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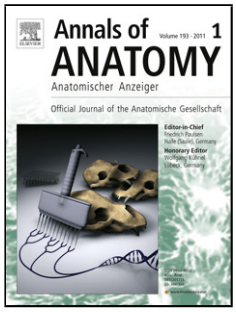

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Plasma of argon enhances the adhesion of murine osteoblasts on different graft materials

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Abstract

Objective: plasma of argon treatment was demonstrated to increase material surface energy leading to stronger and faster interaction with cells. The aim of the present *in vitro* study was to test the effect of plasma treatment on different graft materials.

Materials and Methods: synthetic hydroxyapatite (Mg-HA), biphasic calcium phosphate (BCP), cancellous and cortical xenogeneic bone matrices (CaBM, CoBM) were used representing

commonly used classes of bone substitute materials. Fifty serially numbered disks with a 10mm-diameter from each graft material were randomly divided into two groups: Test group (argon plasma treatment) and Control group (absence of treatment).

Cell morphology (using pre-osteoblastic murine cells) and protein adsorption were analyzed at all samples from both the test and control group.

Differences between groups were analyzed using the Mann–Whitney test setting the level of significance at $p < 0.05$.

Results: plasma treatment significantly increased the protein adsorption at all samples.

Similarly, plasma treatment significantly increased cell adhesion in all groups.

Conclusions: data confirmed that non-atmospheric plasma of argon treatment led to an increase of protein adsorption and cell adhesion in all groups of graft material to a similar extent.

Clinical relevance: plasma of argon is able to improve the surface conditions of graft materials.

Keywords: Apatites, Biophysics, Bone biology, Bone graft(s), Plasma of argon

Introduction

The amount of bone regeneration is strictly correlated to the interaction between the graft material and the osteo-regenerative cells. According to each individual healing pattern, the process of new bone formation and graft material resorption depends on the physico-chemical properties of the material itself. Cell adhesion is mediated by molecules of extracellular or provisional matrix (fibronectin, vitronectin, collagen, laminin or fibrin) (Vallée et al. 2014), which is adsorbed to the graft surface from blood, body fluids and culture media. On surfaces that are highly hydrophilic, these molecules are adsorbed in a denatured and rigid state. On the contrary, materials which are hydrophobic might prevent the adsorption of such proteins. Moderately hydrophilic and positively charged surfaces may, in contrast, promote cell adhesion (Bacakova et al. 2011). The extent and strength of cell adhesion plays a role in regulating the activity of proliferation and differentiation of cells, hence proper in vitro experimental settings are relevant for predicting the actual in vivo performance of bone interface materials (Schierano et al. 2015).

Today clinical research generally focuses on more rapid and less invasive procedures (Mussano et al. 2016; Sacerdote et al. 2013) whereas basic and pre-clinical research is focusing on enhancing the activity of graft materials that are in contact with bone forming cells. This aim to improve ‘‘bioactivity’’ of bone substitute materials has been addressed using a variety of different approaches (Galván-Chacón & Habibovic 2017). Acceleration of osseointegration of graft particles may depend

on the optimization of the biomaterial rather than on an actual increase in the rate of bone response. In this sense, alteration of the physical surface characteristics might positively affect early bone response.

Plasma can be categorized as either thermal or cold. At the same time, according to its pressure, plasma can be applied in a vacuum or at atmospheric pressure (Kalghatgi et al. 2008). Plasma, when applied with vacuum, is an electrically neutral, ionized gas comprising of ions, electrons, neutral particles, ultraviolet irradiation, free radicals and chemically reactive neutral particles. If Plasma is produced under ambient pressure conditions, no ultraviolet radiation is produced. When plasma is produced in a vacuum chamber, atmospheric gases evacuate below 0.1 torr. These low pressures allow a relatively long free path of accelerated electrons and ions, therefore preserving the integrity of the material (Moisan et al. 2002). Whilst removing all chemical traces left from former treatments, plasma of argon treatment effectively produces cleaner and better-controlled surfaces compared to other cleansing methods (Aronsson et al. 1997).

From a physico-chemical point of view, plasma treatment increases the surface energy and therefore reduces the contact angle. This promotes cells to spread (Duske et al. 2012). Additionally, plasma treatment was demonstrated to activate surfaces at the atomic and molecular level, producing hydrophilic surfaces and enhancing their wettability (Swart et al. 1992). Appropriate plasma processes can render surfaces hydrophilic and modify the oxide layer that interacts with proteins and cells of surrounding tissue. Thus, plasma application can lead to an improved adhesion of cells (Zhao et al. 2005). In this respect, several studies have demonstrated that the cleaning of a dental implant surface with plasma simultaneously improves the surface's hydrophilic characteristics. (Coelho et al. 2012; Duske et al. 2012). Additionally, bioactivation of titanium abutments seems to enhance soft and hard tissue stability (Canullo & Götz 2012)

Hence, the purpose of the present study was to test the effect of plasma treatment on different (graft) materials regarding a change in surface characteristics and assessing protein adsorption and osteoblast growth.

Materials and methods

The number of needed specimens was estimated by applying the conceptual approach used elsewhere (Canullo et al. 2016). In a previous pilot study, mean initial cell adhesion values of 181±37 ug/sample and 135±26 ug/sample after 2 hours ($P<0.05$) were planned by setting the effect size (dz)

at 1.438, the error probability (a) at 0.05, and the power at 0.95 (1-b error probability), resulting in 12 samples for each sub-group (G* Power 3.1.7 for Mac OS X Yosemite, version 10.10.3). Four different classes of graft materials were used:

1. Synthetic pure hydroxyapatite discs (Sintlife, Finceramica, Faenza Italy) (Mg-HA)
2. Biphasic calcium phosphate (60% HA, 40% β-TCP) discs (SUNSTAR Degradable Solutions AG, Schlieren, Switzerland) (BCP)
3. Cancellous xenogeneic (porcine) bone matrix discs (Sp-Block, Tecnoss, Coazze, Italy) (CaBM)
4. Cortical xenogeneic (porcine) bone matrix discs (Cortical Lamina, OsteoBiol, Tecnoss, Coazze, Italy) (CoBM)

Therefore, taking into consideration that four types of materials were to be tested with two different assays (protein adsorption and cell adhesion) under two conditions (either plasma treatment or not), as each condition required 12 samples, the total number of specimens necessary was determined as 192 serially numbered blocks with 10mm in diameter. As for cell morphology characterization two samples per material were further required as this analysis was only qualitative.

All specimens were manufactured for the purpose of the study and were non-commercial products. The synthetic graft material discs (Mg-HA and BCP) were pressed from spherical granules and showed a macroscopic flat surface (600-900 microns for magnesium-enriched-hydroxyapatite; 450-1000 microns for BCP). The xenograft discs (CaBM and CoBM) were produced by trimming from an organic porcine bone. A flow-chart graphically depicts the allocation of the samples (Fig.1)

Protein adsorption

To determine the quantity of proteins adsorbed by the grafts, a 2% solution of fetal bovine serum (FBS) in phosphate buffered saline (PBS) was used to incubate the samples for 30 minutes at 37°C. A total of 96 samples were then washed twice with PBS and the adsorbed proteins were eluted from the grafts using Tris Triton buffer (10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% glycerol and 0.1% SDS) for 10 minutes (Mussano, Genova, Verga Falzacappa, et al. 2017). The total protein amount was quantified using Pierce™ BCA Protein Assay Kit (Life Technologies, Monza, Italy) according to the manufacturer's recommendation. The obtained values were subtracted from the respective values of the non-treated grafts (control group).

Cell culture

To investigate the biological response, a pre-osteoblastic murine cell line MC3T3-E1 (ECACC) (Genova et al. 2016) was used. Cells were maintained in Alpha MEM (Minimum Essential Medium) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin, under a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were passaged at subconfluency to prevent contact inhibition.

Cell adhesion assay

Cell adhesion was evaluated on 96 samples using a 48-well plate as support. Cells were detached using trypsin for three minutes. They were then counted and carefully seeded at a concentration of 3 x 10^3 cells/disk in 100µl of growth medium on each sample. The 48-well plates were kept for 12 minutes at 37°C in 0,5% CO_2 (Federico Mussano et al. 2018; Mussano F, Genova T, Laurenti M et al. P 2018). The cell number was evaluated using Cell-titer-GLO (Promega, Milan, Italy) following the manufacturer's instructions.

Cell morphology

To analyze cell morphology, the osteoblasts were seeded on eight samples (two per type), which were washed in PBS after 24 hours. The blocks were then fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes. After two washes with PBS, cells were permeabilized with 0,1% Triton X-100 (Sigma-Aldrich, St.Louis, MI, USA) in PBS (Avanzato et al. 2016; F. Mussano et al. 2018). Following the manufacturer's protocol, cells were stained with Rodhamine-Phalloidin (Life Technologies Monza, Italy) and 1uM Dapi (Life Technologies) to detect the cytoskeleton and the present nuclei (Mussano, Genova, Rivolo, et al. 2017) . Image acquisition was performed using a Nikon Eclipse Ti-E microscope with both a 20X and 40X objective (Plan Fluor Nikon, Tokyo, Japan) connected to it. Image analysis was performed by means of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Statistical Analysis

Due to the nonparametric nature of the data collected, differences between groups were analyzed using the Mann–Whitney test and by means of GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) (Petrillo et al. 2017). Data were collected and stored in a biology laboratory abiding by the protocols and regulation of the University of Turin. All of the statistical comparisons were conducted with a 0.05 level of significance.

Results

Plasma treatment significantly increased the total protein adsorption on all grafts: 18.7±1.2 ug/sample (control) vs. 30.2±2.7 ug/sample (test) for BCP (*p* value: 0.0286), 15.0±0.5 ug/sample (control) vs. 29.7±1.7 ug/sample (test) for Mg-HA (*p* value: 0.0285), 22.2±5.2 ug/sample (control) vs. 191.8±37.9 ug/sample (test) for CaBM (*p* value: 0.0003), 52.2±10.0 ug/sample (control) vs. 211.25±41.6 ug/sample (test) for CoBM (*p* value: 0.0044) (Fig. 2).

Cancellous and cortical BM grafts showed higher values in total protein adsorption compared to BCP and Mg-HA samples, both in the control and the test group.

Similarly, on all different graft surfaces, plasma treatment significantly increased the cell adhesion levels: 212.0±11.0 cells/field (control) vs. 262.0±13.3 cells/field (test) for BCP (*p* value: 0.0003), 94.7±14.1 cells/field (control) vs. 124.7±8.7 cells/field (test) for Mg-HA (*p* value: 0.0001), 104.7±11.3 cells/field (control) vs. 362.5±37.9 cells/field (test) for CaBM (*p* value: 0.0044), 167.7±28.1 cells/field (control) vs. 384.5±38.8 cells/field (test) for CoBM (*p* value: 0.0102). Both xenografts presented a higher cell adhesion capacity compared to the two synthetic materials in the test group (Fig. 3). Interestingly, the synthetic BCP showed the highest cell adhesion in the non-treated groups (control) compared to the other materials.

In all samples, the cells displayed a tip-shape morphology which was particularly evident when seeded on the surface of the mg-HA sample. In group BM, a higher number of cells were embedded in the pores of the rough surface (Fig. 4).

Discussion

The study showed that the samples treated with plasma of argon presented with increased values of cell adhesion and protein adsorption when compared to samples from the control group.

Despite morbidity of the donor site, autogenous bone might be considered the gold standard for bone regeneration due to the presence of viable bone cells and growth factors which allow to consider this material osteoinductive (Roato I. et al. 2018; Mussano, Genova, Corsalini, et al. 2017)(Chiapasco et al. 2009). However, in order to minimize the surgical procedures side effect of the donor site, various bone graft substitutes with osteoconductive properties were developed. They can be classified into synthetic products [pure hydroxyapatite (HA), beta calcium phosphate (BCP)] and xenografts (ABM) (Benic & Hämmerle 2014).

The xenografts can further be divided into collagenated and deproteinized bone mineral. The subgroup consisting of deproteinized bovine matrix was extensively investigated in both pre-clinical

and clinical studies and showed favorable results (Araujo 2008, Jung 2013, Fickl 2009 Chiapasco & Zamboni 2009, Lutz et al. 2015). The absence of a protein component, due to heat treatment, greatly reduces the risk of immunogenicity and disease transmission (Lutz et al. 2015). On the contrary, one drawback of this material may be represented by incomplete resorption (Mordenfeld et al. 2010).

The xenograft used in the present study comprises of collagenated bone of porcine origin. The analyzed material demonstrated to preserve collagen and micro-porosity similar to the human bone (Figueiredo et al. 2010). This may be attributed to the manufacturing methods which do not include de-calcination and might be a reason for remodeling processes histologically demonstrated in animal studies (Nannmark and Sennerby 2008). Both the cortical and cancellous form of this bone substitute were investigated in clinical studies confirming its biocompatibility (Barone et al. 2005, Orsini et al. 2006).

In contrast to xenogeneic substitutes, synthetic bone graft materials are based on calcium and phosphates in order to mimic the inorganic phase of human bone. Both biphasic calcium phosphate (BCP) and pure hydroxyapatite (HA), which is a stable form of calcium phosphate, are intensively studied materials. Broad evidence supports good clinical outcomes in terms of amount of regenerated bone and graft material resorption (Sisti et al. 2011, Cordaro et al 2008, Froum et al 2008). Nevertheless, synthetic materials also present drawbacks. In fact, HA and BCP were shown to present a partial resorption rate (Valdivia-Gandur et al. 2016).

Despite their properties, all above mentioned materials are integrated within human bone through the same process, primarily involving proteins (fibronectin, vitronectin, actin, vinculin). These molecules are present in the blood and are adsorbed to the surface of the graft. They provide mechanical attachments for the extracellular matrix, which is mandatory for cell adhesion (Genova et al. 2017) (Großner-Schreiber et al. 2006). Besides these requirements, material hydrophilicity is the main prerequisite for graft integration.

The present study highlights that graft materials (independent of their physical characteristics) can benefit from plasma-of-argon treatment in terms of protein adsorption. So-called bio-functionalization increased protein adsorption by up to 60% compared to untreated graft samples for all tested classes of graft materials. Similar behavior was observed analyzing cell adhesion: comparing test and control samples, bio-activation through plasma of argon allowed at least 30% higher cell adhesion for all graft materials, independent of the product investigated.

Outcomes of the present study confirm that bio-activation of graft material can positively influence cell and protein behavior at substitute surfaces. So far, this could only be shown by modifying roughness, morphology and chemical composition of the graft material (Jiang et al. 1999, Qiao et al. 2017, Huges & Grover 2017). According to the available literature, the present study seems to be the first investigation describing the effect of bio-physical surface modification by the use of plasma-of-argon produced in a vacuum chamber.

One pre-clinical study reported that TCP activated by plasma of argon failed to present better outcomes in terms of bone regeneration compared to untreated graft material (Beutel et al. 2014). However, Beutel et al. used a plasma at atmospheric pressure. Evidence confirms that the effect of plasma treatment is directly correlated to the gas utilized, time of exposure, and power and pressure used (Moisan et al. 2002, Duske et al 2012). Differences between the two studies might be explained by the effect of the vacuum.

Intragroup differences in terms of protein adsorption and cell adhesion were noticed. In fact, only slight differences were measured regarding cells adhesion and protein adsorption within the control group between synthetic products and xenografts. Within the test group the differences between the different materials were considerably larger. However, statistical comparison could not be performed due to the absence of a normalization effect. These differences might be due to the product manufacturing specific for the present experiment. The synthetic materials were fabricated by compacting particles into disks, thus the initially porous structure of the material was pressed to a smooth disk surface. In case of synthetic products, compression of single granules has flattened the scaffold tridimensional geometry, creating a very smooth and therefore smaller accessible surface. In contrast, both cancellous and cortical xenografts were cut out from the processed animal bone, maintaining their porous and rough surface. Because of these large differences in macroscopic surface morphology and accessible surface between the different graft materials, a comparison of the results for protein and cell absorption between the different material groups was not considered appropriate.

To mimic relevant clinical conditions, the present study adopted low concentrations of proteins and cells. Moreover, due to the small surface of the disks, using high concentrations of proteins or cells might have jeopardized the study outcomes due to a saturation effect, as previously demonstrated in a study on titanium disks (Canullo et al. 2013). However, this might represent a limitation only for in vitro studies. In fact, the presence of higher physiological protein and cell concentrations does

not prevent the effect of bio-functionalization as demonstrated by clinical and *in vivo* histological studies (Garcia et al. 2016, Canullo et al. 2016, Canullo et al 2017a, Canullo et al. 2017b).

An obvious limitation of the present study is represented by the fact that simulating clinical conditions *in vitro* is not possible as a whole, since a great number of heterogeneous proteins contemporarily interact *in vivo*. An additional limitation was the use of a murine cell line. However, the outcomes reported here seem to encourage testing of this approach in a pre-clinical study model.

Conclusions

Within the limitations of the present study, the data obtained confirm that non-atmospheric plasma-of-argon treatment is capable of increasing protein adsorption and cell adhesion to different classes of graft materials. Further research is required to assess whether these results might be beneficial in clinical settings. Biofunctionalization of bone grafts might lead to improved integration and degradation of these substitute materials, primarily in elderly patients and/or patients exhibiting deficient healing patterns.

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All authors explicitly declare no conflict of interest.

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Figure legend

Figure 1. Flow diagram showing the respective distribution of the randomized samples to the analyzing method.

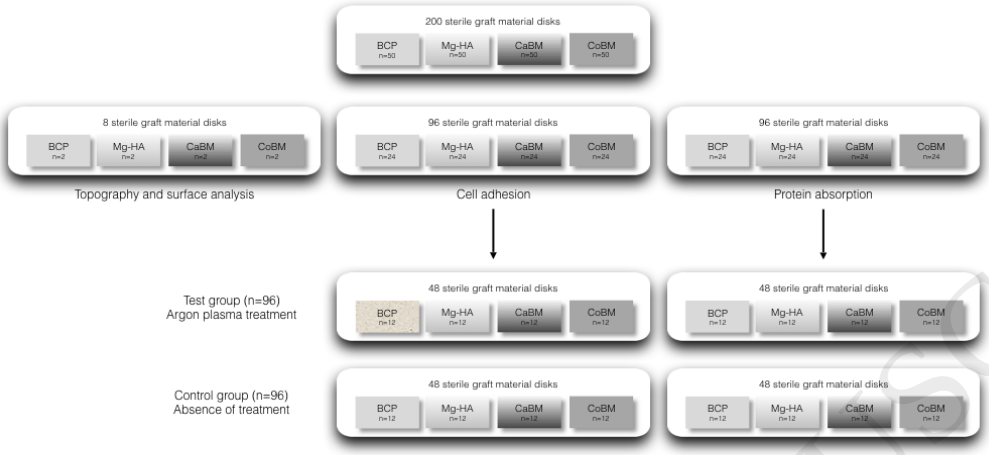

Figure 2. Diagram showing the amount of protein adsorption per sample. Values represent mean ± SD; the symbol (*) indicates a statistically significant difference, considering a p-value < 0.05.

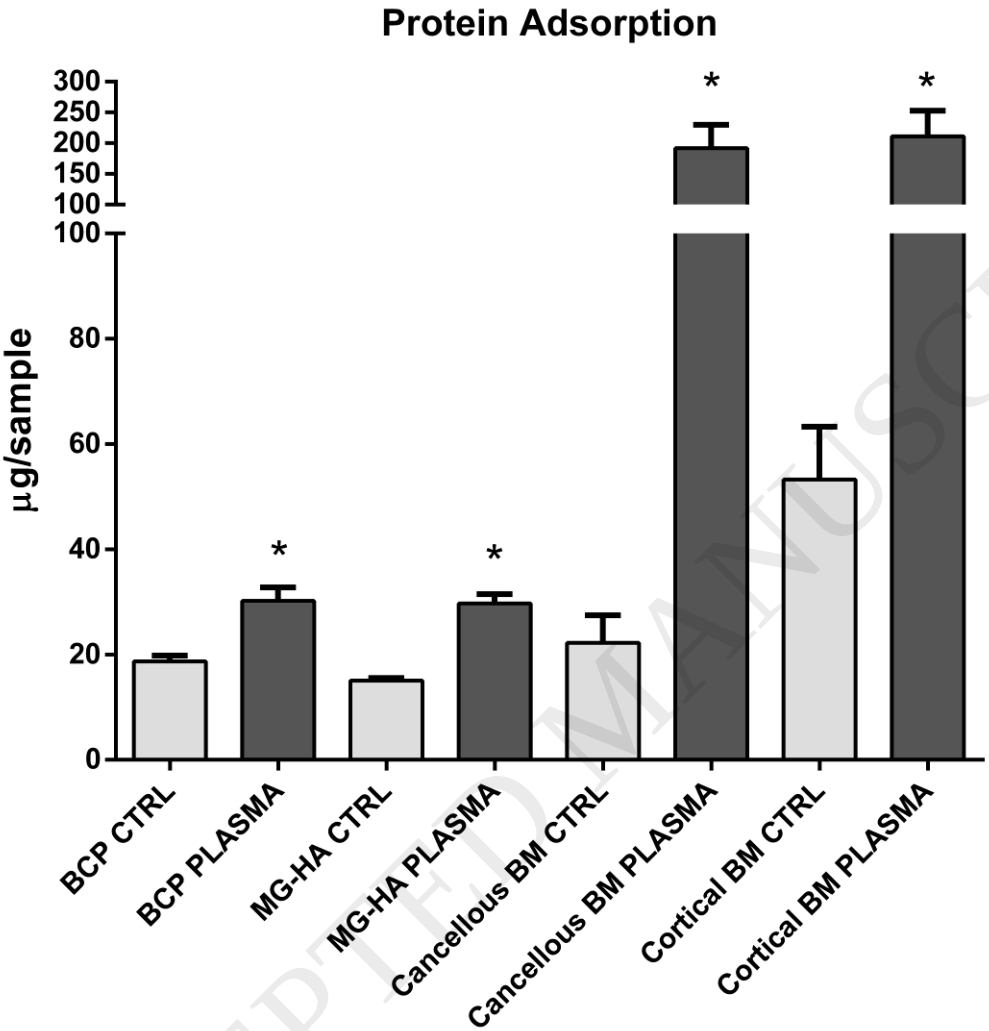

Figure 3. Diagram showing the number of cells measured in all groups. Values represent mean ± SD; the symbol (*) indicates a statistically significant difference, considering a p-value < 0.05.

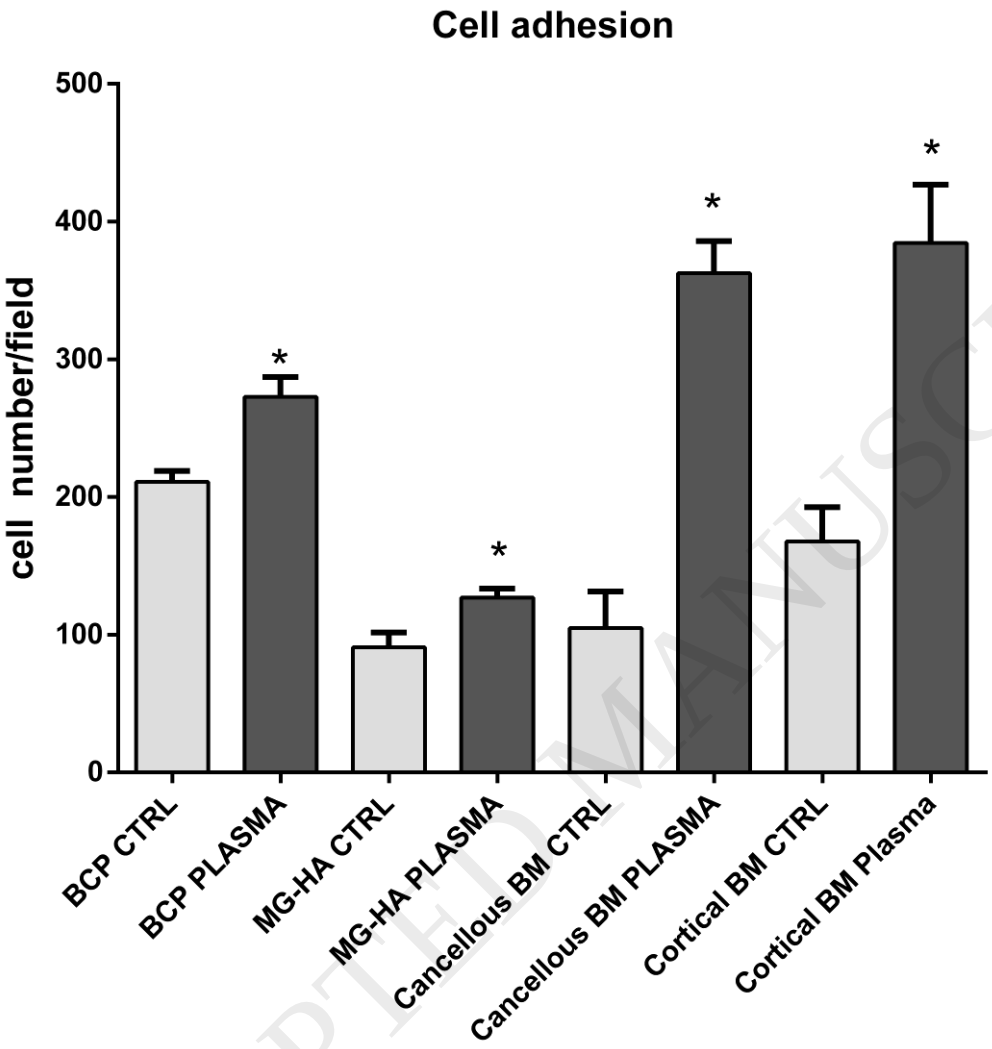

Figure 4. Cell Morphology. SEM-image of different grafts after plasma treatment: *BCP (A); Mg-HA (B); CaBM (C); CoBM (D) at 100X.*