UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Osteoblastic response to biomaterials surfaces: mineralization evaluation and extracellular matrix proteomic analysis

Resposta de osteoblastos a superfícies de biomateriais: avaliação da mineralização e análise proteômica da matriz extracelular

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Orientador: Prof. Dr. Rodrigo Cardoso de Oliveira

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"Volta teu rosto sempre na direção do sol e,
então, as sombras ficarão para trás."
(Provérbio chinês)

ABSTRACT

Osteoblastic response to biomaterials surfaces: mineralization evaluation and extracellular matrix proteomic analysis

Dental implants are designed to replace tooth loss, due to periodontal diseases, trauma or decay. Among the biomaterials used for this purpose, titanium and zirconia have been investigated for some years, with excellent mechanical properties and biocompatibility. Surface treatments such as anodizing, with the incorporation of Mg, Ca and P in the structure of the titanium oxide films, were used in order to increase tribocorrosion resistance and improve the osseointegration process. The cellular response to surfaces is mediated, among other factors, by the extracellular matrix (ECM) . However, very little is still known about the ECM proteomics during mineralization. Our objective was a longitudinal comparison of osteoblastic behavior on different materials, in terms of mineralization volume and actin cytoskeleton status, associated with the proteomic analysis of the extracellular matrix. The three types of biomaterial surfaces (pure titanium, anodized titanium and zirconia) were imaged by confocal 3D microscopy and analyzed in terms of roughness. MC3T3 cells were cultivated on the biomaterials for 7, 14 and 21 days, with osteogenic medium containing calcein. The cells were then fixed, stained with Rhodamine phalloidin and DAPI, and imaged by confocal laser scanning microscopy. The quantification of mineralization and actin cytoskeleton was performed by a novel technique, based on the acquired 3D images. For the proteomic analysis, the specimens were washed, decellularized and the ECM was collected in buffer solution. The anodized titanium surface is more porous when compared to that of cp-Ti and zirconia, and superior mineralization was obtained over it after 21 days of culture. The actin microtubular volume was increased on the three materials on the first 14 days, but on the 21th day there was a reduction over anodized titanium and zirconia, related to mineralization phase.. Conclusion: The greater mineralization obtained over anodized titanium after 21 days demonstrated an improved response provided by the surface modification. The innovative volume quantification technique adopted was useful in providing information about the cellular status and biomaterial performance. Alpha-1_4 glucan phosphorylase and Glycogen phosphorylase brain form were down-regulated on

zirconia after 7 and 14 days of culture, and up-regulated on Anod Ti on the 7th day, suggesting the influence of material surface roughness and chemical composition on energy metabolism. Proteins related to bone development, like TGF-β3, were found exclusively on cp-Ti on the 21st day. The small number of identified proteins demonstrates that the chosen decellularization process was effective at reducing the proteome dataset. Altogether, our results reveal new insights regarding osseointegration and how material surfaces affect this process.

Key words: Osteoblasts. Biomaterials. Bone Mineralization. Extracellular matrix. Proteomics.

RESUMO

Resposta de osteoblastos a superfícies de biomateriais: avaliação da mineralização e análise proteômica da matriz extracelular

Implantes dentários são projetados para substituir a perda de dentes, que pode ser causada por doenças periodontais, traumas ou cáries. Entre os biomateriais utilizados para este fim, titânio e zircônia têm sido investigados durante alguns anos, com excelentes propriedades mecânicas e biocompatibilidade. Tratamentos de superfície como a anodização, com a incorporação de Mg, Ca e P na estrutura dos filmes de óxido de titânio, foram utilizados a fim de aumentar a resistência à tribocorrosão e melhorar o processo de osseointegração. A resposta celular às superfícies é mediada, entre outros fatores, pela matriz extracelular (ECM). No entanto, muito pouco ainda é conhecido sobre a proteômica da matriz óssea durante a mineralização. Nosso objetivo foi a comparação longitudinal do desempenho de osteoblastos em diferentes materiais em termos do volume da mineralização e do status do citoesqueleto de actina, associada à análise proteômica da matriz extracelular. Imagens dos três tipos de superfícies de biomateriais (titânio puro, titânio anodizado e zircônia) foram adquiridas por microscopia confocal 3D e analisadas em termos de rugosidade. Células MC3T3 foram cultivadas na superfície dos biomateriais durante 7, 14 e 21 dias, com meio osteogênico contendo calceína. As células foram então fixadas, coradas com faloidina-rodamina e DAPI, e levadas ao microscópio confocal de varredura a laser. A quantificação da mineralização e do citoesqueleto de actina foi feita por uma nova técnica, baseada em imagens 3D. Para a análise proteômica, os espécimes foram lavados, descelularizados e a matriz extracelular foi coletada em solução tampão. A superfície de titânio anodizado é mais porosa quando comparada com a de cp-Ti e zirconia e apresentou mineralização superior após 21 dias de cultura. O volume dos microtúbulos de actina foi aumentado sobre os três materiais nos primeiros 14 dias, mas no 21º dia houve uma redução relacionada ao aumento da mineralização sobre o titânio anodizado e zirconia. Conclusão: a mineralização superior obtida sobre o titânio anodizado após 21 dias de cultura demonstrou a melhoria provocada pela modificação de superfície. A nova técnica adotada para a quantificação do volume foi útil para fornecer informações sobre o status celular e o

desempenho dos biomateriais. Alpha-1_4 glucano fosforilase e glicogênio fosforilase forma cerebral foram sub-expressas sobre a zircônia após 7 e 14 dias de cultura e sobre-expressas sobre o titânio anodizado no 7º dia, sugerindo a influência da rugosidade e composição química da superfície dos materiais no metabolismo de energia. Algumas proteínas relacionadas com o desenvolvimento ósseo, como a TGF-β3, foram encontradas exclusivamente sobre o cp-Ti no 21º dia. A pequena quantidade de proteínas identificadas demonstra que o processo de descelularização adotado foi eficiente em reduzir o conjunto de dados da análise proteômica. Em suma, nossos resultados revelam novos detalhes sobre a osseointegração e como a superfície dos materiais podem afetar esse processo.

Palavras-chave: Osteoblastos. Biomateriais. Mineralização óssea. Matriz extracelular. Proteoma.

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1 Introduction

1 INTRODUCTION

Dental implants are designed to replace tooth loss, which can be caused by periodontal diseases, trauma, or decay. Among the biomaterials used for this purpose, titanium is the most commonly used. Its biocompatibility and mechanical properties have been proved excellent over more than 50 years. However, all metal implants are subjected to gradual degradation when placed in contact with the electrolytic environment of the human body (1). Degradation may occur by electrochemical corrosion, often together with mechanical wear caused by micromovements induced by mastication (2). As a consequence, an increase of metallic ions and/or nanometric metallic-based particles may reach the circulatory system and accumulate in other organs (2). Besides, particles may be stored locally in the gingival tissue surrounding the implant, causing inflammation and undesirable color changes (3).

Superior tribocorrosion performance for metal implants can be achieved by surface modifications. Changes in composition, morphology or structure can be made, while keeping the substrate mechanical properties. Anodizing treatments has been widely used on this purpose, providing better hard tissue compatibility and accelerating bone formation over implants (1). Indispensable elements in bone formation, Ca and P ions may be incorporated on the deposited layer, leading to better tribocorrosion resistance (4). Besides cellular behaviors such as adhesion, spreading and IFN-γ cytokine secretion are affected , which may lead to shorter rehabilitation times (5)

Ceramic implants pose an alternative to avoid corrosion issues related to metallic counterparts. A review article by Hisbergues (2008) emphasized zirconia properties like resistance to corrosion and aesthetic appeal, as well as the fact that healthier gum tissue is developed around ceramic implants (6). The grey color of the titanium implants may become visible when placed to restore anterior teeth, especially in cases of thin gingival tissue (3). More research is needed over the long-term performance of zirconia implants, as its use in dental clinic is recent (3,7–9).

The major factor impacting the long-lasting performance of an implant is the osseointegration, which is the capacity of building new bone around it. This characteristic may be evaluated by the amount of mineralization obtained when bone cells are cultivated over the material (10).

Short-term responses such as cell number and alkaline phosphatase expression do not necessarily correspond to greater mineralization. Aiming to analyze calcified nodules directly, Ahmad et al. (1999) introduced fluorescent marking by calcein, in a study comparing Tivanium and Zimaloy with glass, as substrates for a 6-weeks long osteoblastic culture (11).

In another study on mineralization obtained over titanium, de Oliveira et al. (2007) demonstrated that a chemically caused nanotopography on the titanium surface improved the osteoblastic response, possibly due, between other factors, to the fact that the modified surface is more hydrophilic than the untreated surface (12).

Hempel et al (2010) compared the cellular response caused by two surface treatments on titanium and zirconia. The osteoblasts grown on the zirconia showed greater accumulation of calcium in the mineral nodules than those grown on titanium. There was little difference between the treated and untreated zirconia surface (13).

Several processes are triggered when osteoblasts meet a surface, leading to adhesion, proliferation and differentiation, many of them mediated by the cytoskeleton. Besides, cell morphology is determined by the cytoskeleton, which is known to change during the osteoblastic mineralization process (14).

Confocal laser scanning microscopy (CLSM) has been largely used to analyze cell morphology, structures and functions. Its ability to provide high resolution and high contrast 3D image stacks allow feasible and trustworthy volume measurements. Additionally, fluorescent labelling of intracellular proteins or organelles have been proven specific and reliable (15). Kihara et al. (2004) used CLSM to visualize calcein-stained mineralization nodules and compared them with conventional Alizarin staining, confirming the quantitative correlation between calcein deposition and calcium contents (16). However, the CLSM images were used to quantify the fluorescent intensity, not the nodular size. The use of confocal volume quantification to measure the amount of mineralization obtained by cultured osteoblasts has not yet been described in the biomedical literature.

Cellular responses to material surfaces are determinant for a successful implant performance. After adhering, proliferating and spreading over the implant surfaces, it is mandatory for the osteoblasts to be able to generate bone around the implant, a process called osseointegration, which guarantees the long-term stability of the implant (14).

Bone formation is a not fully understood process, which involves mineralization of the extracellular matrix by the deposition of hydroxyapatite, rich in calcium and other minerals. The extracellular matrix (ECM) provides structural support for the cells within a tissue, besides directing important events such as cell proliferation, survival, differentiation, and migration. Composed of water, collagens, glycoproteins and proteoglycans, the ECM is a complex structure that is constantly being remodeled (17). Grzesiak et al. (2017) concluded that ECM synthesis and mineral deposition during the osteogenic differentiation involves cell death and mineralization nodules derive from calcium rich cellular remnants (18).

The field of proteomics has contributed to the understanding of many cellular processes. Still, very little is known about the ECM proteomics during mineralization. The protein profiles of the ECM and matrix vesicles (MVs) of mineralizing osteoblasts was described for the first time in the work of Xiao et al. (2007). The cells were cultivated on conventional culture plates, and the cellular content was separated from the ECM proteins by gel electrophoresis. However, this work had no relation to biomaterials interaction (19).

Comparison of the whole proteomic profile of human osteoblasts grown over two biomaterials was conducted by Jinling Xu (2008), but only for an initial phase (4 days of incubation) (20). A description of the protein content of an isolated ECM was presented by Rashid et al. (2012) using an *in vitro* model of fibrotic liver tissue, where the samples were decellularized (21). This interesting approach directs the proteomic analysis to a focused dataset.

Many efforts have been undertaken towards elucidating cell attachment and proliferation over biomaterials, but less work has been done investigating the effect the surface properties can exert on the late steps of osseointegration, such as the mineralization phase. Our objective was to compare, in a longitudinal manner, the cellular response to anodized titanium (Anod Ti) and yttria stabilized zirconia (Y-TZP) with that obtained over commercially pure titanium (cp-Ti) by 1) measuring the mineralization and actin cytoskeleton volumes, based on 3D confocal microscopy; and 2) analyze the proteome of the decellularized ECM, by mass spectrometry. It will certainly contribute to elucidate the influence of biomaterials surfaces on the process of osseointegration.

2 Articles

2 ARTICLES

This thesis was divided in two articles, written in accordance to *JAOS* and *Bone* Guidelines, respectively:

• ARTICLE 1 – On a novel method for evaluation of the mineralization process of osteoblasts on biomaterials surfaces.

• ARTICLE 2 – Longitudinal comparison of the ECM proteins from osteoblasts cultivated on different biomaterials.

3 Discussion

3 DISCUSSION

Dental implant failures cause patient discomfort and bone loss is often clinically observed. It is therefore important to assure good osseointegration capability and appropriate mechanical properties to withstand the masticatory pressures. Titanium is the gold standard for dental implant industry, but new materials as well as surface treatments have been investigated aiming at a long-lasting performance allied to improved aesthetics. In this context, we decided to compare three different biomaterials: commercially pure titanium (cp-Ti grade 2), anodized titanium surface and yttria stabilized zirconia (Y-TZP) in a longitudinal study.

Surface treatments like polishing, sandblasting, acid etching, coatings, nanotextures and biofunctionalization have been proposed and tested over titanium and zirconia (12,22–26). As mentioned earlier, the anodization process improves cell attachment and proliferation (27), but less work has been done on the final steps of osseointegration and the bone quality obtained.

Techniques used to evaluate mineralization includes von Kossa staining (28), colorimetric assays based on alizarin red staining (29), or EDX (30). Direct fluorescent labelling of hydroxyapatite crystals by calcein was used for visualization (11,16), fluorometric quantitation (10,31) or nodule area measurement (12,32), but no work has been published measuring the mineralization volume based upon confocal 3D imaging.

When comparing anodized titanium (TiUnite®), machined titanium (Ti-m), sandblasted and acid-etched zirconia (TZP- proc), and machined zirconia (TZP-A-m), Kohal et al. (2013)(23) observed contradictory results. Similar to our results, the cell responses to zirconia surfaces were comparable to those obtained over titanium, but in the *in vivo* experiment TZP-proc performed worse than a standard titanium implant surface modification. Hempel et al. (2010) also compared titanium and zirconia with two surface modifications, and significantly higher calcium accumulation was obtained on both zirconia surfaces when compared with titanium (33).

In our work, cp-Ti was the roughest surface, while zirconia was the smoothest. Notwithstanding, the amount of mineralization obtained over both materials were equivalent. Our results suggest that roughness alone may not be a determining parameter concerning cell behavior, agreeing with the conclusion presented by Setzer

et al. (2008) (29). Besides, this result strengthens the use of zirconia in the clinic by suggesting that both materials would present a similar osseointegration. On the other hand, anodized titanium presented a greater mineralization volume compared to the other two biomaterials, probably due to its chemical composition or porosity, as suggested in the works of Alves et al. (24,34).

Also, Anselme et al. (35) stated that there was a negative correlation between roughness and cell adhesion and proliferation, yet the roughness organization and surface chemical composition were relevant, as it seems to be the case for anodized titanium in our work.

In the present study, all biomaterials showed a good proliferation of MC3T3 cells. It has been reported that osteoblasts undergo shape changes as they differentiate: the cell loses its flattened, elongated shape and adopts a cuboidal morphology. The actin restructuring observed after 14 days of culture is in agreement with the results shown in previous study by Titushkin et al. (2007)(36). In a study on the elasticity of the extracellular matrix, Meng et al. (2009), stated that only mineralizing osteoblasts have the cytoskeleton remodeled when compared to the inactive ones, and also that mineralization requires a correct and completely developed ECM to occur (37).

TAKAI et al. (2005) cultivated osteoblasts for 1 hour on glass coated or not by collagen type I, fibronectin, vitronectin, poly-L-lysine, and fetal bovine serum to assess the influence of the extracellular matrix proteins on the cellular elasticity and cytoskeleton. In adhesion processes mediated by integrins (in the case of collagen type I, fibronectin, vitronectin and fetal bovine serum) the cytoskeleton proved more robust and the cells were firmer. On the contrary, the cells grown on glass and poly-L-lysine, where adhesion occurs by non-specific connections, presented the cytoskeleton with fewer actin fibers and smaller elastic modules (38).

Among many other functions like adhesion (39), migration and cellular morphology, the cytoskeleton is responsible for vesicle transport. Osteoblasts release matrix vesicles (MVs), which are the initial sites where crystals of apatite bone mineral are formed (40).

Many efforts have been undertaken to understand the process and mechanism of MV formation and release. Some results have suggested that the MV release is mediated by the actin cytoskeleton. A correlation between the release of MVs and changes in cellular actin distribution has been reported by Hale et al. (41). Furthermore,

actin microfilament disassembly is involved in the mechanism of MV formation, as Thouverey et al. demonstrated (42).

Our current data have shown a weak inverse correlation between mineralization and actin cytoskeleton volumes, reinforcing the role of the actin fibers depolymerization in the formation of MVs. This result makes it of interest to investigate other techniques like fluorescent colocalization or proteomics to further elucidate this mechanism.

Much of the pivotal role ECM proteins play in osseointegration remains unclear. As previously mentioned, ECM components participate in cellular signaling, besides providing structural support. Many research efforts are focused on ECM description, trying to elucidate its complex composition and dynamical behavior (17). We employed a proteomic approach to evaluate differences in ECM protein expression from osteoblasts cultivated on three different implant surfaces, and how it changes over time.

Our results revealed that the great majority of the ECM proteins are expressed and regulated equally on the three biomaterials tested, albeit distinct chemical compositions and surface roughness. Some proteins identified were uniquely found on a biomaterial at certain time points, whereas only two proteins were up or down-regulated.

Two enzymatic proteins were identified: Probable imidazolonepropionase (Q9DBA8, unique to zirconia after 7 days of culture), and N-alpha-acetyltransferase 20 (P61600, identified exclusively on Anod Ti at the 14th day). The first is involved in step 3 of the subpathway that synthesizes N-formimidoyl-L-glutamate from L-histidine. The latter is responsible for N-terminal peptidyl-methionine acetylation and is required for maintaining the structure and function of actomyosin fibers and for proper cellular migration. It has been demonstrated that ECM remodeling is a pre-requisite to pre-osteoblast phenotype response in a recent work by Da Costa Fernandes et al. (2018). The cellular response to zirconia involved a cascade of signaling molecules related to cellular anchoring (43). The fact that enzymes were identified in this work certifies that the samples were properly handled, as they are very sensitive to heat.

Some proteins identified in the collected ECMs are classified as membrane receptors and growth factors or associated to molecular signaling pathways, normally found in the extracellular region. B9 domain-containing protein 1 (Q9R1S0) is required for sonic hedgehog/Shh signaling and was uniquely found on zirconia on the 7th day. The role of the Hedgehog-Gli1 pathway in the response of osteoblasts to different

titanium topographies was evaluated by Lin (2017), and on the nanostructured surface studied, the mRNA expression of Sonic hedgehog (Shh) was higher when compared to that on the smooth and microstructured surfaces.(44). In our work, the zirconia surface presents irregularities in the nanoscale, which may have caused a similar effect.

Breakpoint cluster region protein was unique to zirconia at 7th day. Being a GTPase-activating protein for the GTPases of the Rho family, it is involved in the regulation of Rho protein signal transduction, negative regulation of cell migration, regulation of cell cycle, actin cytoskeleton organization and intracellular protein transmembrane transport, among other functions. In our work, this protein was found associated with Cell cycle progression protein 1 (Q640L3). The latter is a membrane protein involved in positive regulation of cell cycle and cell proliferation, acting as an assembly platform for Rho protein signaling complexes.

Low-density lipoprotein receptor-related protein 4 (Q8VI56, Lrp4) was uniquely identified on zirconia at the 7th day. In this work, it was associated with Agrin, known to induce an increase in cytoplasmic calcium ions, specifically modulating calcium ion homeostasis in neurons. Xiong et al (2015)(45) identified Lrp4 as a critical player in bone mass homeostasis, acting as a receptor of sclerostin to inhibit Wnt/β-catenin signaling and bone formation.

The work of Sophocleous et al. (2017) indicates that Cannabinoid receptor 2 (P47936, Cnr2) plays a role in regulating bone mass and bone cell activity. Combined deficiency of the Cnr1 and Cnr2 receptors in female mice protects against age-related bone loss due to a reduction in osteoclast number when compared with wild-type (49). Our results show Cnr2 uniquely expressed on cp-Ti after 21 days of culture, during the mineralization phase.

Identified only on cp-Ti at the 21st day, transforming growth factor beta-3 (Q91YU7, TGF-β3) is typically found in the extracellular region. Among other functions, it participates in the positive regulation of bone mineralization and positive regulation of collagen biosynthetic process. In a very elegant study of Wang et al (2012)(50), the relations between collagen XXIV, TGF-β e Smad were demonstrated in MC3T3 cells. Interestingly, a recent work by Deng (2017)(51) reports that TGFβ3 could recruit MSCs to initiate bone regeneration.

Another grow factor identified in this work, typical of the extracellular region, Fibroblast growth factor 8 (P37237) was found exclusively expressed on cp-Ti at the

21st day. It plays an important role in the regulation of embryonic development, cell proliferation, cell differentiation and cell migration. Valta et al. (2006) showed that besides inducing osteoblast differentiation, FGF-8 stimulates the proliferation of cultured mouse bone marrow cells efficiently and induced their early stage differentiation (52). The stimulatory effects of FGF-8 on osteoblast proliferation in primary osteoblast cultures was also proved in a study by Lin et al (2009) , while in long-term cultures of osteoblasts, nodule formation was inhibited (53).

Cytoskeleton associated proteins like centriolin (A2AL36, unique to Anod Ti at the 7th day), MAP7 domain-containing protein 2 (A2AG50, unique to Anod Ti at the 14th day), and vimentin were also identified. Vimentins are class-III intermediate filaments, localized in the extracellular matrix or as part of the cytoskeleton. According to Gene Ontology, it is involved in the positive regulation of collagen biosynthetic process and SMAD protein signal transduction. In this work vimentin was identified as exclusive for the cp-Ti group after 21 days of culture.

One association between osteoblasts and vimentin was described by Lian et al. (2009) where vimentin inhibited differentiation in immature osteoblasts by interacting with Activating transcription factor 4 (ATF4) (46). Later, Lian et al. (2012) described this process in detail, stating that TGF-β stimulates vimentin production, leading to suppression of ATF4-dependent osteocalcin (Ocn) transcription and osteoblast differentiation (47). Schmidt et al. (2015) demonstrated that alkaline phosphatase (ALP) mRNA binds to and is stabilized by vimentin (48).

Curiously, various proteins related to bone remodeling were found exclusively expressed on cp-Ti at the 21st day. At the same time point, zirconia presented the same amount of mineralization as cp-Ti, while Anod Ti had a greater amount of mineralization. So why those proteins weren´t identified on zirconia and Anod Ti? Perhaps on both materials the cellular cycle is more advanced when compared to cp-Ti. This hypothesis could only be checked in a study where the time points were closer.

One of the categories with the highest percentage of associated genes in the ClueGo analysis was glycogen catabolic process. On zirconia, two proteins related to energy metabolism had a lower expression when compared to that of cp-Ti at the 7th and at the 14th day, but at the 21th day they became equalized. One protein was up-regulated on Anod Ti after 7 days of culture when compared to cp-Ti, but there was no difference in energy metabolism proteins between them afterwards.

Alpha-1,4 glucan phosphorylase was down-regulated on zirconia on the 7th day, while Glycogen phosphorylase brain form was up-regulated on Anod Ti on the same day and down-regulated on zirconia on the 14th day. Phosphorylases are allosteric enzymes of glycogenolysis, participating on the carbohydrate metabolism, being found in the extracellular region or secreted by the cells. In our data, glycogen phosphorylase brain form was indirectly associated with High mobility group nucleosome-binding domain-containing protein 5 (Q9JL35), found on Anod Ti on the 14th day, through a common link to 14–3-3 protein epsilon (P62259).

These results suggest that the early cellular metabolism on zirconia is somehow slower when compared to that observed on cp-Ti and Anod Ti, maybe associated with the smoother surface. On the other hand, Anod Ti seems to require a higher level of energy at initial times. Perhaps its chemical composition, enriched with Ca, P and Mg, helps promoting the cellular proliferation typical of that phase. No articles were found in the literature specifically describing these two proteins and their relation to osteoblasts metabolism, but Komarova et al. (2000) suggested that the glycolytic component of energy generation in mature osteoblasts may play a key role in adapting to transient challenges such as changes in either O_2 supply to bone or increases in transient demands for energy (54).

Another significant biological process appointed in the ClueGo analysis was the positive regulation of collagen biosynthetic process. Collagens, as is well known, are the main structural components of the ECM, focus of our study.

In spite of the great variety of studies found within the contemporary bio-medical literature regarding proteomics and biomaterials, as evidenced by the recent review by Othman et al. (2018) (55), none was focused on describing the ECM proteins expressed differentially on three different biomaterials. The novelty of the processes used to limit the number of analyzed proteins, comprising decellularization followed by ECM collection by scrapping, improves the validity of our data. Proteomic analysis of whole cell lysates usually involve hundreds of proteins, while in our work, only 24 proteins were found uniquely or differentially expressed. Our results contribute to the comprehension of the complex relationships between ECM composition, cytoskeleton remodeling and mineralizing capacity of osteoblastic cells along time and the effect biomaterial surfaces cause in this process. Further studies are needed to confirm the temporal changes and the implications of the uniquely expressed proteins mentioned.

4 Conclusions

4 CONCLUSIONS

The greater mineralization obtained over the anodized titanium after 21 days demonstrated an improved response provided by the surface modification. The amount of mineral deposition obtained over zirconia was equivalent to that of cp-Ti, which favors the use of zirconia in the clinic. An inverse correlation between mineralization and cytoskeleton volume has been shown, strengthening the hypothesis of actin fibers depolymerization related to mineral deposits through MVs. The innovative volume quantification technique adopted was useful in providing information about the cellular status and biomaterial performance in terms of osseointegration.

The small number of identified proteins demonstrates that the chosen decellularization process was effective at reducing the proteome dataset. Typical ECM proteins were expressed and regulated equally on the three biomaterials tested. Some proteins related to bone development, like TGF-β3, were found exclusively on cp-Ti on the 21st day. Alpha-1_4 glucan phosphorylase and Glycogen phosphorylase brain form, both proteins involved in metabolic processes, were down-regulated on zirconia after 7 and 14 days of culture, and up-regulated on Anod Ti on the 7th day, suggesting the influence of material surface roughness and chemical composition on energy generation. Our results are the first obtained by isolating the ECM generated by osteoblasts over implant biomaterials and reveal new insights regarding osseointegration and how material surfaces affect this process.

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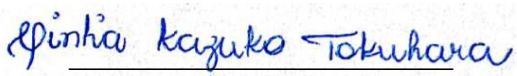
Appendix

APPENDIX A – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

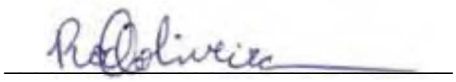
We hereby declare that we are aware of the article **On a novel method for evaluation of the mineralization process of osteoblasts on biomaterials surfaces** will be included in Thesis of the student Márcia Sirlene Zardin Graeff was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, June 6th, 2018.

Márcia Sirlene Zardin Graeff
Author



Cintia Kazuko Tokuhara
Author



Rodrigo Cardoso de Oliveira
Author

APPENDIX B – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We hereby declare that we are aware of the article **Longitudinal comparison of the ECM proteins from osteoblasts cultivated on different biomaterials** will be included in Thesis of the student Márcia Sirlene Zardin Graeff was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, June 6th, 2018.

Márcia Sirlene Zardin Graeff
Author

Cintia Kazuko Tokuhara
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Annexes

ANNEX A – Manuscript submission letter confirmation from JAOS

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1 mensagem

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03-Jun-2018

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Your manuscript entitled "On a novel method for evaluation of the mineralization process of osteoblasts on biomaterials surfaces" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Applied Oral Science.

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