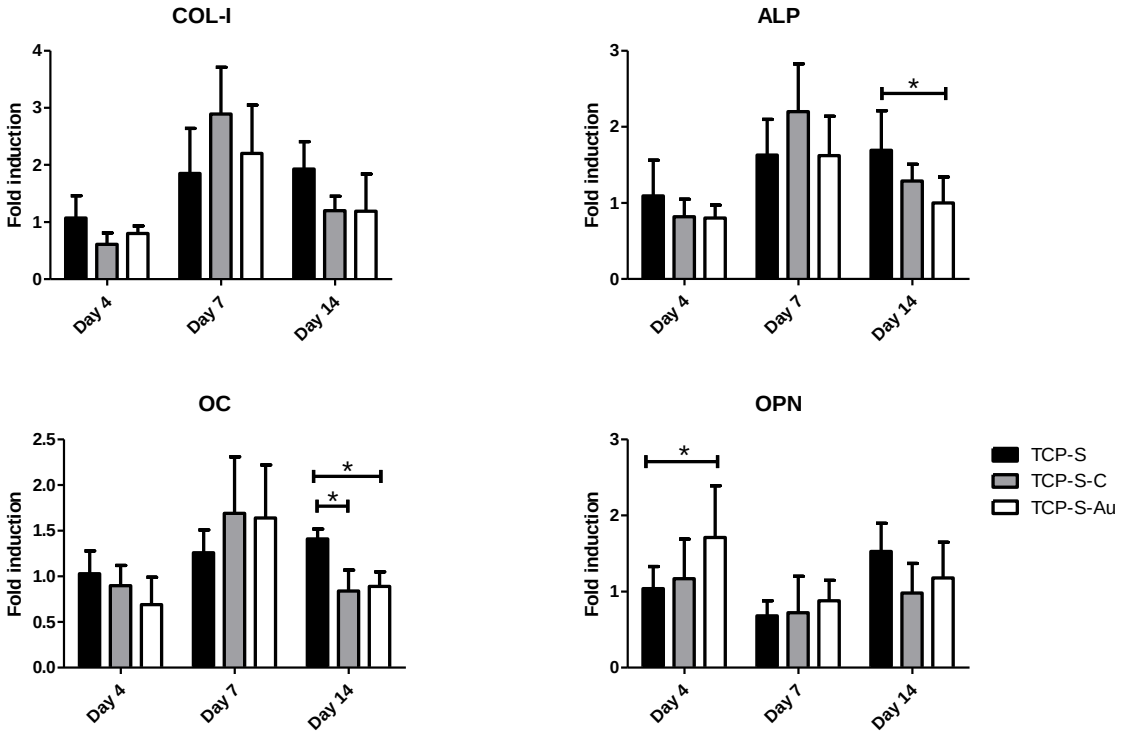

Figure 10 - Osteogenic gene expression of bone specific markers for hBMSCs cultured on TCP-S with different coatings (normalized to the B2M).

ALP, OC, OPN and Col-I expressions. On the other hand, the expression of ALP marker at the protein (Figure 9) and genes level (Figure 10) was contradictory. But generally, although there were some differences, it can be concluded that these coatings themselves do not cause differences in cell behaviour.

As the main goal of this study was to understand the influence of a single parameter in osteogenic differentiation, only the surface chemistry of TCP-S discs was changed between experiments. It is important to test only one variable at a time because if there is more than one being tested, and the outcome differs between experiments, it would be more difficult to safely assume which variable was responsible for a certain change in the outcome. If only one is being tested though, it is easier to conceptually recognize the cause of a certain change and it can also be easier to measure it independently. In most of the studies using different coatings at least two properties were varied at the same time: surface roughness and surface chemistry (12). Almost all concluded that different coatings imply different cell behaviors. However, since two parameters were varied, it was not possible to identify which of the two parameters was primarily responsible for a certain behaviour. Hence, the importance of the studies that vary only one factor at a time.

Nevertheless, other studies should be carried out to prove that these coatings have no different influences on cell behaviour. Beginning with the study of the discs properties, a better option would possibly be to perform the coating procedure on the entire disc instead of only on one side, in order to avoid a direct influence of the chemistry of the calcium phosphate material in the culture medium. Regarding the study of cell shape, it

would be much more credible if a quantitative study with a cell profiler was carried out. As it was mentioned before, the majority of the studied genes could not be lineage-specific. Thus, they may not allow to conclude if these cells were differentiated into bone cells, or not. In order to understand these results, the expression of adipogenic genes should be measured. If their expression became lower or null, it might lead to believe that hBMSCs were in an osteogenic differentiated state. In addition, it would also be important to study the mineralization process, i.e. if these cells (because they differentiate into osteoblast cells) are able to induce bone mineralization. Nowadays these studies are made histologically through staining with Alizarin red. However, due to the nature of this process and the fact that the discs are made of calcium, this process would be ineffective in the present study. Thereby, it is necessary to find alternative strategies to the staining with alizarin red.

To conclude, a better understanding of how stem cells respond to substrate cues is crucial for a deeper scientific knowledge on the mechanisms regulating stem cell fate decisions and thus for their potential in Regenerative Medicine. With this master thesis study it can be concluded that the results given by the set of both the topography and the chemistry of TCP is not inseparable, as can have the same positive influence on cells given by TCP but with certain different chemistries. This means that changing the surface chemistry of the TCP, cells are able to attach and proliferate well too. Whether or not having a certain coating (at least the ones tested), the result it will be the same. These results contribute to the development of design principles for the engineering of surfaces that direct cell adhesion for biomedical and biotechnology applications.

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