Gabriella Mercedes Peñarrieta Juanito

COMPORTAMENTO MECÂNICO, QUÍMICO E BIOLÓGICO DE NOVOS MATERIAIS COM GRADIENTE DE PROPRIEDADES PARA SISTEMA DE IMPLANTES ENDÓSSEOS.

Tese submetida ao Programa de Pós-Graduação em Odontologia, do Centro de Ciências da Saúde, da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutora em Odontologia – Área de Concentração Implantodontia
Orientador: Prof. Dr. Ricardo de Souza Magini
Coorientador: Prof. Dr. João Manuel Mendes Caramês.

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Esta Tese foi julgada adequada para obtenção do Título de "Doutora em Odontologia, - Área de concentração em Implantodontia" e aprovada em sua forma final pelo Programa Pós-Graduação em Odontologia

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Dedicatória

Este trabalho é dedicado a Deus em Trindade e a minha família por serem a minha vida.

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RESUMO

Biomateriais produzidos para a fabricação de implantes endósseos atualmente são estudados para tentar diminuir o tempo de osseointegração, incrementando sua capacidade biológica, assim como melhorando suas propriedades biomecânicas. Para isso o processo de revestimento ainda é muito utilizado para tentar modificar a superfície dos implantes recobrindo eles com outros biomateriais que possam favorecer os objetivos listados acima. No entanto este processo possui problemas como delaminação e perda do recobrimento por fratura ou perdas parciais do recobrimento. Sendo assim, este trabalho tem como objetivo, a produção, análise mecânica e avaliação do comportamento celular de novos materiais biocompostos com gradiente de propriedades (FGM) a base de titânio, zircônia e poliéter-éter-cetona e tendo na sua composição hidroxiapatita e beta-tricálcio fosfato. Para isso 9 grupos foram estabelecidos produzindo 16 discos por grupo para análise mecânico, morfológico por microscopia eletrônica de varredura e análise da viabilidade, atividade de fosfatase alcalina e produção de conteúdo mineral de osteoblastos fetais humanos semeados nos discos. Os resultados mostraram uma melhor resposta na maioria dos grupos bioativos sendo o de Zircônia com hidroxiapatita, titânio com beta tricálcio fosfato e PEEK com hidroxiapatita, evidenciaram maior potencial biológico, concluindo que os novos biocompostos produzidos se apresentaram biocompativeis mostrando aderência, morfologia normal, viabilidade, atividade e mineralização aumentada e em algumas situações obtiveram melhores resultados quando comparados aos materiais base.

Palavras-chave: Biocompostos, Titânio, Zircônia, PEEK, Osteoblastos humanos.

ABSTRACT

Biomaterials produced for the manufacture of endosteal implants are currently studied to try to reduce the time of osseointegration, increasing its biological tissue capacity, as well as improving its biomechanical properties. For this the coating process is still very used to try to modify the surface of the materials covering. However, this process has problems such as delamination and loss of the recoating by fracture or partial losses of the recoating. Therefore this work has as objective, the production, mechanical analysis and evaluation of the cellular behavior of new with gradient properties (FGM), titanium, zirconia and polyether ether ketone PEEK and having in its composition hydroxyapatite and beta-tricalcium phosphate. For this, 9 groups were established producing 16 discs per group for mechanical analysis, morphological analysis by scanning electron microscopy and analysis of viability, alkaline phosphatase activity and mineral content production of human fetal osteoblasts seeded on the discs. The results showed a better response in most of the bioactive groups being Zirconia with hydroxyapatite, titanium with beta tricalcium phosphate and PEEK with hydroxyapatite that showed better potential, concluding that the new biocomposites produced were biocompatible showing adhesion, normal morphology, viability, activity and increased mineralization and in some situations obtained better results when compared to the base materials.

Keywords: Biocomposites, Titanium, Zirconia, PEEK, Human osteoblasts

LISTA DE ABREVIATURAS E SIGLAS

MSC	células estaminais mesenquimais
BMPs	proteínas ósseas morfogenéticas
Wnt	vias Wingless
Runx2	fatores de transcrição relacionados a Runt 2
Osx	osterix
Runx2	fatores de transcrição relacionados a Runt 2
ColIA1	Colágeno tipo I
ALP	Fosfatase Alcalina
BSP	Sialoproteina óssea
OCN	Osteocalcina
FGM	Functional gradient material
Ti	Titânio
Z	Zircônia
YTZP	Zircônia estabilizada com Yttria
PEEK	Poli éter-éter-cetona
HA	Hidroxiapatita
βTCP	Beta tricálcio fosfato
SEM	Scanning eletron microscopy
μm	Micrometro
μl	Microlitro
nm	nanômetro
nmol	nano mol
BSE	backscattering electron
MPa	Mega pascal
GPa	Giga Pascal
BIC	Bone Implant Contact

SUMÁRIO

CAPITULO I

1.1. REVISÃO DA LITERATURA

1.1.1. BIOLOGIA ÓSSEA

1.1.1.1. Tecido ósseo

O osso é um tecido conjuntivo mineralizado que exibe quatro tipos de células: osteoblastos, células do revestimento ósseo *(bone lining cells)*, osteócitos e osteoclastos (BUCKWALTER *et. al.* 1996; P. A. DOWNEY AND M. I. SIEGEL, 2006). O osso exerce funções importantes no corpo, como locomoção, suporte e proteção de tecidos mole, armazenamento de, cálcio e fosfato e abrigos de medula óssea (A. G. ROBLING, A. B. CASTILLO, C. H. TURNER, 2006; DATTA e*t. al*, 2008). Apesar da aparência inerte, o osso é um órgão altamente dinâmico que é reabsorvido continuamente por osteoclastos e neoformado por osteoblastos. Há evidências de que osteócitos atuam como e orquestradores deste processo de remodelação óssea (B. CLARKE, 2008; G. KARSENTY, H. M. KRONENBERG, AND C. SETTEMBRE, 2009; S. L. TEITELBAUM, 2007; L. F. BONEWALD, 2011). A função das células de revestimento ainda não são tão claras, mas essas células parecem desempenhar um papel importante no equilíbrio da reabsorção e formação óssea (V. EVERTS et. al. 2002).

O remodelamento ósseo é um processo altamente complexo onde o osso antigo é substituído por osso novo, em um ciclo composto por três fases: (1) iniciação da reabsorção óssea por osteoclastos, (2) a transição (ou o período de reversão) da reabsorção para nova formação óssea e (3) formação óssea por osteoblastos (N. A. SIMS AND J. H. GOOI, 2008; K. MATSUO AND N. IRIE, 2008). Esse processo ocorre devido a ações coordenadas de osteoclastos, osteoblastos, osteócitos e células de revestimento ósseo que juntos formam a estrutura anatômica temporária chamada de unidade multicelular básica (BMU) (H. M. FROST, 1969; E. M. HAUGE, *et. al*. 2001; T. L. ANDERSEN *et. al*. 2009).

A remodelação óssea normal é necessária para a cicatrização da fratura e adaptação de esqueleto para uso mecânico, bem como para homeostase de cálcio (S. L. DALLAS *et. al*. 2013). Portanto, o equilíbrio entre a formação óssea e a reabsorção é necessário e depende da ação de vários locais e fatores sistêmicos, incluindo hormônios, citocinas, quimiocinas, e estimulação biomecânica (L. G. RAISZ AND G. A.

RODAN, 1998; T. C. A. PHAN, J. XU, AND M. H. ZHENG, 2004; J. C. CROCKETT, *et. al.* 2011).

Estudos recentes mostraram que o osso influencia na atividade de outros órgãos e ossos também são influenciados por outros órgãos e sistemas do corpo (S. FUKUMOTO AND T. J. MARTIN, 2009), fornecendo novas informações à complexidade e a natureza dinâmica de tecido ósseo.

1.1.1.2. Osteoblastos

Os osteoblastos compreendem aproximadamente 4-6% do total das células ósseas e são amplamente conhecidos por sua função na formação óssea (M. CAPULLI, R. PAONE, AND N. RUCCI, 2014). Estas células apresentam características morfológicas de células sintetizadoras de proteínas por possuir abundante retículo endoplasmático rugoso e proeminente aparelho de Golgi, também como várias vesículas secretoras (P. D. DAMOULIS AND P. V. HAUSCHKA,1997). Sendo evidente que os osteoblastos secretam o osteóide em direção à matriz óssea (P. DUCY, *et. al* 1997).

Osteoblastos são derivados de células estaminais mesenquimais (MSC), as MSC como osteoprogenitoras precisam da expressão de genes específicos, seguindo etapas oportunas programadas, incluindo a síntese de proteínas ósseas morfogenéticas (BMPs) e membros de as vias Wingless (Wnt) (T. KOMORI, H. YAGI, S. NOMURA et al., 1997; M. FAKHRY *et al*, 2013). Os fatores de transcrição relacionados a Runt 2 (Runx2), e osterix (Osx) são cruciais para a diferenciação osteoblástica (P. D. DAMOULIS AND P. V. HAUSCHKA,1997; M. FAKHRY *et al*, 2013). Além disso, Runx2 é um gene mestre da diferenciação de osteoblastos (T. KOMORI, H. YAGI, S. NOMURA et al.1997). Runx2 demonstrou estar relacionado com a presença de genes relacionados com osteoblastos, como ColIA1, ALP, BSP, BGLAP e OCN (K.NAKASHIMA, X. ZHOU, G. KUNKEL *et al*. 2002).

Uma vez que um grupo de progenitores osteoblásticos expressando Runx2 e ColIA1 foi estabelecida durante a diferenciação de osteoblastos, há uma fase de proliferação. Nesta fase, os progenitores mostram atividade de fosfatase alcalina (ALP) e são considerados pré-osteoblastos (M. CAPULLI, R. PAONE, AND N. RUCCI, 2014). A transição de preosteoblastos a osteoblastos maduros é caracterizado por

aumento da expressão de Osx e na secreção de proteínas da matriz tais como osteocalcina (OCN), sialoproteína óssea (BSP) I / II e colágeno tipo I. Além disso, os osteoblastos sofrer alterações morfológicas, tornando-se maior e definido (D. A. GLASS II, P. BIALEK, J. D. AHN *et al*. 2005; H. HU, *et al*, 2005).

Há evidências de que outros fatores, como fator de crescimento dos fibroblastos (FGF), microRNAs e conexin desempenham papéis importantes na diferenciação dos osteoblastos (H. C. ANDERSON *et al*., 2003; K. KAPINAS *et al*. 2010; Y. ZHANG, *et. al*, 2011).

A síntese de matriz óssea por osteoblastos ocorre em dois passos principais: deposição de matriz orgânica e subsequente mineralização. No primeiro passo, os osteoblastos secretam proteínas de colágeno, principalmente colágeno tipo I e proteínas não colágenas (OCN, osteonectina, BSP II e osteopontina), e proteoglicanos incluindo decorin e biglycan, que formam a matriz orgânica. Posteriormente, a mineralização de matriz óssea ocorre em duas fases: a vesicular e a fase fibrilar. A fase vesicular ocorre quando as porções com um diâmetro de 30 a 200 nm, chamadas vesículas de matriz, são liberadas da membrana dos osteoblastos. Na recém-formada matriz óssea em que se ligam a proteoglicanos e outros componentes orgânicos. Por causa de sua carga negativa, o proteoglicanos sulfatados imobilizam íons de cálcio que são armazenados dentro das vesículas da matriz. Quando osteoblastos segregam enzimas que degradam os proteoglicanos, os íons de cálcio são liberados dos proteoglicanos e atravessam os canais de cálcio (H. C. ANDERSON *et al*., 2003; Y. YOSHIKO *et al*., 2007).

Esses canais são formados por proteínas chamadas anexinas (H. C. ANDERSON *et al*., 2003). Por outro lado, os compostos que contêm fosfatos são degradados pela ALP secretada por osteoblastos, liberando íons de fosfato dentro das vesículas da matriz. Então, o fosfato e íons de cálcio dentro do nucleato das vesículas, formam fosfatos de cálcio principalmente os cristais de hidroxiapatita (V. E. ARANA-CHAVEZ, A. M. V. SOARES, AND E. KATCHBURIAN, 1995). A fase fibrilar ocorre quando a supersaturação de íons de cálcio e fosfato dentro das vesículas de matriz leva à ruptura dessas estruturas e os cristais de hidroxiapatita se espalham para a matriz circundante (M. J. GLIMCHER,1998).

Osteoblastos maduros aparecem, alguns desses osteoblastos mostram processos citoplasmáticos em direção à matriz óssea e alcança os processos de osteócitos. Nesta fase, os osteoblastos maduros podem sofrer apoptose ou tornar-se osteócitos ou células de revestimento ósseo (G. BOIVIN AND P. J. MEUNIER, 2002; G. BOIVIN, Y. BALA, A.DOUBLIER *et al*, 2008).

1.1.2 MATERIAIS PARA IMPLANTES ENDÓSSEOS

Na caracterização de materiais utilizados na indústria biomédica a biocompatibilidade é de grande importância. A história da implantodontia mostra que os materiais empregados para fabricação de implantes endósseos apresentam diferentes níveis de biocompatibilidade. Um material de implante ideal deve possuir adequadas propriedades biológicas, físicas e químicas. Assim deve apresentar biocompatibilidade elevada, tenacidade, resistência à corrosão, desgaste e fratura adequados entre outras. (Parr et al., 1985; Smith 1993). Os materiais utilizados para a fabricação dos implantes endósseos podem ser categorizados de acordo com sua composição química em metais, cerâmicas ou polímeros (Sykaras et al., 2000). Com base no tipo de resposta biológica, estariam classificados em: biotolerante, bioinerte e bioativo (LeGeros e Craig 1993). Devido as elevadas taxas de sobrevivência clínica a longo prazo relatadas para titânio e suas ligas, considerou-se o titânio o material "padrão-ouro" para a fabricação de implantes endósseos em muitas situações clínicas (Jemt et al., 1996).

1.1.2.1 Titânio

Dentre os biomateriais atualmente aplicados em Implantodontia, o Titânio comercialmente puro (Ti-CP) é o mais usado para fabricar implantes dentários propriamente ditos, enquanto as ligas de titânio (ex. Ti6Al4V) são mais utilizadas para fabricação dos pilares (*abutments*) e infraestruturas protéticas (SYKARAS *et al*. 2000; SAMPAIO *et. al*. 2015). O Titânio e suas ligas possuem um significante desempenho clínico, devido às suas propriedades físico-químico-mecânicas, tais como baixa densidade (aprox. 4,5 g/cm^3), baixo módulo de elasticidade (110-140 GPa), alta resistência mecânica, alta resistência à corrosão e excelente biocompatibilidade (NIINOMI, 1998; CRUZ et al., 2011). Ligas de titânio têm mostrado integração e ótima adaptação com o osso e tecidos moles adjacentes. No entanto, existe a preocupação de que as ligas de titânio possam liberar quantidades significativas de elementos de liga, como alumínio (Al) e vanádio (V) prejudicando o processo de osseointegração e promovendo desde efeitos citotóxicos como um potencial mutagênico e carcinogênico devido a

presença de vanádio. Na verdade, a resistência à corrosão e a biocompatibilidade do titânio e suas ligas são resultantes da composição e estrutura do filme de óxidos de titânio na superfície. Presença da película protetora (filme passivo e compacto) de óxido de titânio (BRANEMARK et al., 1987; CRUZ et al, 2011; SOUZA et al, 2012). O filme de óxido de titânio sobre Ti-CP apresenta-se como uma fina camada compacta com espessura variável em 10-20 nm sob condições ambientais. Esta camada também é chamada de filme passivo à medida em que apresente baixa reatividade química com o meio. E muitas vezes, chamada de película protetora por evitar ou diminuir a corrosão do material de titânio (CRUZ et al., 2011; LANDOLT et al., 2006).

A degradação do titânio e suas ligas aplicadas para sistemas de reabilitação oral e implantes dentários depende principalmente das condições do meio oral (BHATTARAI et al., 2008; CRUZ et al., 2011). Estas condições envolvem pH ambiental ácido, presença de substâncias reativas ao titânio e efeito adicional de solicitações mecânicas oriundas da mastigação. Um ambiente fisiológico corrosivo em combinação com cargas cíclicas (fadiga) e degaste em superfícies de infraestruturas protéticas e conexões de sistemas de implante dentários podem aumentar significativamente as taxas de corrosão e, consequentemente, a degradação dos materiais propiciando liberação de íons metálicos (LANDOLT et al., 2006; SOUZA et al., 2012; LEWIS et al., 2005). A degradação da camada protetora torna o titânio vulnerável a uma acelerada degradação química e mecânica (SOUZA et al., 2012). Um estudo *in* vitro avaliou o efeito citotóxico de diferentes concentrações de titânio comercialmente puro em osteoblastos (PIOLETTI et al., 1999). As partículas de titânio tinham um efeito indireto na viabilidade dos osteoblastos. Observou-se também que o as partículas de titânio induziram um processo de morte celular programada (apoptose) quando cultivado com osteoblastos. Uma maior concentração de desgaste de titânio influencia a viabilidade de osteoblastos e estes osteoblastos liberam produtos citotóxicos (PIOLETTI et al., 1999) Sridhar et al. relatou que esta liberação de íons para a cavidade oral pode estar relacionada ao desenvolvimento de peri-implantitis (SRIDHAR et al., 2015). Razões pelas que atualmente tenta se produzir novos biomateriais a base de titânio.

1.1.2.2. Zircônia

Zircônia, o dióxido de metal (ZrO_2), foi identificado como tal em 1789 pelo químico alemão Martin Heinrich Klaproth em um produto de

reação, obtido depois de aquecer algumas gemas. O zircônio puro não foi produzido até 1914.

O dióxido de zircônio é um óxido de zircónio cristalino branco. O interesse inicial em usando zircônia como um biomaterial cerâmico derivado de sua boa química e estabilidade dimensional, bem como da sua resistência mecânica, combinado com um módulo de Young (200 GPa) da mesma ordem de grandeza que ligas de aço inoxidável (ASSAL 2013). A zircônia foi usada no final dos anos sessenta como aplicação biomédica (HELMER E DRISKELL, 1969). No entanto, na década dos 80 já se utilizava a zircônia para fabricar cabeças de bola para reabilitações de joelho (CHRISTEL et al., 1988). O dióxido de zircónio, vulgarmente conhecido como zircônia, é um material cerâmico que teve um rápido aumento no uso em medicina e odontologia. Dentro da odontologia, cerâmicas foram introduzidas em implantes sob a forma de revestimentos em implantes endósseos baseados em metal para melhorar a osseointegração (OSMAN E SWAIN 2015). A zircônia também foi usada para pilares e estrutura para próteses dentárias fixas (NAKAMURA et al., 2010; PICONI et al., 1998). Embora os pilares cerâmicos associados a coroas totalmente cerâmicas tenham se mostrado como adequados materiais em situações estéticas de maior exigência, a presença da junção de fixação do pilar suscitou preocupações (CANULLO et al., 2007). Houve uma forte renovação do interesse na cerâmica para aplicação dentária com o desenvolvimento de ciência dos biomateriais e tecnologia industrial. A zircônia policristalina tetragonal estabilizada com Ytria exibe propriedades mecânicas melhoradas, como corrosão, resistência e resistência à flexão quando comparada com outras cerâmicas, fazendo dela adequada para a fabricação de implantes dentários (DENRY E KELLY 2008). Um estudo mostrou que o contato osso-implante (BIC) era semelhante ao comparar o titânio com implantes de zircônia demonstrando assim que a zircônia pode ser potencialmente utilizada como material para implantes (VAN DOOREN et al., 2012). Alguns estudos mostraram que o revestimento de zircônia na superfície dos implantes de titânio favorece a aposição do osso, e concomitantemente melhora da osseointegração, quando comparados a implantes de titânio sem revestimento (FRANCHI et al., 2004).

Apesar de comprovadas várias características vantajosas da zircônia, alguns problemas já foram reportados com os pilares comerciais de titânio revestidos tais como: processamento laboratorial e diferenças de propriedades entre titânio e zircônia, assim como a desvantagem da zircônia referida a sua degradação conhecida como

envelhecimento (*aging*) relacionada a ambientes húmidos que prejudicam as propriedades mecânicas da zircônia (DEVILLE, CHEVALIER, GREMILLARD, 2006). As diferentes propriedades entre YSZ e pilar de titânio podem provocar fissuras na interface levando a um prejuízo na integridade desta interface e consequente falha por esforço mecânico.

1.1.2.3. Poli éter-éter-cetona (PEEK)

Atualmente, polímeros são cada vez mais utilizados, entre outras razões pela facilidade de produção e custo relativamente baixo comparado a outros materiais, sendo assim, na indústria biomédica é um dos materiais mais utilizados. O poli éter-éter-cetona (PEEK) é um polímero muito estudado e produzido desde a década dos 80 na área da ortopedia, cirurgia e reabilitação craneana, vertebral e oral devido à sua biocompatibilidade e baixo módulo de elasticidade em comparação com o titânio (SKINNER HB, 1988; LEE *et al*., 2012). A estabilidade térmica do PEEK tem sido estudada para aplicações industriais em altas temperaturas tendo em vista as suas propriedades biocompatíveis e biomecânicas após esterilização. O PEEK reforçado com fibras de carbono (CFR-PEEK) tem apresentado melhor desempenho mecânico mantendo a sua biocompatibilidade o que pode ser adequado para revestimento de pilares protéticos. (KOCH FP *et. al.* 2010). Porém mesmo com essas propriedades tão atraentes presentes neste biomaterial, as propriedades biomecânicas do PEEK ainda não são melhores quando comparadas a zircônia ou alguns metais, assim como a sua biocompatibilidade não se encontra tão esclarecida para se afirmar que seja maior do que ao titânio ou zircônia. Por isso, estudos propõem melhorar essas dificuldades com revestimentos metálicos e cerâmicos, para melhorar resultados mecânicos e biológicos quando comparado a forma sem revestimento (COOK SD, RUST-DAWICKI AM, 1995; KOCH FP *et. al.* 2010).

1.1.2.4. Materiais com gradiente de propriedades (FGM)

Como referido, a grande diferença de propriedades entre implante e osso gera tensões excessivas na interface osso/implante

durante a distribuição das cargas da mastigação (DÍAZ et al., 2009; CRUZ et al., 2011). Materiais compósitos convencionais possuem vantagens quando comparados a materiais puros mas apesar de todas essas vantagens são sujeitos a uma transição acentuada de propriedades na interface que pode resultar em componentes falha (por delaminação) em condições extremas de trabalho. Esta desvantagem dos compósitos convencionais é eliminada pela forma modificada de compósitos chamados materiais de gradiente funcional (FGMs). Esses materiais substituem a interface nítida com a interface gradiente, o que resulta na transição suave de propriedades de um material para outro. Estes materiais avançados com gradientes projetados de composição, estrutura e propriedades específicas na direção preferida são superiores ao material homogêneo composto de constituintes similares. As propriedades mecânicas, como módulo de elasticidade de Young, razão de Poisson, módulo de cisalhamento de elasticidade, a densidade do material e o coeficiente de expansão térmica variam de forma suave e contínua nas direções preferidas nos FGM.

Osso, dentes, pele e bambu são alguns exemplos de materiais com gradiente funcional. Em implantodontia também existe a ideia de utilizar materiais com módulo de elasticidade similar ao do osso para a reabilitação implanto-suportada sugere uma distribuição mais suave de tensões aos tecidos de suporte com decréscimo de tensões nesta interface. Os FGM consistem de um compósito não convencional ou material híbrido contendo um número de constituintes que apresentam um gradiente de composição de uma superfície do material para o outro, em seguida, resultando num material com propriedades graduadas continuamente. Sendo assim, os problemas em próteses dentárias e sistemas de implantes com a utilização de diferentes materiais de contato podem ser ultrapassados pela utilização de um FGM. Análises experimentais e teóricas revelaram que os gradientes de composição de superfície pode melhorar o desempenho mecânico de um material demonstrando melhorias na resistência de união de interfaces usando a abordagem FGM (SHAHISTHA. et. al. 2014; MIRANDA G. et. al. 2016; ZHANG et al., 2012; HENRIQUES et al, 2011). O desenvolvimento de materiais com gradiente de propriedades pretende reduzir a degradação por fadiga e desgaste dos materiais envolvidos e melhorar o desempenho clínico destes materiais híbridos, como já foi demostrado em algumas pesquisas (HENRIQUES et al., 2012; KIM, LIU, ZHANG, 2010).

Depois de ter mostrado as desvantagens apresentadas pelos materiais base de titânio, zircônia e PEEK este trabalho de tese julga importante realizar o estudo biomecânico, químico e biológico de novos materiais com gradiente de propriedades que poderiam oferecer resultados melhores mecânica e biologicamente oferecendo resultados previsíveis e atraentes para investigações futuras que poderiam ser levados à clínica nas cirurgias orais e ortopédicas.

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CAPITULO II

2. Artigos Científicos

2.1. Primeiro Artigo: Submetido 08 de Dezembro - *Journal of Biomedical Materials Research: Part A* (aceito, *ahead of print* 25 março)

Bioactivity of novel functionally structured titanium-ceramic composites in the presence of human osteoblast cells

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Abstract

The aim of this study was to analyze the osteogenic cell behavior on the surface of novel functionally graded titanium-based composites containing bioactive ceramics. Titanium grade V discs (8 x 3 mm) embedding gradual content of hydroxyapatite (TiAlV-HA) or beta-tricalcium phosphate (TiAlV-βTCP) were produced by hot-pressing technique. Titanium-ceramic composite discs and Ti grade V (control group) were placed in contact with human osteoblast culture assays. The morphology and adhesion of osteoblasts were inspected by field emission guns scanning electron microscopy (FEGSEM) while cell viability was assessed by fluorometric method. Alkaline phosphatase (ALP) activity and fluorescent microscopic analyses were used to evaluate mineralization on the test and control discs. FEGSEM images showed cells adhered to Ti6Al4V/ceramic and Ti6Al4V surfaces over a

period of 24 h and therefore an intense proliferation of osteoblasts and spreading cells was noticed for 7 days. Cell viability increased with time on all the surfaces although TiAlV-βTCP revealed significant higher percentage of cell viability than that recorded for TiAlV-HA ($p < 0.01$). TiAlV-βTCP also showed the highest hydrophilic character. ALP levels increased on the Ti/ceramic surfaces when compared to the control group. Also, a qualitative analysis of mineralization evidenced an increase in mineral content TiAlV-HA or TiAlV-βTCP groups. Novel functionally graded composites based on Ti grade V and hydroxyapatite or βTCP show a higher bioactivity in presence of osteoblasts than that recorded on Ti grade V. Also, such functionally graded materials can prevent risks of failures by detachment of bioactive ceramic materials during implant placement. Keywords: FGM, titanium, bioactivity, functionally graded materials, βTCP, Hydroxyapatite

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1. Introduction

Biomaterials used in oral and orthopedic implantology are currently developed to achieve stable, predictable and rapid integration to different types of bone tissues [1].

Titanium and its alloys are widely used to produce implant and prosthetic structures due to their excellent biocompatibility, corrosion resistance and strength [2]. Also, titanium modifications by coating and different surface treatment with bioactive ceramic materials have been developed to enhance biocompatibility and osseointegration process. However, failures related to ceramic detachment by chipping and bone-implant adverse reactions have been reported in literature [3].

The most widely used materials for the manufacture of dental implant fixtures are commercially pure titanium (cpTi) grade II or grade IV: cpTi grade IV has higher strength (tensile strength at around 560 MPa) than cpTi grade II (tensile strength at around 360 MPa). On the other hand, cpTi grade IV has lower purity (98.9% titanium)
and corrosion resistance compared with cpTi grade II (99.8% titanium). The
biocompatibility of titanium and its alloys is attributed to the protective titanium oxide thin layer formed on the titanium surface, which provides excellent corrosion resistance and chemical interactivity with proteins and human cells. The dissolution reaction of the titanium oxide film

depends on the environment pH and consequently the proton concentration. *In vitro* studies of biocompatibility are carried out in osteoblasts, for the research to be the most similar to human bone and it is preferable to use healthy human osteoblasts, since osteoblasts of animals or oncological tissues could have different behavior. [4][5] Despite their favorable use for load-bearing implants, bonding of Ti and its alloys to bone takes time in the early post implantation stage [4]. Many surface modifications of implant and prostheses have been developed to improve the physchochemical properties if titanium-based surfaces and therefore to stimulate osteogenic cell reactions [6,7].

Hydroxyapatite (HA) and beta tricalcium phosphate (βTCP) are the most extensively studied calcium phosphates and therefore they are widely used in various applications, such as in biomaterials, ion exchangers, adsorbents, and catalysts [8–10]. HA is also considered suitable for bone repairing, particularly in orthopedic implants to accelerate growth of surrounding bone because of the structural and chemical similarities between HA crystal and bone calcium phosphates. However, these bioactive materials cannot be used clinically such as monolithic implant materials due to the low strength and fragile behavior that increases the risks of early failures [11–13]. Furthermore, bioactive ceramic materials can be used as coating of Ti based implants to stimulate migration and adhesion of cells and proteins, as well as to accelerate bone growth. Several methods have been used to coat Ti-based implant surfaces with HA and TCP [8,10] although the major concern is the prevalence of fractures at the Ti/ceramic interfaces by chipping on friction during implant placement into bone.

Concerning the fractures at ceramic coatings on titanium, some studies have reported the development of composites for implant and prosthetic materials [10,11,14,15].

Bioactive ceramic materials have been used to produce titanium/ceramic composites; however, the strategies found in literature are based on homogenous composites with chemical, biologic and mechanical limitations. A few studies have recorded promising mechanical behavior results for functionally graded materials (FGM) by using hot pressing technique [16,17]. In fact, there is a lack of data on the biological behavior for such novel composites. For this reason, this *in vitro* study proposed to study the bioactive behavior for osteoblast cells on functionally structured composites composed of titanium and hydroxyapatite or beta tricalcium phosphate. It was hypothesized that functionally graded titanium-based composites embedding bioactive

ceramics can enhance bioactivity and osteoblast cell migration to implants.

1. Materials and Methods

2.1. Preparation of samples.

Ti grade V (Ti6Al4V) powders were mechanically mixed with HA or βTCP in a proportion at 95 wt% Ti6Al4V and 5 wt% bioactive ceramic material in a stainless steel containing steel milling balls at 25 rpm for 21 h. The powder mixture was dehydrated at 110°C for 1 h, and then placed into graphite molds. The composites were then produced by hot-pressing, in primary vacuum, using a high frequency induction furnace as illustrated in Figure 1. The molds containing powder mixtures were heated up to 1200 oC at 31 oC/min. At 1100 oC, the pressure on the sample was raised up to 20 MPa, and then maintained during 30 min. 16 Disc samples were obtained with 8mm in diameter and 3mm in height and divided in three groups: Ti6Al4V, TiAlV-HA and TiAlV-βTCP. The substrate surface was air braded with 250 μm alumina particles at an impact angle of 90 oC on 5 bar at 80 mm away from the surface to obtain a standard rough surface. The discs were grit-blasted to obtain similar roughness for all the samples. The coupons were ultrasonically cleaned in ethanol for 15 min, and in distilled water for 10 min before sterilization at 121°C in autoclave for 20 min. To evaluate morphology, roughness and hydrophilicity, the hydrophilicity of the materials was related to the contact angle at lower angle value, more hydrophilic is the material.

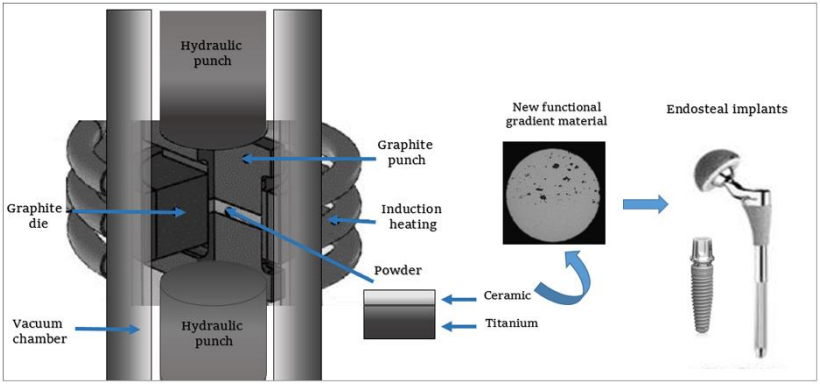

Figure 1: Schematic illustration for samples production by hot pressing sintering.

2.2. Cell culture

Human fetal osteoblasts (hFOB 1.19, ATCC R CRL11372, Manassas, VA, USA) were used for the biological assays to mimic the initial human osseointegration process since osteoblasts of animals or oncological tissues could have different behaviors [4][5].

Such cell is a conditionally immortalized and stably transfected with a gene encoding a temperature-sensitive mutant (tsA58) of SV40 large T antigen. The cells grown at a permissive temperature at 33.5 oC exhibit rapid cell division, whereas little cell division occurs at a restrictive temperature at 39.5 oC [18]. Osteoblasts were washed in a 37°C water bath for 2 min and transferred to a 25 cm3 culture flask for culture at 37°C in an atmosphere of 5% CO2 and 100% humidity. The culture medium was composed of 1:1(vol) mixture of Ham's F12 medium (Biowest, France) and Dulbecco's Modified Eagle's Medium DMEM (Biowest, France), enriched with 0.3 mg/ml antibiotic solution G418; fetal bovine serum FBS (Biowest, France) to achieve a final concentration at 10%. After reaching 80% confluence, cells were washed three times with phosphate buffer saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2HPO4) and harvested in 0.25% trypsin-0.53 mM EDTA (Gibco, Gaithersburg, Maryland) at 37°C for 5 min. Then, 8 mL of complete growth medium was placed in 75 cm3 culture flask. The medium was changed every 48 h. All tests were performed on the fourth subculture.

2.3. Morphological analyses of surfaces and cells

Groups of coupons were removed after 24 h, or 7 days incubation and washed with PBS to remove non-adherent cells. Adhered cells onto the surfaces were fixed in 1.5% glutaraldehyde solution for 10 min, washed with sterile distilled water, and then kept in buffer solution of 0.14 M sodium cacodylate until dehydration procedure. For dehydration, the surfaces containing cells were washed with PBS and immersed progressively in ascending concentration of ethanol ranging from 50 up to 100%. The coupons were covered with a 15 nm thin film (of Au-Pd (80-20 wt %); using a high resolution sputtering coater (208HR Cressington Company, USA), coupled to a MTM-20 Cressington high resolution thickness controller (Cressington Company, USA).

Morphological analyses were carried out by ultra-high resolution Field emission Guns scanning electron microscopy (FEG-SEM), (NOVA 200 Nano SEM, USA). Secondary electron images were performed at

different magnifications ranging from 500 and 5,000X at 10kV. Atomic contrast images were performed by backscattering electron (BSE) mode at 15 kV.

2.4. Cell Viability Assays

Four groups were considered for the biologic assays: Ti6Al4V, TiAlV-HA, TiAlV-βTCP and positive control (medium with cells free of test materials). The coupons were placed on the bottom of 48 well culture plates. The bottom of the well plates had a treated surface that increases the wettability of the surface to allow a standard cellular adhesion.

The cells were seeded at a density of 1 x 10^4 cells/well in 0.5 ml medium. After 1, 3, 7 and 14 days of culture, the supernatant for each well was then removed, and 300 µl Cell-Titer Blue buffer (Promega, Madison, WI, USA) was added into the wells. This assay provides a homogeneous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurin to measure the metabolic capacity of cells an indicator of cell viability. Viable cells retain

the ability to reduce resazurin into resorufin, which is highly fluorescent. Non-viable cells rapidly lose metabolic capacity that do not reduce the indicator dye, and thus do not generate a fluorescent signal. After incubation at 37°C for 4 h, the cell viability assay (n=8) per group was performed by spectrophotometry at 560/590 nm using a Luminescence *spectrometer* (*PerkinElmer* LS 50B, UK)

2.5. The alkaline phosphatase activity and mineralization assays

The alkaline phosphatase (ALP) assay is one of the most widely used biochemical markers for this type of cell since it is abundantly expressed in osteoblasts and allows a quantitative measurement of osteoblast activity. ALP activity was measured following the manufacturer's instructions (ab83371 ALP assay Fluorometric, Cambrige, UK).

ALP activity was evaluated for the same groups that were analyzed considering cell proliferation (n = 3) per group.

At each experimental time point, the coupons were washed twice with PBS and the cell culture medium was removed. The cell layers were detached and samples were washed. For the analysis, a standard curve in ranging from 0.0 to 0.4 nmol 4-MUP was also considered. ALP concentration was measured by spectrophotometry at 360/ 440 nm using *Fluorescence spectrometer* (*PerkinElmer* LS 45, UK).

For 7 and 14 days of culture, groups of coupons were removed and transferred to new well plates. Coupons were washed with PBS to

remove non-adherent cells and fixed in 1.5% glutaraldehyde solution for 10 min. After fixation and washing in sterile distilled water, cell culture mineralization was evaluated by fluorescent staining of mineral content deposited by cells following OsteoImage test protocol (Lonza, USA). Such
measurement was performed by spectrophotometry at an excitation wavelength at 492 nm and emitted wavelength at 520 nm. These images was obtained using an Leica TCS SP5 confocal microscope (Leica Microsystems)

2.6. *Statistical analysis*
The statistical analysis for viability and ALP activity was performed using the normal distribution analysis evaluated by the Shapiro-Wilk test. A factorial analysis of variance (ANOVA) was used to determine significant differences among the evaluated groups. Then, the Post Hoc (Tukey) test was applied to compare the groups´ viability and ALP activity, considering $*p<0.01$ statistically significant. All analyses were performed using SPSS statistics 17.0 (SPSS, USA).

3. Results

3.1. Functionally graded titanium-ceramics
The cross-sectioned area of the functionally graded samples was inspected by FEGSEM, as seen in Figure 2A.

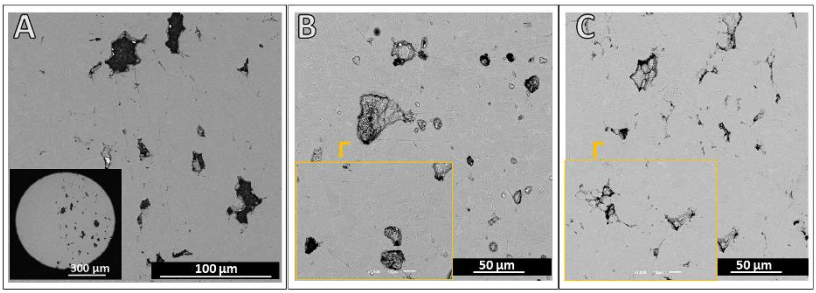

Figure 2. FEGSEM images of the cross-sectioned (A) Ti6Al4V and functionally graded Ti6Al4V containing (B) 5% HA (TiAlV-HA), or (C) 5% βTCP (TiAlV-βTCP).

The presence of the bioactive materials based on hydroxyapatite or beta tricalcium phosphate is shown in Figures 2B and C. Results of the mechanical behavior of the material in shown in Figure 3. Shear bond strength of the materials are shown in Figure 3A while hardness is revealed in Figure 3B. Ti6Al4V showed the highest mean values of shear bond strength at about 548 MPa considering there is no transition zone within bioactive ceramic. Considering the presence of a transition zone containing bioactive ceramics, TiAlV-HA revealed higher mean values of shear bond strength (aprox. 210 MPa) than that recorded on TiAlV- βTCP (aprox. 188 MPa). The hardness of the functionally graded materials containing bioactive ceramics increased when compared to Ti6Al4V (340 HV). TiAlV-βTCP revealed higher mean values of hardness (aprox. 520 HV) than that recorded on TiAlV-HA (aprox. 473 HV).

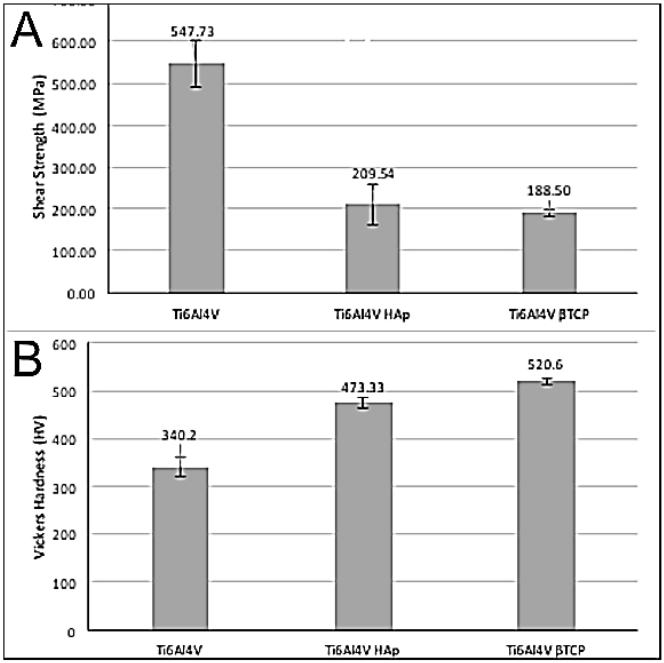

Figure 3. (A) Shear bond strength (MPa) and (B) hardness (HV) recorded on Ti6Al4V, TiAlV-HA and TiAl-βTCP.

Table I. Values of Average Roughness (R_a) and Water Contact Angle

Group	Roughness (R_a)	SD	Water Contact Angle (8)	SD
Ti6Al4V	2.36	0.22	111.96	1.45
TiAlV-HA	2.08	0.11	81.74	1.89
TiAlV-βTCP	2.19	0.08	75.29	1.23

Average roughness (R_a) values were quite similar among the groups although Ti6Al4V revealed the highest water contact angle (WCA) values, as seen in Table I. Surfaces of TiAlV-bTCP showed the highest hydrophilic character considering the lowest water contact angle values.

3.2. Cell Morphology

Adhesion and morphological aspects of the osteoblasts on the surfaces are shown in Figure 4.

The morphology of the adherent cells for 24 h cell culture in all groups. Cells were spreader on TiAlV-HA or TiAlV-βTCP than on Ti6Al4V, possibly indicating that the bioactive ceramics enhanced the stimulation of cell adhesion.
Also, it was noticed fine lamellipodia and filopodias cellular extensions (red arrows) as a sign of the spreading behavior of the cell on the materials. For 7 days, the spreading of the osteoblasts increased on all the surfaces as seen in Figure 4 (right panels). That veil-shaped cells morphology revealed a high interactivity between cell and surfaces. The agglomeration of cells was more evident on TiAlV-HA and TiAlV-βTCP than that on Ti6Al4V.

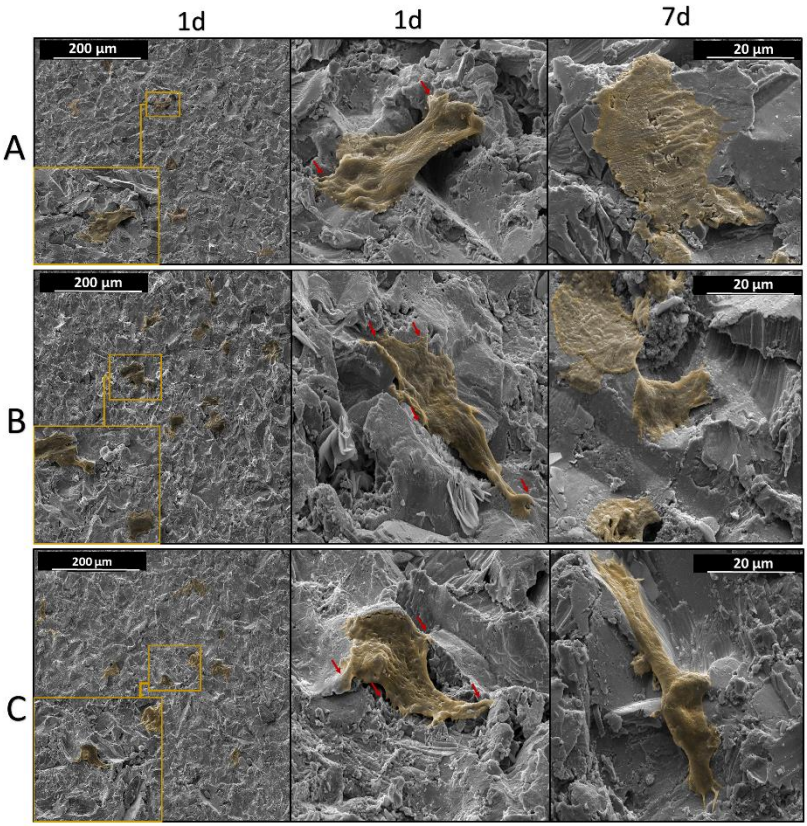

Figure 4. FEG-SEM images of the osteoblast cultured (colored brown) in discs (A) Ti6Al4V; (B) Ti6Al4V containing 5% HA (TiAlV-HA) and (C) βTCP (TiAlV-βTCP); (left panels) 500x and (inside the yellow square) 2000x for 1 day; (center panels) 5000x for 1 day; (right panels) 5,000x for 7 days of cell growth.

3.3. Cell Viability

The mean values of cell viability results for 1, 3, 7 and 14 days of cell culture are shown in Fig. 5. Results showed normal distribution with statistic difference significantly within time, according to ANOVA and post hoc Tukey tests (*). The cells favorably raised on the surfaces, showing considerable increments with culture time. At the first day, TiAlV-βTCP surfaces revealed the highest proliferation values with statistically significant differences compared to the other groups.

Ti6Al4V and TiAlV-HA groups showed a small increase in cell proliferation over the period of cell culture compared to that for control and TiAlV-βTCP groups. At all times, test groups revealed the highest cell viability without having statistical difference with the control group for the third day.

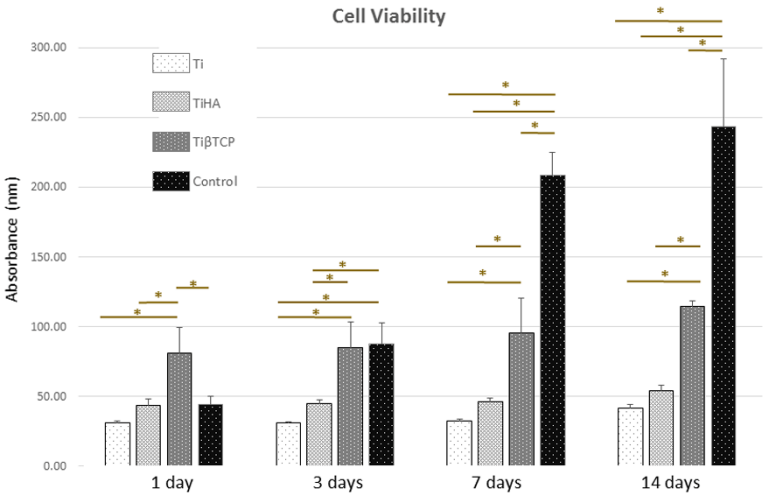

Figure 5: Viability and proliferation cell in 1, 3, 7, 14 days *p<0.05

3.4. Alkaline phosphatase and bone cell mineralization activity

ALP activity of osteoblasts cells on the surfaces for 7 or 14 days are shown in Fig. 6. Ti6Al4V group revealed the lowest ALP activity mean values for both times. Within the test groups, the ALP activity recorded on TiAlV-βTCP group for 7 and 14 days was significantly higher than that recorded on the other groups. There was no noticeable statistical difference between TiAlV-βTCP and the control group for 7 or 14 days of cell culture.

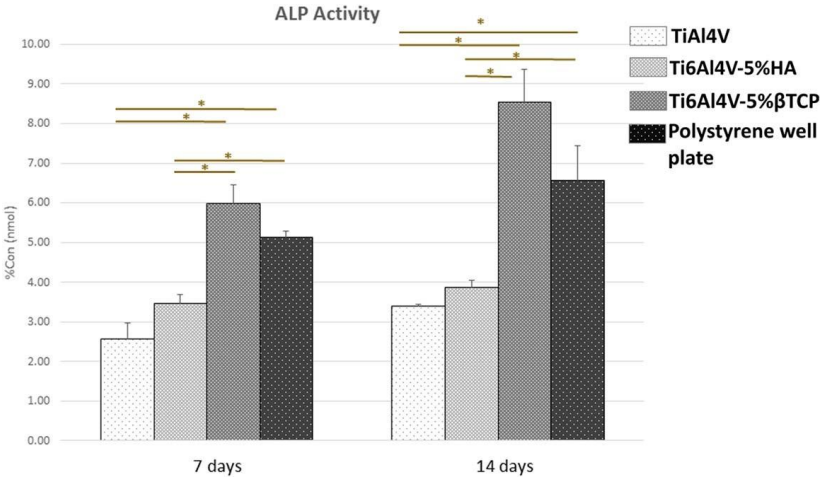

Figure 6: Mean values of fluorescence intensity in % concentration (nmol) revealing the ALP activity of osteoblast cells grown on cp Ti, Ti/HA, Ti/βTCP and polystyrene well plate (control group) for 7 and 14 days. *p<0.05

3.5. Bone cell mineralization

Images obtained by confocal fluorescence microscopy showed mineral content on all the surfaces after 7 days of cell culture (Fig. 7).

Mineral nodules produced in the presence of cultured osteoblasts were noticed on the surfaces although they were more intense on TiAlV-βTCP and TiAlV-HA surfaces while the lowest values were noticed on Ti6Al4V surfaces.

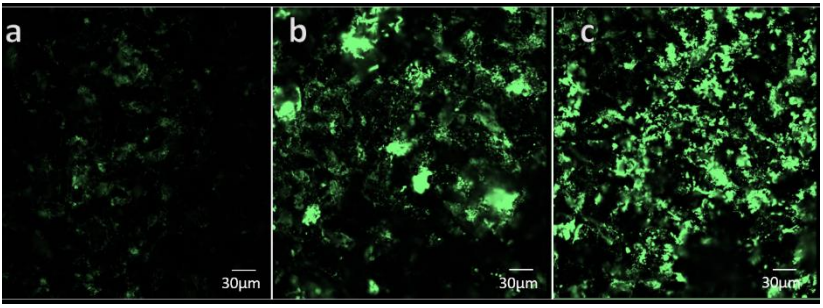

Figure 7: Confocal fluorescence microscopy images obtained by OsteoImage-stained method. Bone mineral nodules on (a) Ti; (b) Ti/HA; (c) TiβTCP groups for 14 days of cell culture, scale bar 30 μm.

4. Discussion

In this study, two novel functionally graded titanium-based structures embedding bioactive ceramics were produced by hot pressing technique. The results of the present study demonstrated a significant statistically stimulation of human osteoblast growth behavior by the presence of bioactive ceramics embedded into titanium.

In this study, we used 5% hydroxyapatite or β-TCP to improve the biocompatibility of titanium-based composites without compromise mechanical behavior of the resultant structure [16]. Hydroxyapatite and β-TCP were gradually incorporated in the composition of the titanium substrate to produce a functionally graded titanium structure leading to absence of titanium-ceramic homogeneous interface. The results for hydrophilicity showed that the Ti grade V group had a higher water contact angle (WCA) values while the functionally graded TiAlV-βTCP composites revealed the lowest WCA values and consequent hydrophilic nature. Previous studies reported the modification of titanium surfaces using bioactive ceramic coatings to improve bioactivity and to shorten osseointegration time [16]. However, mechanical issues such as ceramic delamination occur during implant-bone friction on implant placement [6,7,12,16,19]. Several types of bioactive ceramics and glass-ceramic have been used in the field of biomaterials although technological issues are related to the absorption rate associated with bioactivity and bone remodeling [6,7,12]. Bioactive ceramic and glassceramic particles are produced with defined characteristics of size, microstructure, chemical composition and morphology to enhance bone integration and remodeling.

Additionally, the mechanical properties of such ceramic materials are required for incorporation onto implant surfaces and subsurface [17,18]. Adverse local tissue reactions have been observed after delamination of coating ceramic material [19]. In implant dentistry, the implant placement is performed into bone socket by applying torque on the implant connection that is dependent on the bone quality and type of implant. The friction and stresses generated to the implant-bone interface can promote the disruption of the bioactive ceramic on the implant surface in the case of ordinary coatings. In order to evaluate osteogenic cell behavior, immortalized fetal human osteoblasts (HFOB 1.19) were used in this study to mimic the *in vivo* scenario of interaction between implant surface and osteoblasts of healthy patients. The cellular morphology detected by microscopic assessment in our results is similar

to that shown in other studies that also used HFOB 1.19 osteoblasts [5,18,19]. The use of high resolution FEGSEM images allowed us to evaluate the cell morphological details such as spreading, cellular extensions (filopodia) and cell stretching onto the morphological structures of the test surfaces, as seen in Figure 4. The cellular viability results obtained in our study showed that Ti grade V had the lowest percentage of cell proliferation. These results are similar to those of previous studies regarding the presence or not of bioactive ceramics in titanium [20,21]. At all times of cell culture assessment, the control group (well plates) showed the highest values of cell viability, due to the high roughness of the polystyrene based material which is proper for cell culture. However, Ti6Al4V, TiAlV-HA and TiAlV-βTCP samples possessed similar
roughness mean values for comparison of results concerning the differences in chemical composition of the test surfaces. In fact, the presence of bioactive ceramics plays an important role on the migration of osteogenic cells during the osseointegration process as reported by previous studies [21,22]. Those previous studies also agree that ceramic materials such as hydroxyapatite or betatricalcium phosphate stimulate the osteogenic cell migration when compared to control groups.

Studies on alkaline phosphatase (ALP) activity are also required because they have a high relationship with bone formation and healing. Regarding ALP activity in our study, test groups containing bioactive materials had higher cellular activity when compared to that recorded for Ti grade V group. These data show a correlation with fluorescence microscopy images obtained for analysis of mineral content. Also, the group containing beta tricalcium phosphate had even higher ALP values when compared to the control group. The results found in our study corroborate the results reported in previous studies. However, it is important to state that several studies are carried out with different cell types, medium, coating or modified surfaces [20].

Qualitative analysis of mineralization patterns by fluorescence method demonstrated a greater amount of mineral nodules on the test groups containing HA and βTCP, although that was more noticeable for surfaces containing βTCP. In literature, there is a lack of mineralization studies on titanium surfaces modified with bioactive materials.

Further *in vitro* and *in vivo* studies should be performed to validate the biocompatibility and mechanical behavior of the novel functionally graded structures for applications in biomedical implants for cranio-maxillofacial, oral, spinal, knee and hip prostheses.

It should be emphasized that most previous studies have assessed only the biological behavior on ceramics coatings without mentioned the cross-sectioned microstructure or titanium-coating interface. That is quite different of the purpose of the present study, which shows a novel strategy to avoid interfaces susceptible to fractures by delamination during implant placement. Also, the resorption rate into bone tissue is faster for βTCP than that recorded for HA [3]. For instance, the release of βTCP is followed by the process of mineralization and bone growth in the pores of the rough titanium surfaces. The low resorption rate of HA provides a higher mechanical stability of the functionally graded transition zone although the bone growth into the porous titanium surfaces can be delayed. In fact, a functionally graded materials composed of a mixture between such bioactive materials could be a potential strategy to control bioactivity and mechanical stability of the implant to bone interface.

5. Conclusions

The addition of hydroxyapatite and βTCP into titanium grade V as bioactive materials was performed in the present study by using a novel strategy to create a functionally graded composite implant. Osteoblast cells adhered to the surfaces of test materials revealed a typical morphology and spreading behavior for bioactive surfaces. The functionally graded structure embedding betatricalcium phosphate provided a higher viability and alkaline phosphatase activity with noticeable formation of mineral matrix when compared to commercially pure titanium containing or not hydroxyapatite.

Further *in vitro* and *in vivo* studies are required to predict the long term mechanical stability of the functionally graded materials. Also, other parameters should be investigated to improve the biological and mechanical behavior of such novel structures. For instance, the chemical composition, proportion and design of the structure can play a significant role on osseointegration and mechanical behavior of titanium-based implant and prostheses.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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2.2 Segundo Artigo: Submetido

A new gradated zirconia (YTZP) implant material with embedded HA and βTCP: *in vitro* bioactivity and mechanical properties

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Abstract

Hydroxyapatite and calcium phosphate based coatings have been proposed to improve the bioactivity of zirconia. Since these are associated with coating detachment, we have developed a new manufacturing strategy to overcome these limitations, modifying zirconia substrate instead of adding a coating. Briefly it consists in gradation from YTZP in inner part to YTZP/HA and YTZP-βTCP in outer layer, obtained by powder metallurgy. The aim of this study was to characterize the behavior of osteoblasts in contact with these novel materials, as well as to characterize their mechanical properties.

Human osteoblasts were cultured on zirconia-based discs for 15 days and cell morphology, viability, alkaline phosphatase activity (ALP) and mineralization patterns were evaluated. A comprehensive mechanical characterization was performed including FEGSEM-Electronic Dispersive spectroscopy, Grazing Incidence X-Ray Diffraction analysis, Vickers hardness, shear assessment and wettability were analyzed. Cell viability increased over time and was statistically evident on gradated zirconia containing HA or βTCP. ALP levels increased on gradated zirconia surfaces, although increased mineral content over

time was more evident on YTZP/HA. Preliminary mechanical Characterization demonstrated no substantial degradation of the composite specimens as compared to monolithic YTZP. In particular, shear strength between the outer layer and the bulk inner area seem very promising, mainly when compared to traditional bioactive coatings solutions. Gradated zirconia containing hydroxyapatite revealed an increased viability, activity and mineralization of human osteoblasts *in vitro* when compared to conventional zirconia surface, without substantial loss of mechanical properties.

Key words: Cell viability, Alkaline phosphatase, Dental implants, Human fetal osteoblast, Zirconia FGM composite.
Keywords (MeSH): Zirconia, Functionally-graded materials, Hydroxyapatite, β-tricalcium phosphate, hFOB cells

1. Introduction

Although titanium has been the material of first choice for dental implants in the last 30 years, poor aesthetic outcomes, susceptibility to corrosion, and toxicity have stimulated the development of alternative metal-free materials.1 Zirconia has been increasingly used as a metal substitute for manufacturing orthopedic and dental implants and prosthetic structures. High fracture toughness (8-10 Mpa.m1/2), flexural strength (1200 MPa), thermal stability, low thermal conductivity, chemical resistance, and biocompatibility are the main noticeable physicochemical properties recorded for zirconia in literature.[1-4]
These features have been validated in retrospective clinical studies focusing on the perimplant soft and hard tissue compatibility.[1, 5-8.] The use of a zirconia as dental implant reveals clear esthetic outcome in the case of soft tissue recession or thinning gingival biotype.[1, 9.] Also, zirconia has demonstrated significantly decreased adhesion of oral biofilms when compared to metallic materials for abutments.[10-15,16.]
Zirconia-based materials are considered as biologically inert, meaning they are not able to initiate an adverse reaction in host tissues after implantation.[17,18] The capability of osseointegration of zirconia is considered to be comparable to that for titanium implant surfaces.[19, 20]
A few recent studies in literature have reported the modification of zirconia surfaces by coating with bioactive ceramics to enhance migration of cells and deposition of bone matrix on implants.[21-23.]
The fundamental purpose of surface modification techniques is to promote a high osteogenic stimulation by providing chemical affinity of

implant surfaces to the surrounding bone environment[21, 22], thus promoting either improved primary and secondary stability of implants. Ordinary surface coating methods include plasma-spraying, electrophoretic deposition, dip-coating and spin-coating. [23]
Even though hydroxyapatite and calcium phosphate coatings have a high bioactivity, those bioactive layers showed low tensile strength (< 51 MPa) and fracture toughness (0.28 to 1.41 Mpa.m1/2). That results in crack propagation in the bioactive ceramic layer after residual stress from processing and then the detachment of the bioactive ceramic layer due to friction and torque forces during implant placement.[21, 24, 25]. Chipping or delamination of the bioactive ceramic coating layers has discouraged the production of bioactive coatings for dental implants.26 Concerning limitations of bioactive coatings, a novel material design by creating a functionally gradated material has been proposed in the present study. Such strategy involves an outer implant layer and a transition zone composed of a zirconia composite containing 10 %(vol.) HA or β-tricalcium phosphate (βTCP), while the implant core is composed of YTZP. Thus, the main aim of the present study was produce a gradated zirconia embedding bioactive ceramics to overcome surface chipping and to enhance bioactivity of dental implants.

2. Materials and methods

2.1. Materials and processing

Three types of materials were used in this study, namely yttria stabilized zirconia (YTZP), hydroxyapatite (HA), and beta-tricalciumphosphate (βTCP) as described in Table 1.

Table 1 - Powders dimension and their distribution, according to suppliers/manufacturers.

Material/Commercial designation/ Supplier	**Particle size, d50 (μm)**
YTZP/TZ-3YB-E/Tosoh corporation	60 (agglomerate)
HA/ nanoXim.Hap203®/Fluidinova S.A.	10 (particle)
β-TCP/Trans-Tech,Inc	2.26 (particle)

The preparation of samples is illustrated in Figure 1. Concerning deagglomeration, HA or βTCP powders (powder A) were separately immersed in ethanol and dispersed under a high energy ultrasonication process (40KHz, 200W) for 30s. 27 Then a weighted amount of YTZP granules (powder B) was added to the solution while bioactive materials (powder A) were in suspension, for a homogeneous mixing. The volume of alcohol was strictly controlled to prevent the occurrence of decantation. After that, the solutions were heated until ethanol evaporation on a furnace at 60 ºC for 1.5 h. YTZP powders were mechanically mixed with 10% (v/v) HA or βTCP in a stainless steel jar containing steel milling balls at 25 rpm for 21 h.

Powders were then pressed at 200 MPa under uniaxial pressing and sintered at heating and cooling rates at 8 °C/min up to 1500°C (Zirkonofen 700 furnace, Italy) for 2 h.28 Cylindrical samples were obtained with 8 mm in diameter and 3 mm height and divided in three groups: YTZP (Z), YTZP containing 10% HA (ZHA) or 10% βTCP (Z-βTCP). Specimens were wet ground on SiC papers down to 4000 mesh and then polished till a mirror-like finishing using aluminum oxide suspension (1 μm). Surfaces were ultrasonically cleaned in 100% ethanol for 10 min and then in distilled water for 10 min. For biological assays, specimens were sterilized at 121 ºC in autoclave for 20 min

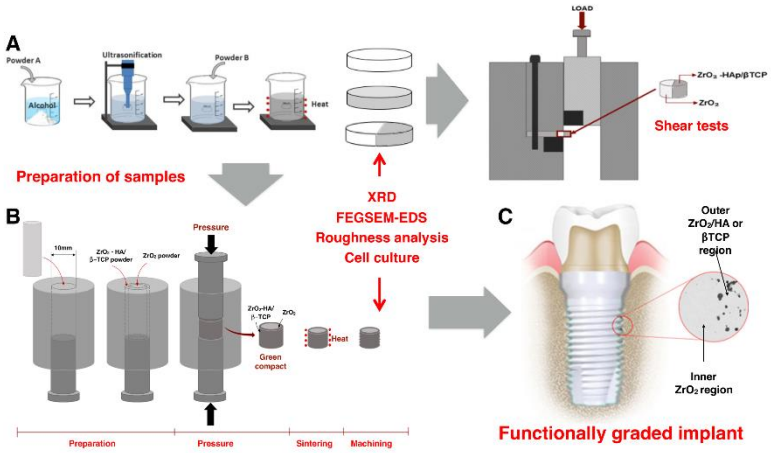

Figure 1. Schematic illustration of the preparation and analyses of the functionally gradated zirconia samples. (A) Mixing and (B) hot-pressing process of the samples for physicochemical and biological assays. (C) Gradated Z-ZHA and Z-ZβTCP for implants.

2.2. Optical and scanning electron microscopy

Porosity analyses of the gradated YTZP-based specimens were performed using an optical microscope (Leica DM 2500 M, Leica Microsystems, Germany) connected to a computer to image processing, using Leica Application Suite software (Leica Microsystems, Germany). Six optical micrographs were obtained at x50 magnification, for each specimen (n = 60) involving the entire specimen area. Black and white images were analyzed by , with the Black areas represented the pores while white areas the bulk material was indicated by the white region following the software Adobe Photoshop (Adobe Systems Software, Ireland). Porosity percentage was measured by using Image J software (National Institutes of Health, USA).

Specimens were sputter-coated with a 15 nm thin film composed of Au-Pd (80-20 wt %); using a high-resolution sputter-coater (208HR Cressington Company), coupled to a MTM- 20 Cressington High Resolution Thickness Controller. Surfaces were inspected by secondary (SE) and backscattered (BSE) electrons mode at 10-20kV by using Field Emission Guns Scanning Electron Microscopy (FEGSEM, FEI NOVA 200, USA). Secondary electron images, i.e topographic images, were performed at different magnifications ranging from x500 up to x10000 at an acceleration voltage of 10 kV. Atomic contrast images were obtained using the Backscattering Electron Detector (BSED) at an acceleration voltage of 15 kV. Grain size of the powders and microstructure of the gradated specimens were assessed by FEGSEM.

2.3. Chemical analyses

Energy-dispersive X-ray spectroscopy analysis (EDAX, Pegasus X4M, USA) was used to evaluate the chemical composition of the raw materials powders and gradated YTZPbased specimens. Grazing Incidence X-Ray Diffraction (GIXRD) technique was used to obtain more contribution from the calcium phosphate phase present in the composites. An incident angle of 3 degrees at a scan range from 20 up to 65 degrees with a step size of 0.02 degrees and 1 s step time. The samples were analyzed in a diffractometer (D8 Discover, Bruker, Germany) using Cu Ka radiation (k51.5406 Å). Finally, the peaks were identified using the X'Pert High Score Plus Software (Panalytical, The Netherlands) within JCPDS patterns database.

2.4. Mechanical assays

Vickers hardness tests were performed using a Vickers micro-hardness tester (DuraScan, Emcotest, Austria) on 4.9 N (500g) loading for 15 s. Calculation of the average hardness values was obtained from five indentations on each of the three different groups (n = 25).

Shear strength tests of the produced gradated composites (Z-ZHA; Z-ZβTCP) and bulk materials (Z; ZHA; and ZβTCP) were performed (n = 8). Specimens were positioned with half of the surface area in a metallic holding device while the other half surface area was in contact with the machine piston (Fig. 1). In case of the gradated YTZP-based specimens, shear tests were performed on the transition zone between zirconia and composite (Fig. 1). Tests were conducted using a servo hydraulic machine (Instron 8874, USA) with a capacity load cell of 25 kN, a crosshead speed at 0.02 mm/s, at room temperature (≈25 ^{o}C). Maximum shear stress was determined by dividing the maximum load by the cross section area.

2.5. Roughness and wettability analyses

All specimens were air braded with alumina particles (250 μm) on a pressure of 2 bars at a distance of 50 mm away from the surface. After surface grit blasting, specimens were ultrasonically cleaned in isopropyl alcohol during 10 min. The roughness of the specimens was measured at a measurement length of 2 mm, cut-off value of 0.8 mm at 0.1 mm/s by using an optical profilometer (Mahr Perthen, Germany). Two roughness parameters were evaluated: average roughness, *Ra*, (average of the height values recorded for peaks and valleys distance from the profile mean line) and the maximum distance between peaks and valleys of the profile, *Rt*. Roughness mean values were obtained from three measurements on different surface areas for each specimen (n = 15).

The surface wettability of the specimens was assessed by water contact angle (WCA) measurements using a digital goniometer (OCA 20, Data Physics, Germany). A volume of 5 μL water was placed on different areas of the sample surfaces for WCA measurements at room temperature and humidity.

2.6. Cell culture

Human Fetal Osteoblasts hFOB 1.19 (ATCC; American Culture Collection, Manassas, VA, USA) were used in this study. Osteoblast human cells were conditionally immortalized and stably transfected with

a gene encoding a temperature-sensitive mutant (tsA58) of SV40 large T antigen. Osteoblasts grown at a permissive temperature of 33.5 oC exhibited rapid cell division, whereas little cell division occurred at a restrictive temperature of 39.5 oC. 29 Cells were cultured in 5% CO2 and 100% humidity at 33.5 oC in culture medium composed of mixture (1:1 v/v) of Ham's F12 Medium (Sigma-Aldrich 51651C, Hampshire, UK.) and Dulbecco's Modified Eagle's Medium-DMEM (Biowhittaker, Lonza, Walksville, USA) supplemented with 0.3 mg/ml G418 (Roche, Indiana, USA) and 10% Fetal bovine serum (Biowest, Nuaillé, France) in 75 cm2 flasks (Corning, Corning NY, USA) until reaching 80 % confluence. Cells were then detached using trypsin-EDTA (Lonza, Veners, Belgium), centrifuged at 800 rpm and re-suspended in culture media at an adequate density for the bioactivity assays. The medium was changed every 48 h and therefore forth passage was used for all tests at 37 oC (restrictive temperature – primary cell behavior).

2.7. Cell Viability and proliferation Assay

Groups of discs composed of YTZP containing or not 10% HA or βTCP (Z, ZHA, ZβTCP) were distributed in 48 well culture plates (Corning, Corning NY, USA) under sterile conditions and incubated in culture media for 1 hour, which was then removed. Cells were seeded on the discs at a density of 1 x 10^4 cells/well and cultured at 37 oC for the assays (restrictive temperature). Cells in culture media free of test discs on the polystyrene well plates were used as positive control for all assays.

Cell viability and proliferation was evaluated using a rezasurin-based viability assay [(Cell- Titer Blue® reagent, (Promega, Madison, WI, USA)], considering the capability of living cells to irreversibly convert a redox dye (resazurin) into a fluorescent end product (resorufin). The conversion rate was measured as fluorescence intensity in arbitrary fluorescence units (AU) for 1, 3, 7, and 14 days incubation. Briefly, a 10% mixture of Cell- Titer Blue® reagent (Promega, Madison, WI) and culture media was prepared according to manufacturer's instructions. Supernatant was removed at each time point, and 300 µl of the Cell-Titer Blue mixture were added to each well. After incubation at 37°C for 4 h, cell viability assay was performed following the manufacturer's instructions and

fluorescence intensity (n = 8) was detected by spectrophotometry at 560/590 nm using a Luminescence *spectrometer* (PerkinElmer LS 50B, UK).

2.8. Cell morphology

Osteoblasts were cultured on other group of samples at a density of 1 x104 cells/well (5% CO2, 37 ºC) for for 24h and 7 days. After that period, culture media was removed and cells were washed twice with PBS. Immediately after the last wash, 1.5% glutaraldehyde solution was used to fixing adhered cells for 10 min. Then, samples were washed with sterile distilled water, and kept in buffer solution of 0.14 M sodium cacodylate until dehydration procedure. A dehydration process was performed by immersing the samples in ascending ethanol concentration ranging from 50 up to 100%. Samples were sputter-coated with a 15 nm thin film composed of Au-Pd (80-20 wt %); and inspected by secondary (SE) electrons mode at 10kV by FEGSEM, as previously described.

2.9. Alkaline phosphatase (ALP) activity

Osteoblast differentiation generally implies expression of Alkaline phosphatase (ALP), which allows a quantitative measurement of osteoblast activity and differentiation. This activity was measured using a fluorimetric enzymatic assay (ab83371 ALP assay Fluorometric, Abcam, Cambrige, UK) following the manufacturer's instructions. ALP

activity was measured for 7 and 14 days of cell culture. A standard curve ranging from 0.0 to 0.4 nmol ALP concentration was performed at each measurement to measure enzymatic activity. Standards and samples were measured by fluorescent intensity at excitation/emission wavelengths of 360/440 nm using a Fluorescence spectrometer (PerkinElmer LS 45, Waltham MA, USA) and mean values were converted to mU/µl of enzyme (ALP) based on the standard regression equation.

2.10. Mineralization assays

Discs were removed after 7 and 14 days incubation, transferred to other plates and washed with PBS to remove non-adherent cells. Cell fixation was performed using 1.5% glutaraldehyde solution for 10 min and then the discs were washed in distilled water. Cell culture mineralization was evaluated by the OsteoImageTM Mineralization Assay (Lonza, USA) according to the manufacturer's instructions. Such assay is based on the fluorescent staining of extracellular mineral content deposited by cells, specifically hydroxyapatite. Mineralization-stained images were obtained at 492/529 nm excitation/emission wavelengths using an Leica TCS SP5 confocal microscope (Leica

Microsystems, USA) coupled to LAS-AF LITE v2.0 software (Leica Microsystems, USA).

2.11. Statistical analysis

The statistical analysis for viability and ALP activity was performed using the normal distribution analysis evaluated by the Shapiro-Wilk test. A factorial analysis of variance (ANOVA) was used to determine significant differences among the evaluated groups. Then, the Post Hoc (Tukey) test was applied to compare the groups´ viability and ALP activity, considering $*p<0.01$ statistically significant. Statistical analyses were performed using the IBM® SPSS® 24.0 statistics software for Mac (SPSS, Chicago, USA).

3. Results

3.1. Morphological and chemical characterization of materials and surfaces

FEGSEM-EDX analyses revealed results on porosity, surface morphology, grain size, and chemical composition of the gradated YTZP-based bulk materials. Ceramic particle morphology and size distribution are shown in Figure 2.

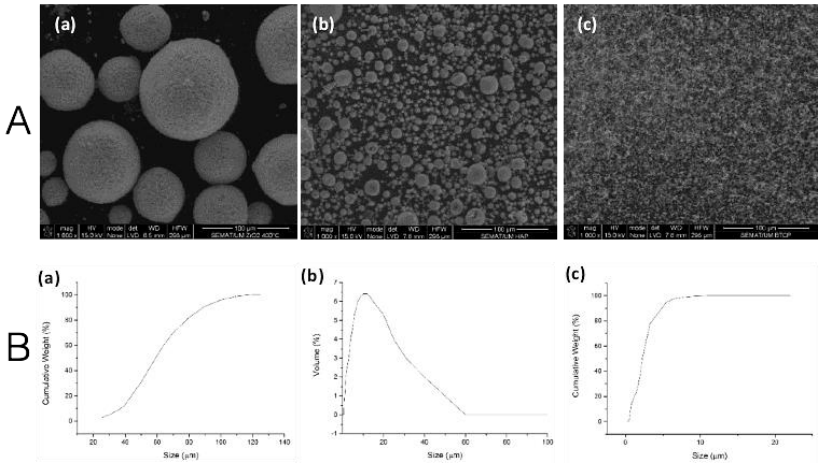

Figure 2. Characterization of the ceramic powders used in sample manufacturing, considering morphology and size distribution. A) SEM images of (a) YTZP, (b) HA and (c) βTCP powders. B) Particle size

distribution of (a) YTZP, (b) HA, (c) βTCP, powders (according to the manufacturer).

Porosity was about 0.3% for bulk and gradated YTZP specimens that indicate a high densification in all samples. Ceramic grains are noticeable in the microstructure of the materials as seen in Figure 3. Grain size mean values recorded for ZHA were higher (0.522 ±0.138 μm) than those for Z (0.344 ±0.156 μm) and ZβTCP (0.292 ±0.098 μm)

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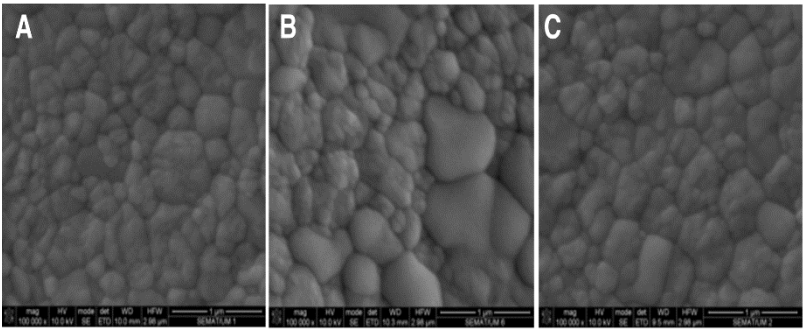

Figure 3. FEGSEM images for grain size measurement for (A) Z; (B)ZHA;(C) ZβTCP surfaces.

The bioactive compounds, HA and βTCP, dispersed in the YTZP matrix are shown in Figure 4. The dispersion of the bioactive ceramic phase was heterogeneous due to the size of the ceramic particles. Agglomeration of βTCP particles was noticed surrounding the YTZP grains.

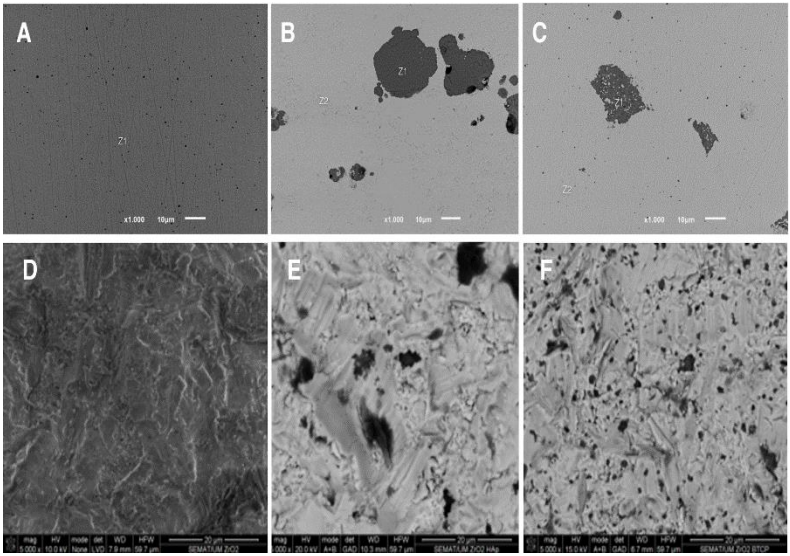

Figure 4. FEGSEM images recorded on polished (A) Z, (B) ZHA and (C) ZβTCP surfaces. FEGSEM images recorded on gri-blasted (D) Z, (E) ZHA and (F) ZβTCP surfaces.

The stoichiometric Ca/P ratio values was maintained as initially established at 1.67 for HA and 1.5 for βTCP. The bioactive phases remained in the composite matrix after grit blasting procedure as shown in Figures 4E,F. EDX analyses confirmed the presence of chemical elements composing the bioactive ceramic phases (Figures 4 B,C,E,F). The overall chemical composition recorded on the marked sites is shown in Table 2.

Table 2. Chemical composition (in at. %) of the test materials as detected by EDX.

	Z	**ZHA**		**ZβTCP**	
Elements	**Z1**	**Z1**	**Z2**	**Z1**	**Z2**
O	52.5	50.2	54.0	50.4	55.6
Zr	47.5	5.8	44.8	10.0	42.8

Ca	-	27.9	1.2	24.9	1.2
P	-	16.2	-	14.7	0.4
Ca/P	-	1.71	-	1.69	-

X-ray diffractograms recorded for ZHA, ZβTCP composite layers and Y-TZP substrate are shown in Figure 5.

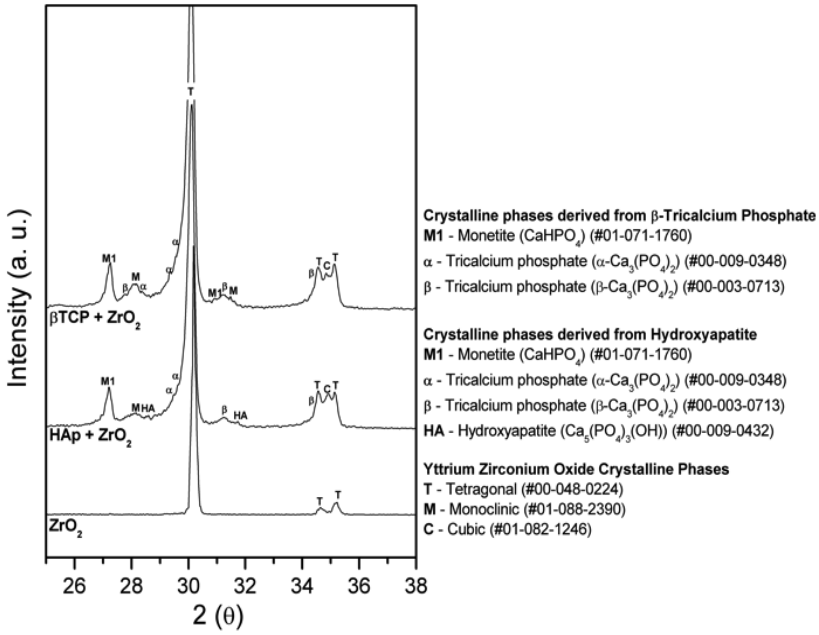

Figure 5. X-ray diffractogram spectra for Z, ZHA, and ZβTCP specimens.

X-ray diffractogram recorded on Y-TZP revealed peaks assigned to tetragonal zirconia phase following the JCPDS #00-048-0224 pattern while spectra obtained for ZHA and Zβ-TCP the composites revealed monoclinic (JCPDS #01-088-2390) and cubic (#01-082-1246) zirconia phases. XRD spectra recorded on β-TCP showed peaks assigned to β-

tricalcium phosphate (β-TCP) (#00-003-0713), α-tricalcium phosphate (#00-009-0348), and Monetite which is a dibasic calcium phosphate anhydrate ($CaHPO_4$) (DCPA) (#01-071-1760). Hydroxyapatite phase was detected on the ZHA composite (#00-009-0432).

The water contact angle (WCA) mean values were recorded at 69.82 ±1.56 degrees for Z group while ZHA revealed WCA mean values at 69.65 ± 1.94 degrees and ZβTCP showed WCA at 65.04 ± 2.21 degrees. There were not statistically differences between the group considering roughness and WCA.

3.2. Mechanical assessment

Vickers hardness results are shown in Figure 6A. A slight decrease in hardness occurred when βTCP was incorporated in the YTZP-based composite that was not noticed for ZHA group (Fig. 6A).

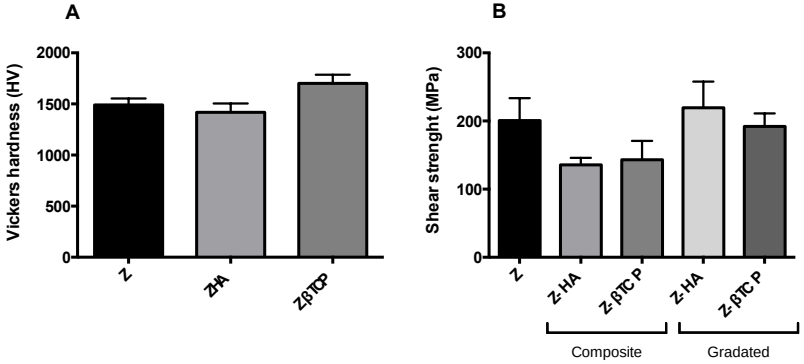

Figure 6. A) Vickers hardness mean values recorded for Z, ZHA, and ZβTCP groups. B) Shear strength values for homogeneous and for gradated composites (Z-ZHA; Z-ZβTCP).

Shear strength mean values for Z, ZHA and ZβTCP are shown in Figure 6A. There was no significant difference in shear strength among the test groups. In fact, results indicated that the mechanical strength was maintained for the novel composites after incorporation of bioactive ceramics.

3.3.Cell Morphology

FEGSEM micrographs obtained on test samples after 24 h cell culture are shown in figure 7 with magnifications showed the morphology of cultured cells on Z (A), ZHA (B), ZβTCP (C) discs. Similar morphology and spreading behavior of osteoblasts was noticed on all zirconia-based materials for 24 h culture. Cells showed a typical elongated veil-shape and filopodias formation.

3.4. Viability and Cell proliferation

Cell viability results of osteoblast cell culture for 1,3,7, and 14 days are shown in Figure 8. These results were normally distributed and statistically different for all times except on ZβTCP for 1 and 7 days ($p = .014$) and on Z for 14 days ($p = .003$). Early cell adhesion was noticed for all surfaces, with measurable viability values after 24 h cell culture. However, statistically significant lower values were recorded for ZβTCP group comparing to the other groups, both for 24h time-point ($p = .011$ and 0.021 on comparisons with Z or ZHA groups, respectively), and for 3 days culture ($p = .013$ and .009 on comparisons with Z or ZHA groups, respectively). Cells proliferated favorably on zirconia-based materials, showing considerable increments with culture time for all groups including the positive control (polystyrene well surface).

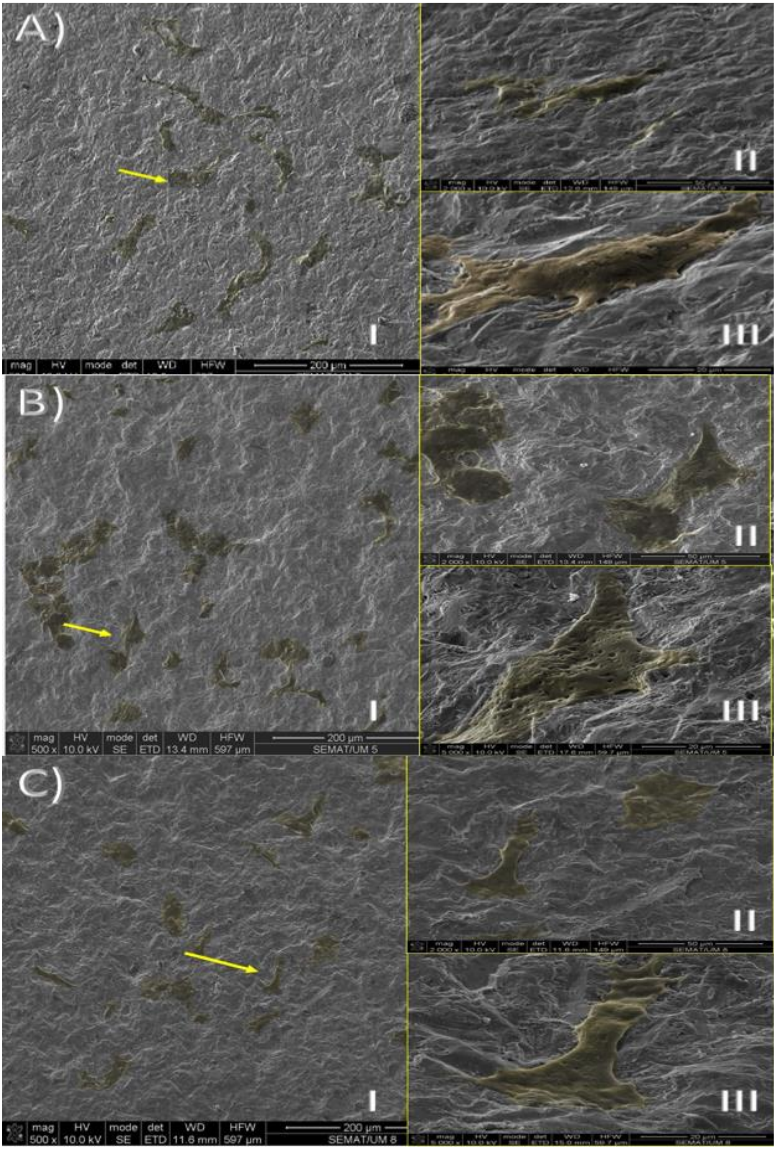

Figure 7. FEGSEM micrographs of the (A) Z group; (B) ZHA group; (C) ZβTCP group surfaces coated with osteoblast for (I) 1 day (x500 magnification); (II) 1 day (x2000 magnification); (III) 1 day (x5000 magnification) (IV) 7 day.

ZHA presented statistically significant higher cell proliferation when compared with the other experimental groups for 14 days culture (p =.000 on comparisons with both pure Z or ZBTCP). Control group values are shown as an positive control reference for each assay, corresponding to cells cultured on polystyrene surfaces.

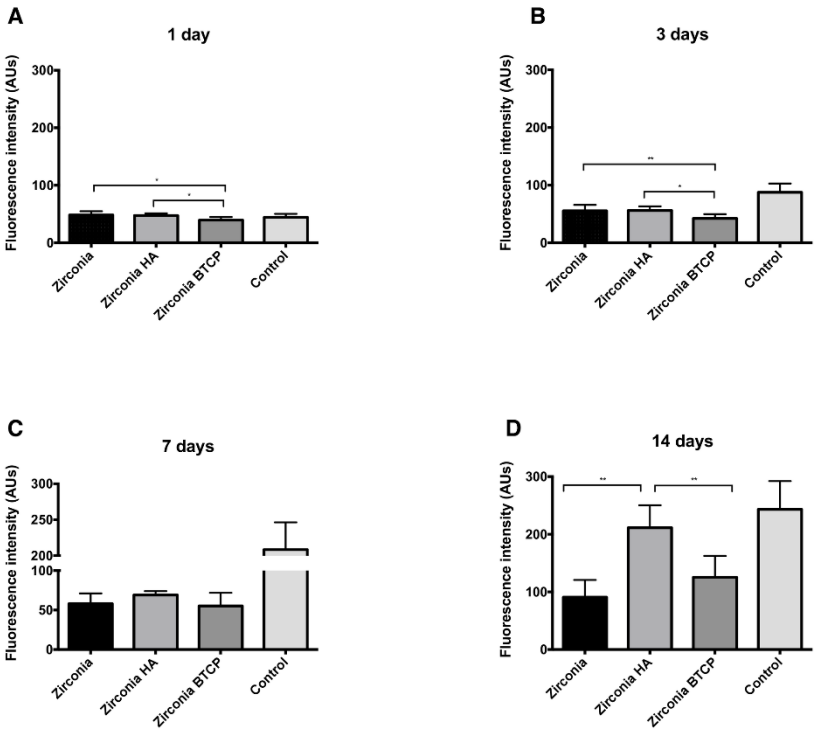

Figure 8. Cell viability and proliferation mean values measured using fluorescence intensity expressed in arbitrary units of resorufin formed by living cells as a surrogate marker. ANOVA with post-hoc Tukey test were used for comparisons between study groups. * - significant (P<0.05); ** highly significant (P<0.01).

Similarly, proliferation ratios were analyzed (Figure 9A). Proliferation ratio values were produced in comparison to values for 24 h cell culture. For 14 days incubation, proliferation ratios measured on ZHA and ZβTCP groups were significantly higher when compared to

those for Z group (p = .00 and .033, respectively). Proliferation ratio for ZHA and ZβTCP groups did not differ significantly at any time-point.

3.5. Alkaline phosphatase activity

ALP activity of cell suspension is shown in Figure 9B, considering measurements for 7 and 14 days cell culture. Z group showed the lowest ALP activity when compared with other groups for both time-points. Within the experimental groups, ZHA group revealed the highest ALP activity both for 7 and 14 days culture. There were significant differences for 7 days culture comparing to values recorded for Z group (p =.03). At both time-points, this increase in ALP activity was of almost 2-fold comparing to monolithic zirconia (Z group).

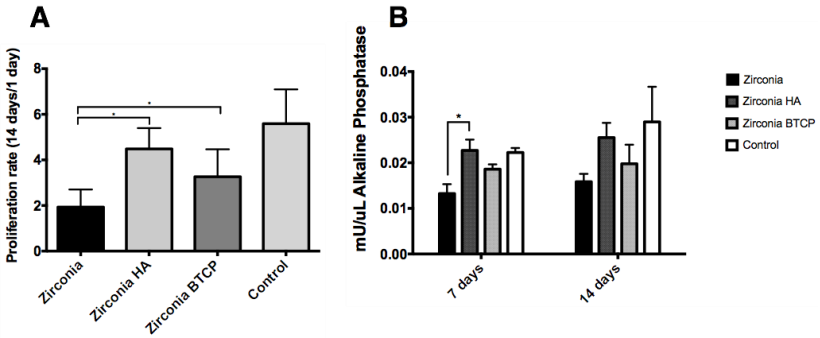

Figure 9. (A) Proliferation rations calculated as the reason of resorufin formation by living cells at 14 days / 1day. (B) Mean values of alkaline phosphatase concentration of cells cultured on different materials for 7 and 14 days, respectively. * - significant (P<0.05).]

3.6. Cell mineralization

Analysis of the mineral hydroxyapatite content by confocal laser fluorescence microscopy (CLFM) showed increased mineralization on all the surfaces. The highest values were recorded on ZHA surfaces for both time-points (Fig. 10). An increase in number and density of mineralization nodules was noted on all study groups for 7 and 14 days culture although it was visibly more intense on ZHA samples (Fig. 10B).

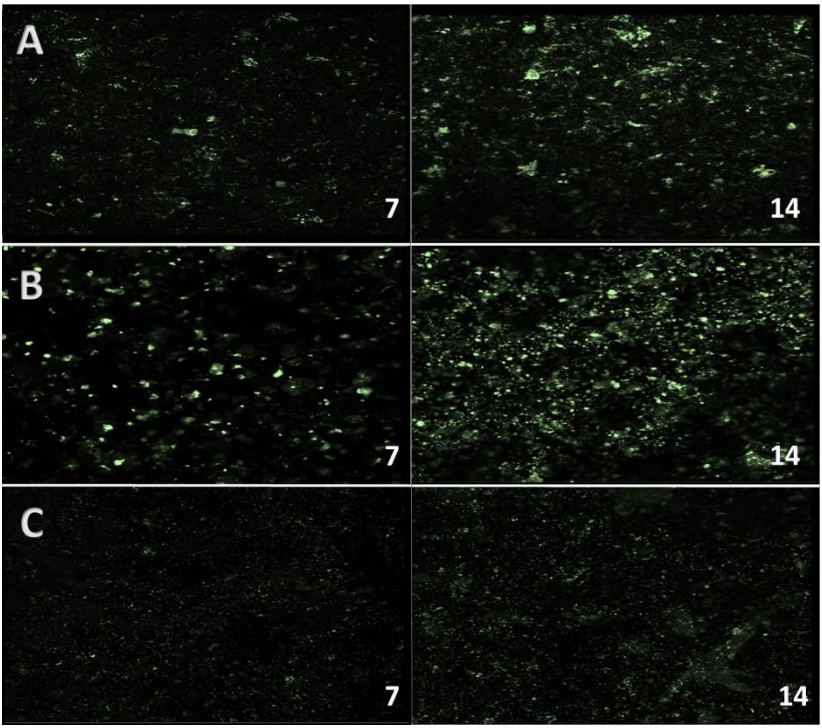

Figure 10. Fluorescence photomicrographs of OsteoImage^{TM}-stained, bone mineral nodules for 7 and 14 cell culture days. (A) Z, (B) and (C) ZβTCP group. Images btained at x200 magnification were representative of 3 replicates.

Discussion

In this study, HA and βTCP were added to YTZP resulting in a novel functionally gradated composite to improve bioactivity of dental implants without significantly compromising strength and damage of the surface during placement into the bone tissue. The functionally gradated implant was investigated focusing on surface bioactivity, mechanical properties, cell viability and proliferation, cell morphology, and bone cell mineralization.

In the present study, the bioactive materials are added to the bulk material only in the outer regions while the core is composed only of zirconia. Then, the green gradated block in pressed and sintered, or hot pressed. A high densification (porosity around 0.3%) of the test materials was noticed in this study. The incorporation of 10%vol

bioactive particles did not negatively affect the composite densification. Different gradations in chemical composition on Functionally Gradated Materials (FGM) are already reported in the literature although the previous studies have focused mainly on thermal and mechanical properties.[42-45] An automatic process for adding powders in different radial directions, for gradated solutions, has been previously proposed to produce gradated composites. [47] Other studies revealed similar or lower densification values for zirconia-HA composites. It may depend on the parameters used namely powders types, mixing processes, thermal treatment, dwell time, and green compact pressure. [30, 48] Considering the microstructure, there was no sharp interface between Z and ZHA or ZβTCP regions since a small fraction of bioactive phase is gradually distributed at the outer region of the implant. The dispersion varied for each bioactive ceramic phase that was related to the particle size. HA particles were well dispersed in the matrix while βTCP agglomerates were detected due to smaller powder particle size (Table 1). A good bonding between bioactive particles and zirconia matrix occurred, as seen in Figure 4.

Considering the crystalline phases detected by XRD spectra (Fig. 5), some reactions between bioactive materials and YTZP are assumed. Inuzuka et al. [30] suggested the following solid-state reactions between HA and the YTZP powders: YTZP particles first react with the HA particles during sintering at 1500 ^{o}C, resulting in conversion of the HA phase into β-TCP. The cubic zirconia phase is then transformed from the tetragonal zirconia phase by uptake of the resultant CaO converted from HA. [30] The monoclinic phase in the composites was considered as a phase containing insufficient solid-state amount of CaO to form the cubic phase. Therefore, the Y_2O_3-stabilized zirconia phase (tetragonal) and the CaO–Y_2O_3-stabilized zirconia phase (cubic or monoclinic) exist in the ZHA composite surface. However, some HA particles did not react with YTZP in our study. Such phenomena was also reported in a previous study on sintering 70/30 ZrO2/HA at 1500 ^{o}C for 5 h.[31] The reactions of βTCP and YTZP also occurred during sintering ZβTCP specimens. Therefore, the crystalline phases identified by XRD analyses revealed that the bioactive materials maintained their Ca/P ratio around 1.7 after production of the functionally graded composite structures. Previous studies have shown a high chemical interaction between the bioactive ceramic phase sites and bone after friction during implant placement,[46] thus improving the primary stability of the implant. Also, the FGM approach can overcome the detachment of the bioactive

materials from the bulk zirconia. Within the first weeks, the replacement of the bioactive materials by human bone might provide a mechanical interlocking leading to an increase in the secondary mechanical stability.

All specimens reached similar R_a and R_z roughness values as expected by using a standard surface modification technique for implant surface. The mean values of roughness and water angle contact obtained in this study are adequate for the osseointegration process.[50,51]

HA and β-TCP coatings have been widely investigated to improve the biological response of metallic and ceramic implants.[32, 33] Several methods have been described in literature for the deposition of Ca-P based coatings on titanium implants including ion beam deposition, plasma spraying, sol-gel methods, laser deposition, radiofrequency sputtering, biomimetic deposition and electrostatic deposition.[23, 33, 38, 57, 65-70] Although most studies show promising results of HA-bioactive surfaces, the surface modification techniques involve coating deposition procedures, whose stability upon insertion may be questionable. Mechanical issues related to coating instability, delamination and entire fracture during implant placement, reveal a significant challenge for surface techniques.

Regarding mechanical properties, no significant differences were found in hardness values for the test groups in the present study. Hardness values are in accordance with other results in literature for YTZP [52] and also in direct correlation with grain size, as seen in Figure 3, whereas hardness increases for smaller grain size. One of the key properties achieved for the novel functionally graded materials was the high strength assessed by shear loading when compared to ordinary HA to zirconia interfaces. The shear strength values recorded on the transition zone of ZHA or ZβTCP were similar to those for zirconia. The incorporation of bioactive ceramic did not induce any detrimental effects on the strength of the composites. The outer layer of composite could eventually have higher proportions of bioactive phase without compromising the overall mechanical properties. It can be explained by thermal compatibility and elasticity of the composites and bulk, as necessary for a proper gradated design.[28] Despite the encouraging preliminary results, additional and more comprehensive mechanical characterization is needed to fully validate this FGM strategy, such as bending strength, fracture toughness, and fatigue strength. Aging behavior of the outer layer of composites should also be performed considering different factors (e.g. loading, acidic substances, and temperature) that can affect the performance of implant and prosthetic structures in biological environment. However, the preliminary

evaluation of this design allows predicting a good mechanical performance of the gradated design. Ongoing studies will establish an optimal equilibrium between mechanical and biological performance.

For in vitro biological assays, hFOB 1.19 osteoblasts were used due to their minimal chromosomal abnormalities and matrix synthetic properties similar to differentiated osteoblasts.[55, 56] In this study, osteoblast behavior was enhanced in contact with YTZP containing bioactive ceramic compounds when compared to homogeneous YTZP structures. Cell adhesion was detected on all zirconia-based surfaces by FEGSEM for 24 h culture, although cell viability was increased in the case of functionally-gradated zirconia containing HA (ZHA). The magnitude of this increase was of about two-fold higher after 14 days when compared to YTZP. The presence of HA based particles in the surface of zirconia is a key factor for their bioactivity, considering cell attachment and proliferation. Such important role of zirconia surface in cell functions was also reported in the study performed by Wang et al., who evaluated bone-like apatite precipitated on nanostructured zirconia coatings formed by plasma-spray techniques on Ti6Al4V.[57] As previously mentioned, HA decomposes above 1300 ºC in the presence of YTZP[58] converting itself into different reaction products. Monetite is known to have faster dissolution rate than that for □TCP, followed by βTCP and lastly by HA [49]. The assembly that exists in ZHA samples (Monetite, □TCP, βTCP, and HA) seems to be more effective than that one in ZβTCP (Monetite, □TCP, and □TCP). While our data did not validate a higher effect of HA for initial cell attachment, cell viability and proliferation were undoubtedly enhanced by our processing method. It suggests that 1) its bioactivity is maintained through this period and 2) HA (and their reaction derivates) is more efficient comparing to βTCP (and their reaction derivates) and pure zirconia in promoting osteoblast cell differentiation and proliferation.

ALP activity is an important parameter that allows for the assessment of the differentiation level of the mineralization in presence of osteoblasts, and therefore it is considered as a marker of the early stage of osteogenic differentiation.[59, 60] Another important later outcome for osteoblast differentiation is their capability to form and mineralize the bone matrix, noticeable by fluorescent staining of mineralized nodules. [55, 61] A hydroxyapatite-specific staining enabling fluorescent imaging of hydroxyapatite mineralization nodules was associated with micrographs for 7 days and 14 days of cell culture. [61, 62] In this study, the highest ALP activity was recorded for ZHA, with statistically

significant increased values (almost 2-fold magnitude) comparing to pure monolithic YTZP and ZβTCP groups. This is concordant with the increased density in hydroxyapatite nodule formation detected on ZHA surfaces for 7 and 14 days of cell culture. It might be speculated that baseline fluorescence noticed on bioactivated samples due to the presence of hydroxyapatite could present a significant bias in these results. However, the hydroxyapatite concentration used in those surfaces was relatively small (10 % vol) to explain the density in mineralization nodules. On the other hand, a noticeable increase in the number and size of mineralization nodules was apparent from 7 to 14 days in cell culture. That corroborates cell proliferation and ALP activity results in this study. Our data showed that both ZβTCP and ZHA zirconia surfaces promoted cell differentiation, and thus the formation of a mineralized bone-like matrix in addition to higher proliferation rates of osteoblast cells. This effect was more pronounced for ZHA samples.

It is well described that Ca-P based surfaces bind to attachment proteins such as fibronectin and vitronectin for the integrin-mediated binding action of osteogenic cells. [63] Therefore, early cell attachment is highly determined by either material chemical composition and/or topographic aspects, which in turn determine selective affinity to protein binding. The nature of binding proteins determines how the contact osteogenesis occurs, by activation of osteoblastic pathways following integrin–mediated signaling.[64] In this study, early cell attachment and viability was comparable for Z, ZHA, and ZβTCP groups. However, ZHA demonstrated higher potential to promote contact osteogenesis in comparison with ZβTCP or Z groups. A recent report demonstrated better cellular results in materials coated with HA and βTCP combined compounds, considering proliferation and ALP activity.[23] However, they were produced by aerosol deposition, while similar results might be obtained with this novel production technique while simultaneously maintaining mechanical properties. The combination of βTCP and HA in this type of substrate should be investigated in future studies.

The present study shows some limitations of an in vitro study that must be considered while analyzing these results. In vitro approaches represent optimal systems for studying cell behavior in contact with materials, without complications and interferences of more complex in vivo systems. However, the information they provide is limited and needs to be further validated in animal studies. It must be also considered that different cell lines and culture conditions, as well as viability and differentiation indicators has been used, which may result

in bias. Human osteoblast cell responses were evaluated in this work. While a successful osteoblast response is critical to osseointegration, current clinical implantology practice shows that soft tissue response is a key factor in achieving a correct sealing of the peri-implant tissues and optimal esthetic results. Additional studies are ongoing to describe the behavior of peri-implant soft tissue cell models, such as human gingival fibroblasts, in contact with these new materials.

CONCLUSIONS

The present study revealed promising physicochemical and biological properties of novel design and manufacturing approach proposed for producing zirconia-based implants containing gradual proportion of HA beta-tricalcium phosphate.

A high densification of the functionally gradated zirconia-based structures were recorded in the present study with a formation of typical polycrystalline grain size and gradual distribution of bioactive ceramic phases at the outer layer of the test structures. Shear strength between bulk zirconia and the gradual transition zone composed of zirconia and bioactive ceramics was similar to the one recorded for monolithic zirconia structures. That revealed a high strength for the novel functionally gradated zirconia structures. Considering such mechanical outcomes, complementary mechanical assays should be performed in future studies such as bending strength, fracture toughness, fatigue results.

Cell culture assays demonstrated the presence of hydroxyapatite or beta-tricalcium phosphate at the outer layer of the zirconia-based structures significantly stimulated the migration and adhesion of osteoblasts on the novel gradated surfaces. The proliferation rate of cells and mineralization was higher for gradated zirconia embedding hydroxyapatite when compared to zirconia/ beta-tricalcium phosphate or zirconia groups.

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2.3. Terceiro Artigo:

Behavior of human osteoblasts in new PEEK-based Biocomposites

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The polyether ether ketone (PEEK) being the most used polymer in the orthopedic and dental implant applications. Has been extensively studied trying to modify its structure by adding other materials to improve its properties. One of the processes most used to achieve this goal is coatings, however this process presents problems like delamination, chipping, fractures or cracks of the added coating, to counteract this situation, and biocomposites are being increasingly searched. . This material was produced for graduated composition in order to avoid features characteristics known for the coating process.
The objective of this work was to develop new bioactive composites based on PEEK produced by hot pressure and to evaluate their cellular behavior in adhesion, proliferation and activity.
Materials and methods: PEEK and biocomposite discs containing PEEK with 5% hydroxyapatite (PEEK HA) and PEEK with 5% beta-tricalcium phosphate (PEEK βTCP) were produced, the culture of human cells hFOB 1.19 was performed on the discs for evaluation . The morphology and adhesion by scanning electron microscopy, quantitative analysis of cell viability and alkaline phosphatase activity of the cells seeded on the discs was compared to the positive control group of cells directly seeded

in the wells without discs. Results: Results showed cells adhered at day 1 in all groups and interconnected cells on day 7, cells were scattered strongly on materials, cell viability increased over time. From the experimental groups, PEEK βTCP showed higher cell proliferation, but lower values when compared to the positive control group and the PEEK group, with a statistically significant difference between some groups (Anova-Pos hoc-Tukey); The levels of ALP activity were higher in the bioactivated groups when compared to the PEEK group, $p<0.05$. Conclusion: It can conclude that osteoblasts adhered, proliferated and exhibited activity in the materials studied, the cellular response was better in the PEEK groups for viability and PEEK βTCP for activity.

1. Introduction

Biomaterials used in dental and orthopedic implantology are currently studied and developed to induce and obtain stable, predictable, and rapid integration with the tissues to be implanted. In order to evaluate this behavior initially, in vitro studies of biocompatibility are carried out in osteoblasts, for the research to be the most similar to human bone and it is preferable to use healthy human osteoblasts, since osteoblasts of animals or oncological tissues could have different behaviors [1,2].

Nowadays, the studies to analyze new metal free biomaterials have been increased by problems related to tribocorrosion and ion liberation [3–5], the polymers are being widely evaluated and produced in the industry for its easy production and low cost. Among the most outstanding polymers is the polyether ether ketone (PEEK) most promising materials today due to its properties PEEK is a semi-crystalline thermoplastic material considering polymer with suitable mechanical properties, fatigue resistance, high temperature durability, high mechanical, thermal (over 300 °C) and chemical resistance in corrosive environments, lower cost of production among others [6,7]. PEEK is a material used in different areas such as aerospace and marine industry and most recently used in the biomedical industry for biomedical applications and applied in the areas of orthopedic, cranial, vertebral and maxillofacial surgery and rehabilitation, among others [8,9].

The biocompatibility tests performed allowed the FDA to consider it as a suitable material to be implanted in the bone [9–11]. But

even with these attractive properties present in this biomaterial the biomechanical properties of PEEK are still not better when compared to zirconia or some metals and their biocompatibility does not surpass that of some ceramics. Which is why studies aim to improve these difficulties with metallic and ceramic coatings that show better mechanical and biological results when compared to uncoated. Many surface modifications have been developed to improve cell reactions, [12–15]. Hydroxyapatite (HA) and beta tricalcium phosphate (βTCP) are the most extensively studied calcium phosphates and are widely used in various applications, such as in biomaterials, ion exchangers, adsorbents, and catalysts [16–18]. HA is considered suitable for bone repair, particularly in orthopedic implants to accelerate growth of surrounding bone because of structural and compositional similarities of HA crystal and calcium phosphates with bone. However, these bioactive materials cannot be used clinically such as solid implant materials because of its worse mechanical properties compared to titanium [19–21]. Bioactive materials are coated on PEEK based implants to enhance bone bonding with implant surfaces, as well as accelerating bone growth and hindering resorption of the surrounding bone. Various methods have been employed to coat HA and TCP on PEEK-based implant surfaces [3,14,11], but there is still some mechanical problems when these materials are implanted lose bond strength when installed in the bone, losing surface coating layers because of friction and torque at the time of implant installation [20]. To improve the time and characteristics of osseointegration, modifications were made in the composition and methodology of processing; these bioactive materials were incorporated into the composition of the PEEK based material and were not limited to the surface, known as biocomposites materials [3,4,10,11]. In order to obtain these materials hot pressing techniques showed promising results compared with conventional techniques such as compacting by Pressure [6]. For this reason, this study to added 5% of hydroxyapatite and beta tricalcium phosphate in the manufacture of PEEK by hot pressing, obtaining a functional material with gradient properties (FGM) in order to analyze the cellular response of fetal human osteoblasts immortalized in PEEK and bioactivated PEEK.

2. Materials and Methods

2.1. Preparation of samples.

A poly-ether-ether-ketone (PEEKOPTIMA450,Victrex,England) and HA and βTCP powders were mechanically mixed in proportion at 5% bioactive material and 95% PEEK, inside a closed stainless steel jar

along with steel milling balls. The jar rotated with constant speed (25 rpm) during 21 hours. The obtained powder mixture was dehydrated at 110°C for 1 hour, being afterwards divided and placed inside graphite dies. The composites were then produced by hot-pressing, in primary vacuum, using a high frequency induction furnace. The die (with the powder mixture) adequate volume of peek particles was heated up to 380 °C with a heating rate of 80 °C/min the pressure on the sample was raised to 20 MPa, being maintained during 30 min, then, the temperature was decreased down to 300 °C where a pressure of 25MP a was applied and maintained for 4s. After, the temperature of the system cooled down to room temperature undervacuum, this protocol has already been established [6].

Obtaining three types of material groups: PEEK pure group (PEEK), PEEK bioactivated with 5% Hydroxyapatite (PEEK HA), and PEEK with 5% beta tricalcium phosphate (PEEK βTCP). For each group 16 discs of 3mm in height and 8mm of diameter were elaborated, The substrate surface was air braded with alumina particles (250 μm) with an impact angle of 901 under a pressure of 5 bars at a distance of 80mm from the surface to obtain a rough surface. The discs were blasted in an attempt to obtain similar roughness in order to guarantee similarity of surface roughness between all samples, the specimens were ultrasonically cleaned for 15 minutes in ethanol, and distilled water for 10 minutes and were sterilized in autoclave for 20 minutes at 121°C.

2.2. Cell seeding

Human Fetal Osteoblasts hFOB 1.19 (ATCC ® CRL11372; American Culture Collection, Manassas, VA, USA) were used. This is a conditionally immortalized human cell line stably transfected with a gene encoding a temperature-sensitive mutant (tsA58) of SV40 large T antigen. The cells grown at a permissive temperature of 33.5ºC exhibit rapid cell division, whereas little cell division occurs at a restrictive temperature of 39.5ºC [22]. Cells were cultured at 37°C in an atmosphere of 5% CO2 and 100% humidity in an culture medium composed of 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium DMEM, completed growth medium, add the 0.3 mg/ml G418; Fetal bovine serum to a final concentration of 10%.
After reaching 80% confluence, cells were washed three times with Phosphate Buffer Saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.4 mM KH2HPO4) and harvested using 0.25% trypsin-0.53 mM EDTA (Gibco, Gaithersburg, Maryland) at 37∘ C for 5

min. After cells to detach, add 8.0 mL of complete growth medium and seeded 75 cm2 culture flask. The medium was changed every 2 days. All tests were performed on the fourth subculture at a 1:4 ratio.

2.3. Cell morphology

The cells were seeded at a density of 1 x 10^4 cells/well in 0.5 ml medium using a micropipette. Discs were removed after 1 and 7 days incubation and washed with PBS to remove no adherent cells and fixed using 1,5% glutaraldehyde solution for 10 minutes, after fixation was washed with sterile distilled water, and the samples were kept in buffer solution 0.14 M sodium cacodylate until dehydration.

The samples were washed with PBS and dehydrated in ascending concentration of ethanol (50%, to 100% progressively). The samples were covered with a very thin film (15 nm) of Au-Pd (80-20 weight %); using a high resolution sputter coater, 208HR Cressington Company, coupled to a MTM-20 Cressington High Resolution Thickness Controller.

Morphological analyses were realized in an Ultra-high resolution Field Emission Gun Scanning Electron Microscopy (FEG-SEM), NOVA 200 Nano SEM, company. Secondary electron images, i.e topographic images, were performed at different magnifications (500X, 2000X and 5000X) at an acceleration voltage of 10kV. Atomic contrast images were realized with a Backscattering Electron Detector (BSED) at an acceleration voltage of 15 kV.

2.4. Cell Viability Assay

Four groups were considered (PEEK, PEEK HA, PEEK βTCP and positive control only with cells in culture in the wells of the plates. The discs were inserted on the bottom of cell culture plates 48 well. The bottoms of the plates have a treated surface that increases the wettability of the surface to allow a better cellular adhesion: more uniform and consistent. The cells were seeded at a density of 1 x 10^4 cells/well in 0.5 ml medium using a micropipette.

Cell proliferation ability was evaluated by measuring absorbance after 1, 3, 7, and 14 days culture. The supernatant for eight discs was then removed per group, and 300 µl of Cell-Titer Blue buffer (Promega, Madison, WI) was added to each well. After incubation at 37°C for 4 hours, the cell viability assay was performed following the manufacturer's instructions and the fluorescence activity was detected at excitation/emission wavelengths of 560/590 nm in a Luminescence *spectrometer* (*PerkinElmer* LS 50B)

2.5. The alkaline phosphatase (ALP) activity

The alkaline phosphatase assay is one of the most widely used biochemical markers for this type of cell since it is abundantly expressed in osteoblasts and allows a quantitative measure of osteoblast activity. This activity was measured following the manufacturer's instructions (ab83371 ALP assay Fluorometric, Cambrige, UK). It was evaluated in the same groups that were analyzed for proliferation; 9 discs at each time of analysis (3 discs per group) at 3, 7 and 14 days.

At each experimental time point, The disks were washed twice with PBS and cell culture medium was removed, the cell layers were detached and samples were washed For the analysis was also considered a standard curve in the range from 0.0 to 0.4 nmol 4-MUP. ALP concentration was measured by fluorescent intensity at 360/ 440 nm (Ex/ Em) using *Fluorescence spectrometer* (*PerkinElmer* LS 45).

2.6. Statistical analysis

The statistical analysis of viability and ALP activity was performed using normality test for normal distribution dates with Shapiro-Wilk test, a factorial analysis of variance (ANOVA) was used to assess the significant interactions between groups and type or analysis between groups a one-way analysis of variance (ANOVA), and test Pos Hoc (Tukey) was used for a comparison among each specimens for the viability and ALP activity. $*p<0.05$ values were considered to be statistically significant. All analyses were performed using SPSS statistics 17.0 (SPSS, USA)

3. Results

3.1. Cell Morphology

Figure 1 showed the morphology of human fetal osteoblast cultured on PEEK, PEEK HA and PEEK βTCP on the discs. SEM micrographs show material surface was rough and contained irregularities but with similar roughness among groups (Figure 1 left panels). Adherent cells were observed in all groups at 24 hours. At 1 day , adherent cells of different morphologies are observed, showing more scattered cells on PEEK group. At 7 days it can be observed that the cells were very scattered in all groups. with veil-shaped cells morphology, more flat and scattered cells over the material, more evident for PEEK βTCP possibly indicating that the bioactives could induce cell adhesion more favorably.

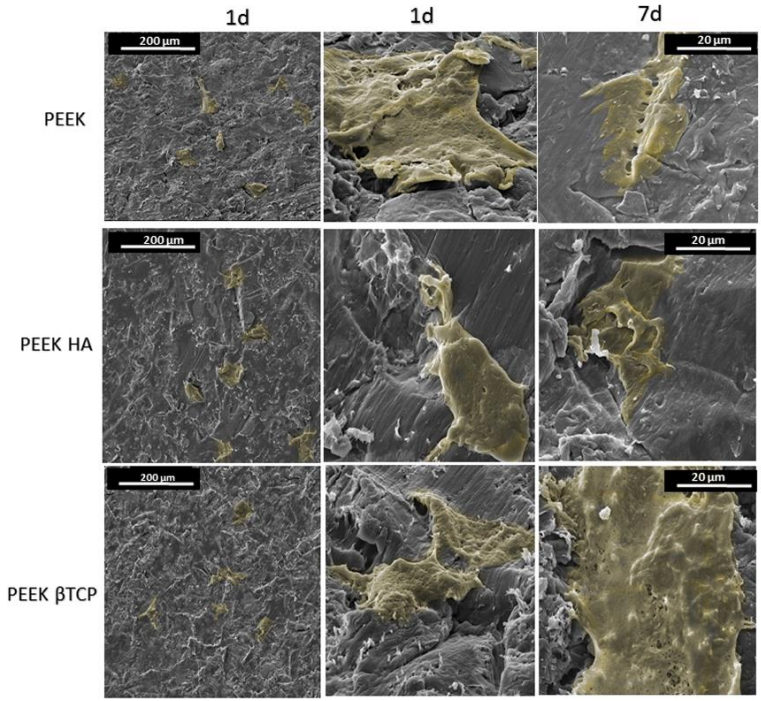

Figure 1: FEG-SEM micrographs of osteoblasts cultured on PEEK; PEEK HA and PEEK βTCP; (left panels) for 1 day 500x; (center panels) for 1 day 5000x and (right panels) for 7 days 5,000x .

3.2. Cell Viability

The cell viability results at 1, 3, 7 and 14 days of cultivation are shown in Figure 2 results presented normally distribution with significant statistic difference in all times, determined by the ANOVA test, and specified by the post hoc Tukey test (*). The cells on the discs proliferated favorably, showing considerable increments with culture time, being more evident in group PEEK except in the group PEEK HA for 3 and 7 day. On the first day, all group presented similar results but PEEK group presented higher proliferation with statistical difference compared to the others groups.

Of the biocomposite groups, a greater proliferation was evidenced in the PEEK βTCP. Likewise, this group presented lower standard deviation when compared to the other groups at all times. At all

times the control group presented the higher cell viability precisely for the reason of being a positive control (Figure 2).

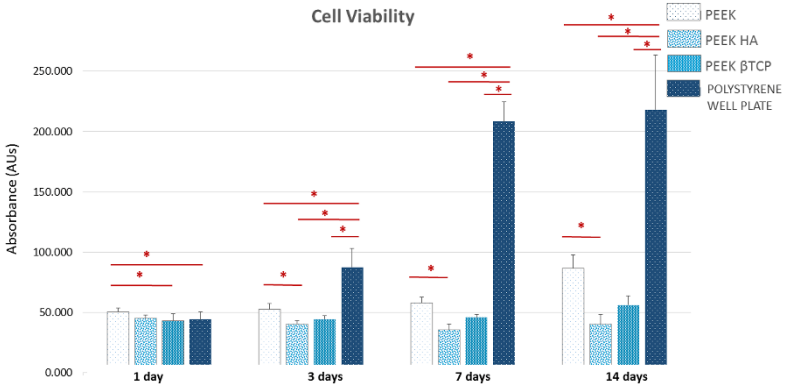

Figure 2: Viability and proliferation cell in 1, 3, 7, 14 days *p<0.05

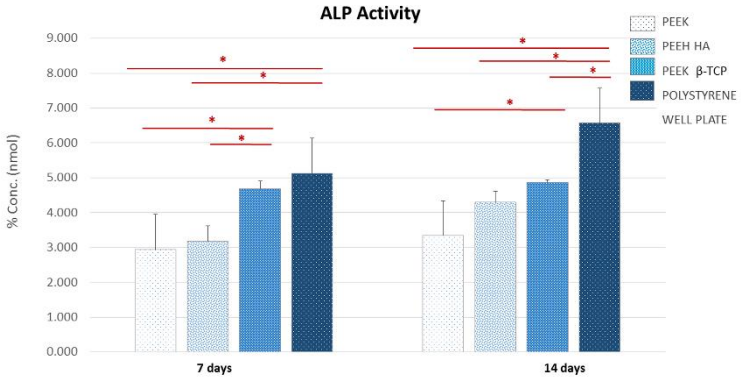

Figure 3: ALP activities of the cells cultured for 7 and 14 days *p<0.05

3.1. Alkaline phosphatase activity

ALP activity of osteoblasts cells on each specimens, as shown in Figure 3. The ALP activities on the discs was measured at 7th and 14th day. The PEEK group presented the lowest ALP activity when compared in both times with all groups. Of the experimental groups, the PEEK βTCP group was significantly higher in ALP activity than on the other groups in both times and did not present statistical difference with the control group at 7 days.

4. Discussion

Endosteal implant is defined as a device made from one or more materials that is intentionally placed within the body, either totally or partially buried beneath an epithelial surface[2]. Great progress has been made over the decades in understanding the role of the implant material, drive improvements in implant surface design, were studied. But it will also lead to the emergence of new materials and therapies that will enhance peri-implant healing in general. One of the main purposes for modifying implant surfaces with coating is to shorten time of osseointegration, and improve the material biocompability [2,23,24].

In the present study we investigated the effects of hydroxyapatite and β-TCP titanium composites in the cellular response, several types of ceramics have been used in the area of bioengineering for analysis of bone behavior and osseointegration, but many of them are of limited use because of the physical and biomechanical properties that they present. The process of coating materials with other particles is also highly researched in order to improve the properties of materials [17,18,11].

These particles are produced with defined characteristics of size, structure and morphology which are suitable for a bone regeneration process, whereby previous studies mention the importance of that this structure be maintained at the time of clinical application, to enhance the properties of materials, coatings with bioactive materials showed promising results when applied to ceramics.[23,24] Furthermore, unfavorable tissue reactions have been observed after separation of the coating material [25]. However, problems related to loss of the coating material, such as what happens in the clinical installation of threadable dental implants, because of the torque and friction required for their installation, which limits the benefits obtained by the bioactive coating

materials. For this reason in this study, bioactive materials (hydroxyapatite and β-TCP) are added in the composition of the titanium substrate obtaining a new material (biocomposites) obtained by hot pressing techniques that showed promising results when compared with conventional techniques. In this study we chose to use 5% of hydroxyapatite and betatricalcium phosphate for the biocompatibility analysis of the produced biocomposites, based on a previous study that showed that this percentage obtained the best mechanical behavior evaluated [11,12,24]. In order to evaluate this method of production, immortalized fetal human osteoblasts were used, which would be more similar to osteoblasts of healthy patients, as opposed to using animal osteoblasts or obtained from oncological processes.

The initial cell adhesion is usually responsible for cellular functions and eventual tissue integration, while cell proliferation is closely correlated with the amount of new bone formation. Also, cell adhesion allows us to evaluate the cytotoxicity of new biomaterials and to analyze whether the composition or new production strategy is pleasant or not for the cells. In this study the osteoblasts adhered in all groups showing the biocompatibility of the materials.

The cellular morphology visualized in our results is similar to other studies that also used HFOB 1.19 osteoblasts or other osteoblast types,[11,22,26] however the possibility of obtaining high resolution images through the use of FEG-SEM allowed us to better appreciate the details such as cellular extensions, cell stretching and connection between them. The cellular viability results obtained in our study are similar to other studies comparing with changes in the surface of peek [3,11,14,27]. At all times, the control group showed higher cell viability, possibly due to the fact that the control group refers to the osteoblasts seeded in the cell culture plates, which have a treatment of the material. In this work it is possible to observe a greater proliferation and cellular activity in the control group, precisely because it is a positive control with cells seeded at the bottom of the well that has a surface treatment ideal for the growth of osteoblasts. However, the standard difference of this group and higher than in others where the results are more similar and constant, this situation may be due to the question of osteoblasts having an irregular behavior when they are in areas with high confluence, being unstable and even programming their apoptosis by inhibition by contact. These findings could also explain why ALP activity is not as different as for groups with disks. However, the alkaline phosphatase activity was lower in the PEEK group when

compared to others groups. Cellular activity is important findings agree with the results of ALP activity when compared to coated material. Jae Hyup Lee and Abu Bakar et al. have demonstrated that the HA-composited PEEK implant facilitates the ossification in vitro and promotes osseointegration in vivo owing to the addition of HA and it is verified that the osteoinductive HA promotes proliferation and osteoblastic differentiation of surrounding bone cells in vitro [28,29].

Conclusion

The present study concludes PEEK βTCP FGM biocomposite exhibited more ability to stimulated ALP activity. Peek demonstrated more viability capacity compared to other groups, human fetal osteoblasts were able to adhere, proliferate to be active on biomaterial surfaces based on PEEK. Moreover, further *in* vitro and *in* vivo studies are necessary to expand the studies with these materials to know their long-term stability. Consequently its behavior after insertion into a living organism under physiological conditions.

Conflicts of interest

The authors declare that there is no conflicts of interest.

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CAPITULO III

3.1. Discussão geral dos resultados e perspectivas futuras

Nos últimos anos, tem se observado um grande interesse pelo uso de materiais com gradiente funcional de propriedades (FGMs) devido às suas numerosas vantagens em relação aos materiais compósitos. No entanto sua pesquisa, desenvolvimento e produção é ainda relativamente novo na área da Implantodontia.

Analisando os resultados de maneira geral pode se observar:

- Que se confirmou que as amostras produzidas proporcionaram materiais FGM, os analises microscópicos evidenciaram que a adição de hidroxiapatita ou β-tricálcio fosfato foi realizada de maneira gradual em direção a superfície onde foram semeados os osteoblastos, como pode se observar na Figura 1.

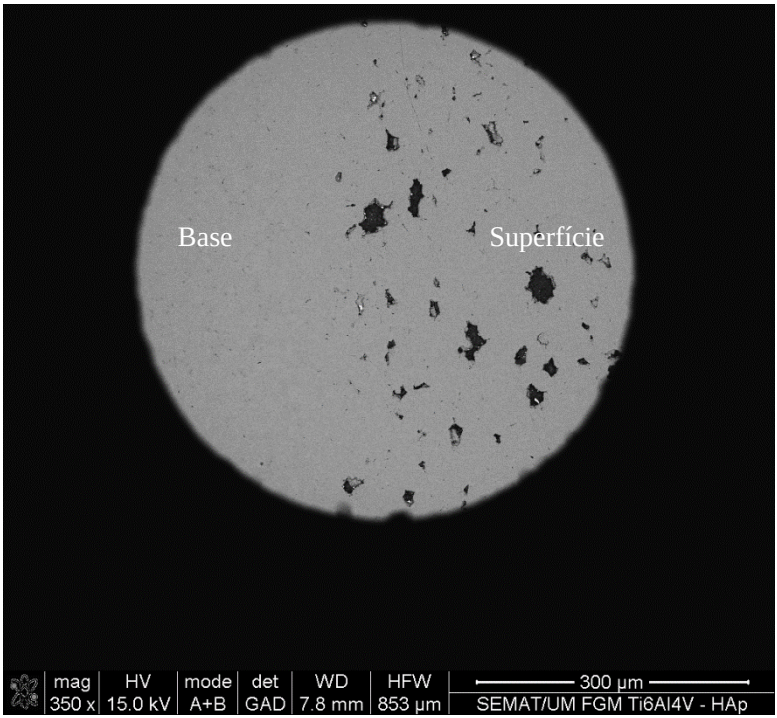

Figura 1: Microfotografia eletrônica de varredura de material FGM x350

- Amostras a base de titânio, zircônia e PEEK tiveram como grupos comportamentos diferentes, pode ser devido à natureza dos materiais assim como a forma de produção deles (temperatura, tempo, etc.).

- O efeito da hidroxiapatita foi benéfica e ressaltante quando adicionada a zircônia, e de βTCP quando adicionado a titânio e PEEK em proliferação e atividade (Figuras 2 e 3), essa diferença deixa interrogantes para realização de pesquisas futuras tentando entender o comportamento desses materiais bioativos.

- Os resultados de proliferação e atividade celular dos grupos de materiais a base de zircônia e titânio tiveram relação, no entanto no grupo PEEK βTCP apresentou menor viabilidade quando comparado a PEEK puro porém maior atividade (Figuras 2 e 3)

- Sabendo que FGMs presentam vantagens quando comparados a biocomposites ou recobrimentos convencionais, propõe-se incrementar os estudos com estes materiais.

- Estudos quantitativos de biocompatibilidade de novos FGMs utilizando hidroxiapatita e βTCP em conjunto com porcentagens em diferentes proporções e proporções já utilizadas na fabricação de substitutos ósseos seria interessante para analisar o comportamento deles interagindo.

- É necessário realizar estudos a longo tempo in vivo e futuramente em humanos para analisas o comportamento dos materiais em sistemas propriamente ditos onde se encontra a interação de outros fatores.

- O material atualmente mais vendido, fabricado e utilizado para fabricação de implantes dentários é o titânio, neste estudo ele mostrou resultados menos atraentes de viabilidade, proliferação e mineralização quando comparados a outros grupos, pelo que deve se motivar a futuros pesquisadores direcionar seus esforços a investigação dos novos FGMs que evidenciam resultados altamente promissores.

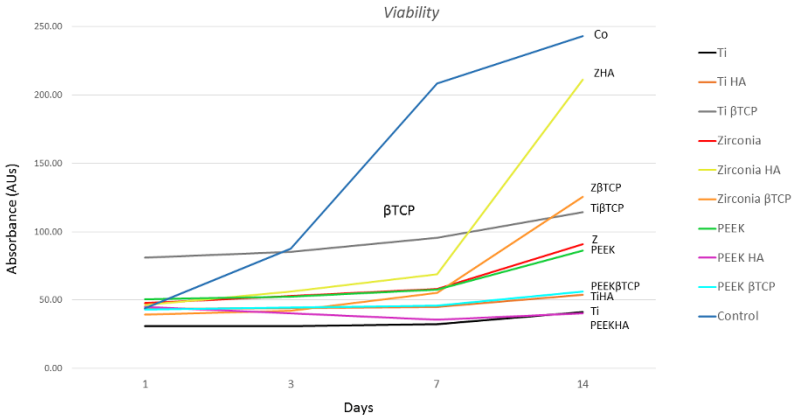

Figura 2: Viabilidade e proliferação celular em todos os materiais de estudo a 1, 3, 7 e 14 dias

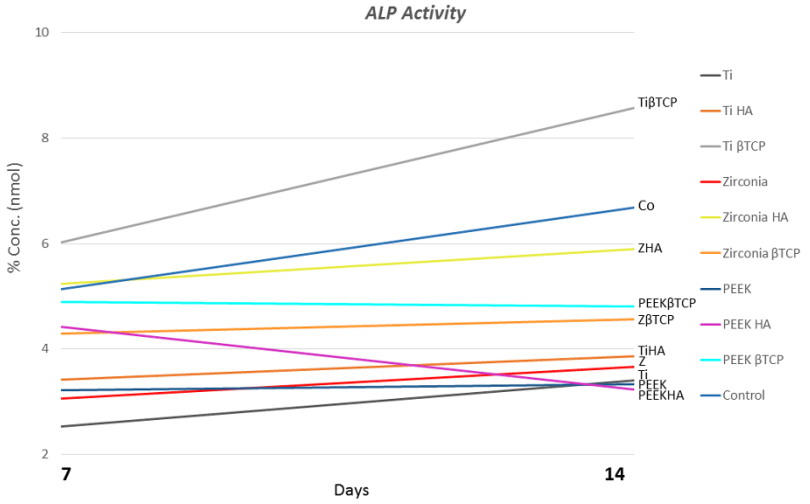

Figura 3: Atividade celular da fosfatase alcalina ALP em todos os materiais de estudo a 7 e 14 dias.

Capitulo IV

4. Produção Científica no Doutorado.

Capítulos de Livro: 3
Artigos: 13
Resumos em anais de eventos: 30

4.1. Capítulos de Livros.

4.1.1. Degradation of dental implant systems after immersion in therapeutic gels. Biodental Engineering IV. 1ed.Londres: CRC Press. Taylor & Francis Group, London, UK., 2014, p. 10-25.

4.1.2. Protocolo cirúrgico na instalação de implante: Conexão hexagonal e cônica. Noções de implantodontia cirúrgica. 1ed.São Paulo: Artes Médicas, 2014, p. 50-63. G.M. PEÑARRIETA JUANITO; ELY, L. B. ; MAGINI, R. S. .

4.1.3 Evaluation of collagen fibers orientation around different connection implants. Biodental Engineering III. 1ed.Londres: CRC Press. Taylor & Francis Group, London, UK., 2014, p. 253-258. ARAUJO, M. A. R. ; D.S.M. CASTRO ; JUANITO, G. M. P. ; M. A. P. P.NORONHA ; C. A. M. BENFATTI, ; R. S. MAGINI ; A. PIATTELLI ; C. R. P. ARAUJO

4.2. Artigos publicados.

4.2.1. LITTUMA, G. J. S. ; SOUZA, H. C. M. ; **JUANITO, G. M. P**. ; MAGINI, R. S. . Lip Repositioning Technique with Smile Elevator Muscle Containment - A New Cosmetic Approach for Gummy Smile: Case Report? has. COMPENDIUM OF CONTINUING EDUCATION IN DENTISTRY (JAMESBURG), 2017.

4.2.2. **GABRIELLA MP JUANITO**, CAROLINA S MORSCH, CÉSAR A BENFATTI, MÁRCIO C FREDEL, RICARDO S MAGINI, JÚLIO CM SOUZA Effect of Fluoride and Bleaching Agents on the Degradation of Titanium: Literature Review. Dentistry, v. 05, p. 1000273-1-1000273-4, 2015.

4.2.3. **G.M. PEÑARRIETA JUANITO**; GEREMIAS, T. C. ; APAZA, K. ; J.F.D. MONTERO. ; C.F. RAFAEL ; R.S. MAGINI . Recobrimento radicular e periimplantar em área anterior usando a técnica de tunelização.. Full Dentistry in Science, v. 6, p. 472-478, 2015.

4.2.4. J.F.D. MONTERO. ; **G.M. PEÑARRIETA JUANITO** ; C.S. MORSCH ; C.F. RAFAEL ; R. S. MAGINI ; A.C. CARDOSO . Prótese cimentada-parafusada. Uma proposta em reabilitação implantossuportada.. Full Dentistry in Science, v. 6, p. 506-512, 2015.

4.2.5. GEREMIAS, T. C. ; J.F.D. MONTERO. ; **G.M. PEÑARRIETA JUANITO** ; C.S. MORSCH ; C.F. RAFAEL ; R.S. MAGINI . Regeneração da parede vestibular em implante anterior com uso de Bio-Oss®. Full Dentistry in Science, v. 6, p. 486-491, 2015.

4.2.6. MORSCH, CAROLINA SCHÄFFER ; RAFAEL, CAROLINE FREITAS ; DUMES, JUAN FELIPE MONTERO ; JUANITO, **GABRIELLA MERCEDES PEÑARRIETA** ; SOUZA, JOÃO GUSTAVO OLIVEIRA DE ; BIANCHINI, MARCO AURÉLIO . Failure of prosthetic screws on 971 implants. Brazilian Journal of Oral Sciences (Online), v. 14, p. 195-198, 2015.

4.3 Artigo aceito para publicação.

4.3.1. **PEÑARRIETA-JUANITO GM**, COSTA M, CRUZ M, MIRANDA G, HENRIQUES B, MARQUES J, MAGINI RS, MATA A, CARAMES J, SILVA FS, SOUZA JCM. Bioactivity of novel functionally structured titanium-ceramic composites in the presence of human osteoblast cells. Journal of Biomedical Materials Research: Part A

4.4. Artigos Submetidos.

4.4.1 **G. M. PENARRIETA-JUANITO**, M B. SORDI, B. HENRIQUES, M. DOTTO, W. TEUGHELS, F. S. SILVA, R. S. MAGINI, J. C. M. SOUZA. Surface damage of dental implant systems and ions release after exposure to fluoride and hydrogen peroxide.

4.4.2. **PEÑARRIETA-JUANITO GM**, M, CRUZ, MARQUES J, SOUZA JCM, MAGINI RS, MATA A, CARAMES J,COSTA M, MIRANDA G, SILVA FS. A new gradated zirconia (YTZP) implant material with embedded HA and bTCP: in vitro bioactivity and mechanical properties.

4.4.3**. G. M. PENARRIETA-JUANITO**, M. ARAUJO, M B. SORDI, R. S. MAGINI, F. S. SILVA, J. C. M. SOUZA. Can disinfectant gels degrade dental implant systems?

4.4.4. M, CRUZ, **PEÑARRIETA-JUANITO GM**, MARQUES J, SOUZA JCM, MAGINI RS, MATA A, CARAMES J, COSTA M, MIRANDA G, SILVA FS. Hard and soft tissue cell behavior on PEEK, Zirconia and Titanium implant surfaces.

4.4.5. SOUZA MT, **PEÑARRIETA-JUANITO GM**, HENRIQUES B, SILVA FS, NOVAES DE OLIVEIRA AP, SOUZA JCM. Mechanical and Biological behavior of LZSA and LZS glass-ceramics in presence of osteogenic cells

4.5. Artigos em escrita para submissão.

4.5.1. **JUANITO, G. M. P**., SCHMITZ, J. K. ; SORDI, M. B. ; C. A. M. BENFATTI, ; SOUZA J, C, M ; MAGINI, R. S. Effect of sodium fluoride on the surface of titanium and zirconia.