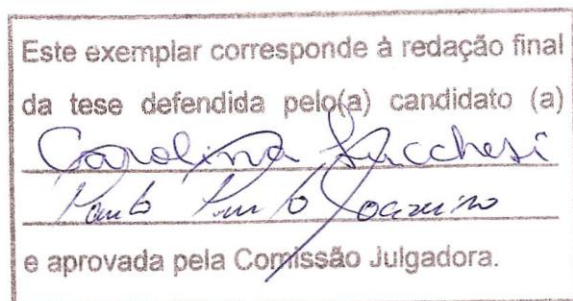


UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA



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“COMPORTAMENTO DE CÉLULAS OSTEÓBLÁSTICAS
SOBRE BIOMATERIAIS POLIMÉRICOS”



Tese apresentada ao Instituto de
Biologia para obtenção do Título de
Doutor em Biologia Celular e Estrutural,
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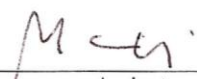
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LISTA DE ABREVIÇÕES E SIGLAS

PCL – Poli (ε-caprolactona)

PLGA – Poli (ácido láctico-co-ácido glicólico)

PHB – Poli(hidroxibutirato)

PHBV – Poli(hidroxiubutirato-co-hidroxi valerato)

PHA - Polihidroxialcanoatos

3D – tri-dimensional

HA - Hidroxiapatita

MSC – Células Tronco Mesenquimais, do inglês *Mesenchymal Stem Cells*.

DNA – Ácido Desoxirribonucleico

GS – Sulfato de Gentamicina

MEC – Matrix extracelular

SFF – Forma sólida livre, do inglês *solid free form*

SLS – Sinterização seletiva a laser, do inglês *Selective laser sintering*

RP – Prototipagem rápida, do inglês *rapid prototyping*

MTT - (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromide), tetrazolium

DMEM – Meio Eagle modificado por Dulbecco, do inglês *Dulbelcco's modified Eagle's médium*

UV - ultravioleta

MEV – Microscopia eletrônica de varredura

BMP – Proteína morfogenética óssea, do inglês *Bone Morphogenetic Protein*

OP – Proteína osteogênica, do inglês *osteogenic protein*

MMP - metaloproteinase

COP – Centro de ossificação primária

PBS – Tampão fosfato salino, do inglês *phosphate buffer saline*

FBS – Soro fetal bovino, do inglês *fetal bovine serum*

DMSO – Dimetil Sulfoxido

ARTIGOS QUE COMPÕEM ESTA TESE

Lucchesi, C.; Barbanti, S.H.; Joazeiro, P.P.; Duek, E.A.R. Cell Culture on PCL/PLGA Blends. *Journal Applied Polymer Science*, v.115, n.5, p.2609-2615, 2010. DOI 10.1002/app.29919

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RESUMO

Os polímeros biorreabsorvíveis, tais como, PHB, PCL e PLGA, têm sido estudados como dispositivo para engenharia de tecidos por serem biocompatíveis, suportarem o crescimento e diferenciação celular e os produtos de sua degradação serem atóxicos. No entanto, a escolha do biomaterial depende das necessidades exigidas para uma determinada aplicação.

Os suportes para engenharia de tecidos devem se basear na construção de réplicas biológicas *in vitro*, como que o biomaterial se tornasse parte integrada para transplante *in vivo* para a recuperação de perdas ou mau funcionamento de tecidos ou órgãos, devendo subsequente, atuar sem agredir o restante do organismo, isto é, sem o risco de rejeição ou complicação.

Muitas estratégias têm sido desenvolvidas com o intuito de substituir tecidos ou órgãos danificados, incluindo a aplicação de suportes tridimensionais (3D), os quais devem possuir características estruturais e mecânicas para guiar a proliferação e espalhamento de células *in vitro* e *in vivo*. Os suportes, feitos de materiais sintéticos ou naturais, servem como substitutos para a matriz extracelular (MEC) nativa. Ênfase especial é dada as técnicas com controle computadorizado, como a fabricação sólida com forma livre (SFF), conhecida como prototipagem rápida (RP), a qual permite preparar suportes 3D com geometrias complexas, tanto externamente como internamente, além de ser uma técnica rápida e de baixo custo.

Além disso, grande parte dos polímeros possuem superfície hidrofóbica, característica inadequada para a maior parte dos diferentes tipos celulares, o que dificulta aplicação na engenharia de tecidos. Uma alternativa a este problema é o tratamento da superfície por plasma. Este tratamento induz modificação restrita ao topo da superfície, conferindo caráter hidrofílico à superfície, dependendo do gás utilizado.

Neste estudo, arcabouços de polímeros biorreabsorvíveis PCL, PLGA e PHB foram preparados por diferentes técnicas, casting e sinterização seletiva a laser, avaliando-se o comportamento de células osteoblásticas diferenciadas sobre os biomateriais poliméricos tridimensionais. Inicialmente o trabalho foi desenvolvido com os polímeros PCL e PLGA preparando-se blendas poliméricas, as quais demonstraram melhorar as características gerais dos polímeros, quando utilizados como dispositivos para tecido ósseo, como as propriedades

mecânicas. Com o intuito de aprimorar o design tridimensional do material, optou-se pela realização da técnica de sinterização seletiva a laser. No entanto, a técnica exige uma grande quantidade de material e devido ao alto custo do PCL e PLGA, este foi substituído pelo polímero PHB, o qual é produzido pela indústria nacional Biocycle possuindo um baixo custo e ainda ser biocompatível. Os dados são apresentados em capítulos independentes.

Arcabouços porosos de PCL, PLGA e suas blendas foram preparados pela técnica de evaporação do solvente, onde sais de citrato de sódio com granulometria de 180-250 μm , foram adicionados a solução para a promoção dos poros, sendo posteriormente lavados do arcabouço. Células osteoblásticas provenientes de calota craniana de ratos *Wistar* foram semeadas sobre os arcabouços, sendo avaliado o comportamento de citotoxicidade do material e o comportamento de adesão e morfologia celular através de ensaios bioquímicos e MEV.

Os arcabouços de PHB foram obtidos por uma das técnicas de RP, a sinterização seletiva a laser (SSL), sendo sua superfície modificada por plasma pelos gases oxigênio e nitrogênio. Células osteoblásticas provenientes de calota craniana de coelhos foram semeadas sobre os arcabouços realizando-se o estudo *in vitro*, através de análises bioquímicas pela técnica do MTT, para viabilidade e adesão celular, quantificação de colágeno por Sírius Red e Microscopia Eletrônica de Varredura (MEV). Para o estudo *in vivo*, após o cultivo celular sobre os arcabouços, defeitos ósseos foram provocados em coelhos e os arcabouços contendo as células foram então implantados, avaliando-se a interação PHB/osteoblasto/tecido, através da análise histológica.

Todos os arcabouços estudados, PCL, PLGA e suas blendas, assim como o PHB, não apresentaram índices de citotoxicidade, permitiram às células a capacidade de adesão, proliferação e síntese de matriz, mantendo seu fenótipo osteoblástico. As amostras de PHB tratadas por plasma de Nitrogênio mostrou melhorar a capacidade de adesão celular. Os arcabouços de PHB contendo células mostraram-se os mais adequados para o preenchimento de defeitos ósseos, melhorando o processo de regeneração apresentando uma boa osteointegração. A sinterização seletiva a laser apresentou-se uma excelente técnica para a obtenção de PHB 3D para a Engenharia de Tecidos.

ABSTRACT

The bioresorbable polymers as, PHB, PCL and PLGA have been studied as device for Tissue Engineering for their biocompatibility and to support the cell growth and differentiation and their degradation products are nontoxic. However, the choice of the biomaterial depends on the needs demanded for a certain application.

The scaffolds for tissue engineering have to be designed to mimetize the biological conditions *in vitro* to become part integrated for transplant for the recovery of tissue or organs lost or without function, and subsequently, to work in a cordial way with the remaining of the organism without the rejection risk or complication.

A lot of strategies have been developed with to substitute damaged tissues or organs, and it has been used the application of three-dimensional supports (3D), which should possess structural and mechanical applications to guide the cells proliferation and spread *in vitro* and *in vivo*. The scaffolds, made from synthetic or natural materials, serve as substitutes for the extracellular matrix (ECM) native. Special emphasis is given the techniques with computerized control, as the free solid form (SFF), known as rapid prototyping (RP), which allows to prepare three-dimensional supports with complex geometries, so much externally as internally, besides to be a fast technique with low cost.

Besides, great part of the polymers possess hydrophobic surface, inadequate characteristic for most of the different cell types, which is not desirable for tissue engineering applications. An alternative to this problem is the surface treatment by plasma. Plasma treatment induces restricted modification to the top of the surface, improving the surface hydrophilicity, depending on the gas used.

In this study, scaffolds of bioresorbable polymer PCL, PLGA e PHB were prepared by different techniques, casting and selective laser sintering, being evaluated the osteoblast cells behavior on the 3D polymer scaffolds.

Previously we developed the studies preparing the polymeric blends with PCL and PLGA, which demonstrated improve the general characteristic of the material, as the mechanical properties, as devices for bone tissue. With the intention to improve the design of the scaffolds, we chose for the selective laser sintering technique. However, the technique demands a great

amount of material and due to the high cost of PCL and PLGA, those were substituted by PHB polymer, which is produced by Brazilian industry Biocycle with low cost and still to be biocompatible. For those reasons the data are presented in independent chapters.

PCL, PLGA porous scaffolds and their blends were prepared those scaffolds by casting solvent, and sodium citrate with 180-250 μm were added to the solution for porous formation when the salt was washed later of the scaffolds. Osteoblast cells from rat *Wistar* calvaria were seeded on the scaffolds, being evaluated the behavior of cell adhesion and viability behavior, cell morphology through biochemical assays, and scanning electron microscopy.

Three-dimensional PHB scaffolds were obtained by selective sintering laser (SSL), with the surface modified by nitrogen and oxygen plasma. Osteoblast cells obtained from rabbit calvaria were seeded on the scaffolds to the *in vitro* studies, through biochemical analyses by MTT test for cell viability and cell adhesion, collagen quantification of by Sirius Red colorimetric assay and scanning electron microscopy (SEM). For the *in vivo* studies, bone defects were provoked in rabbits and they were filling out with 3D PHB with osteoblast cells culture prior implant. We evaluated the PHB/osteoblast/tissue, interaction through the histological analysis.

All the scaffolds studied PCL, PLGA and their blends, as well as the PHB did not showed cytotoxicity effects, allowed cells adhere, proliferated, and matrix synthesized, maintaining their osteoblastic phenotype.

The PHB samples treated by nitrogen plasma have been showed to improve the cell adhesion. The PHB scaffolds with cell seeded previously demonstrated to be more suitable for filling out bone defects, improving the regeneration process showing a good osteointegration. The selective laser sintering was excellent technique to obtain PHB scaffolds for Tissue Engineering.

CAPÍTULO 1

I. INTRODUÇÃO

A incidência de defeitos ósseos devido a fraturas, tumores e infecções tem-se elevado com o aumento da expectativa de vida da população, gerando problemas de grande impacto social (World Health Organization, 2003). Aproximadamente 150 doenças ou síndromes relacionadas a osso foram diagnosticadas, sendo geralmente progressivas e causadoras de dor. No que diz respeito à reparação das fraturas entre 5 a 10% dos casos que ocorrem anualmente nos EUA, resultam em reparo ósseo lento e ineficiente (Li & Wosney, 2001); e entre 5-35% das cirurgias de fusão vertebral resultam em insucesso no processo de fusão (Ludwig *et al*, 2000).

Os custos na área da saúde têm se elevado, devido à necessidade frequente de se substituir o tecido ósseo. A cada ano, milhões de cirurgias para implantes são realizadas para o reparo ou cura de um órgão. Os implantes se constituem de enxertos autólogos e homólogos, cimentos ósseos, materiais poliméricos, metais e cerâmicas (Ishaug *et al*, 1997). Atualmente as estratégias que utilizam enxertos autólogos se mostram favoráveis, nesse tipo de reparação, devido às características de osteoindução, osteocondução e por não causarem resposta imune (Marquis *et al*, 2009). Por outro lado, possuem limitação na quantidade de tecido que pode ser extraído, além de morbidez do doador. Esse fato incentivou as buscas por métodos alternativos para reconstituição de grandes defeitos ósseos.

A limitação nas técnicas existentes gera a necessidade de se buscar novos métodos para regeneração óssea, demandando uma variedade de terapias e implantes de materiais dependendo do tipo e tamanho do defeito. Dessa busca, surge a Engenharia de Tecidos, ciência que emerge como uma alternativa promissora para o tratamento de perda ou mau funcionamento tecidual, combinando células, biomaterial e fatores físico-químicos e bioquímicos para melhorar ou substituir funções biológicas (Langer & Vacanti, 1993).

A interação células-biomateriais é de fundamental importância, pois influencia os subseqüentes processos biológicos como diferenciação e proliferação celular (Marquis *et al* 2009). Além disso, o processo de adesão celular é um importante requerimento para o sucesso da incorporação do implante ou a colonização do arcabouço para o reparo do tecido. No presente estudo, o comportamento de células ósseas foi estudado sobre polímeros sintéticos, preparados por diferentes técnicas, com o propósito de preenchimento ósseo em locais de difícil reparo tecidual.

1. BIOLOGIA DO TECIDO ÓSSEO

O tecido ósseo é uma forma especializada de tecido conjuntivo, sendo um importante elemento do esqueleto. Este tecido é composto pela matriz orgânica e inorgânica. A matriz orgânica é composta por aproximadamente 95% de fibras colágenas, tipo I e III, e proteínas não-colágenas, como osteocalcina, osteopontina, proteína osteoprogenitora (OP-1) ou proteína morfogenética óssea (BMP-7), osteonectina, atualmente denominada SPARC (do inglês *Secreted protein acidic rich in cysteine*), sialoproteína óssea, glicosaminoglicano, células, entre outras. Já a matriz inorgânica é constituída de água, 65-70% de compostos de fosfato de cálcio, principalmente na forma de cristais de hidroxiapatita $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HA), e composta ainda por bicarbonato, magnésio, potássio, sódio, citrato em pequenas quantidades (Junqueira & Carneiro, 2004). Diferencia-se de outros tecidos conjuntivos pelo fato de sua matriz tornar-se mineralizada. Este tecido é composto por osteoblastos, osteoclastos, células mesenquimais osteoprogenitoras e osteócitos.

O tecido ósseo é dinâmico, altamente vascularizado e com a capacidade única de regeneração e remodelação sem a ocorrência de fibrose (Buckwalter *et al* 1996; Kneser *et al*, 2006). É responsável pelo suporte mecânico do esqueleto, suportando carga, promovendo proteção aos órgãos internos e os movimentos, juntamente com o sistema muscular. Serve ainda como reserva mineral e tem a capacidade de mobilizar seu estoque rapidamente, quando se faz necessária a homeostase do nível de íons cálcio no sangue. Esta diversidade funcional requerida ao tecido ósseo reflete na complexidade de sua arquitetura (Kneser *et al*, 2006).

Em indivíduos humanos adultos, os ossos têm arranjo trabecular e compacto, além da cavidade medular. Os ossos trabeculares ou esponjosos são organizados em poros assemelhando-se a uma esponja, que permite o alojamento da medula óssea, estando presente na metafise dos ossos longos, crista ilíaca e corpos vertebrais. Já os ossos compactos, mostram-se sólido, com porosidade menor que 10%, sendo revestido extremamente por uma membrana de tecido conjuntivo, o perióstio e internamente, pelo endóstio. Ambos contêm osteoblastos, osteoclastos e seus progenitores. Este arranjo sólido faz parte dos ossos longos, curtos e achatados, sendo de grande importância para o suporte do esqueleto (Ackerman *et al*, 1976; Datta *et al*, 2008).

A capacidade regenerativa dos ossos é dependente da quantidade de massa óssea perdida e/ou danificada. Quando o defeito ocorrido não permite que o tecido ósseo se regenere por ele mesmo, este

defeito é denominado como defeito crítico (Prado *et al*, 2006; Jan *et al* 2006; Schmitz & Hollinger, 1986). O defeito crítico varia de espécie para espécie, sexo, idade, local do defeito e ainda do metabolismo individual. Em geral, em calota craniana de coelhos New Zealand, o defeito crítico é estimado em 15mm de diâmetro (Hollinger & Kleinschmidt 1990), no entanto, existem várias discussões na literatura sobre o tamanho apropriado do defeito crítico. Um estudo realizado por Hokugo *et al* (2007), onde criou-se um defeito crítico com 5mm de diâmetro, verificou-se que não ocorreu a completa regeneração tecidual após 60 dias.

1.1 CÉLULAS ÓSSEAS

As células ósseas descritas são os osteoblastos, osteócitos e osteoclastos, os quais possuem diferentes origens e funções, sendo de crucial importância na remodelação óssea. No entanto, não podemos deixar de citar as células mesenquimais de medula óssea (BMSCs do inglês, *bone marrow stromal cells*), as quais são capazes de se diferenciar em uma variedade de tipos celulares incluindo osteoblastos. Durante este processo de osteoblastogênese, os osteoblastos passam por diversos eventos morfológicos e moleculares, a célula adquire a capacidade de sintetizar e depositar matriz extracelular mineralizada, característica do tecido ósseo (Ducy *et al*, 2000; Lavery *et al*, 2009). Esta transformação é um processo multi-passos altamente dependente de sinais provenientes do ambiente e justamente regulados.

1.1.1 OSTEOLASTOS E OSTEÓCITOS

Os osteoblastos derivam de células mesenquimais provenientes da medula óssea, do periósteo ou endósteo. Quando ativos, possuem proeminente retículo endoplasmático rugoso, assim como, complexo de Golgi, devido à alta síntese de proteínas como, colágeno, SPARC, osteonectina, osteocalcina, glicosaminoglicano entre outras. São ricos em fosfatase alcalina, enzima fundamental na deposição de matriz mineralizada. Encontram-se na matriz da superfície óssea existente (osteoblastos de superfície) ou depositando camadas de tecido ósseo recém sintetizadas sobre estas (osteoblastos mesenquimais) ou acabam morrendo por apoptose (Shapiro, 1988; Marie, 2008; Marquis *et al*, 2009).

Alguns osteoblastos permanecem livres na superfície, enquanto outros se tornam gradativamente aprisionados pela matriz que a própria célula secreta. Este material recém produzido é conhecido como ósteon e o osteoblasto aprisionado na matriz mineralizada, passa a ser denominado osteócito, continuando a secretar matriz em torno de si, porém em quantidade reduzida. Apesar de aprisionado na matriz, os osteócitos não se encontram isolados, ligam-se a osteócitos vizinhos por projeções celulares, através de canalículos, formando junções comunicantes (Alberts *et al*, 2005; Datta *et al* 2008).

Quando avaliados *in vitro*, a diferenciação requer a presença de vários fatores os quais incluem fatores de crescimento, dexametasona, β -glicerofosfato e L-ácido ascórbico (Bruder & Caplan 2000). Outros elementos como a proteína osteoprogenitora, tem sido estudados por Lavery e colaboradores (2009) descrevendo a bioatividade da OP-1 durante o processo inicial de diferenciação de células mesenquimais humanas em osteoblastos *in vitro*, sendo estas informações importantes para uma melhor compreensão das contribuições individuais das BMPs, durante a formação óssea humana (Lavery *et al* 2009).

1.1.2 OSTEOCLASTOS

Os osteoclastos são células multinucleadas gigantes, diferenciadas a partir de células de linhagem hematopoiética da medula óssea, os monócitos/macrófagos (Dougall & Chaisson, 2006), sendo responsáveis pela reabsorção óssea. Seu citoplasma contém retículo endoplasmático e complexo de Golgi bem desenvolvidos, muitas mitocôndrias e lisossomos. Sintetizam e secretam enzimas proteolíticas, as metaloproteinases (MMP), catepsina K e fosfatase ácida, as quais atuam erodindo a matriz protéica (Vaananen, 2005; Zeng *et al*, 2006).

1.2 OSTEOGÊNESE

O processo de formação óssea durante o desenvolvimento do esqueleto envolve uma complexa coordenação de múltiplos tipos celulares e tecidos. O desenvolvimento ósseo ocorre através de dois padrões de formação óssea: intramembranosa e endocondral. No entanto, deve se ter em mente a

distinção em osso como um tecido, contendo células ósseas e matriz mineralizada e osso como um órgão, incluindo o tecido ósseo, cartilaginoso, fibroso, medula e vasos sanguíneos.

Nos mamíferos, a formação óssea é um evento tardio no embrião e ainda um dos últimos estágios no desenvolvimento do esqueleto. Em alguns elementos do esqueleto, como parte do crânio e clavícula, ocorrem grupos de células mesenquimais que se diferenciam diretamente em osteoblastos, formando o centro de ossificação primária (COP) e iniciando a produção de matriz não mineralizada, o osteóide. Com a mineralização, os osteoblastos são aprisionados na matriz transformando-se em osteócitos. Durante o processo de ossificação intramembranosa, ocorre à formação de vários COPs, os quais crescem radialmente, dando origem aos ossos primários, os quais são invadidos por vasos sanguíneos e células mesenquimais, formando-se a medula óssea (Hall & Miyake 1995; Huang *et al*, 1997).

Para o restante do esqueleto, as células da condensação mesenquimal diferenciam-se em condrócitos formando a cartilagem “análoga” aos ossos futuros. Na periferia desta cartilagem, células do pericôndrio diferenciam-se em osteoblastos, enquanto os condrócitos tornam-se hipertróficos. Eventualmente, a matriz ao redor destes condrócitos hipertróficos se calcifica e vasos sanguíneos presentes na matriz trazem os osteoblastos (Horton, 1993; Erlebacher *et al*, 1995). Uma vez que a matriz é depositada, a medula óssea se forma e os primeiros osteoclastos aparecem (Hofstetter *et al*, 1995). Assim, os estágios seqüentes de formação da cartilagem análoga, calcificação da cartilagem e formação óssea, caracterizam a ossificação endocondral. Neste modelo de ossificação, os osteoblastos precedem a diferenciação dos osteoblastos (Karsenty, 1999; Deng *et al*, 2008).

Os primeiros estágios do desenvolvimento do esqueleto em vertebrados são dependentes de genes que controlam a distribuição e proliferação de células da crista neural, escleroma e placa lateral mesodérmica ocorrendo a condensação das células mesenquimais, pela qual estas se diferenciam em osteoblastos, no caso do tecido ósseo (Deng *et al*, 2008). Esta condensação celular é o principal estágio no desenvolvimento do esqueleto e outros tecidos mesenquimais. As primeiras observações de condensação celular foram realizadas por Strelzoff em 1873 e van der Stricht em 1890, os quais descreveram os processos de osteogênese e condrogênese em membros de aves, e Parsons em 1905 publicou um artigo seguindo os mesmos passos. Até aquele momento, raras eram as observações de osteogênese em mamíferos (Fell 1925; Gould *et al*, 1992). Segundo Hall & Miyake (1992), as condensações celulares são iniciadas pelo aumento na atividade mitótica ou pela agregação de células similares ao redor de um centro. Condensação é definida como a agregação de células pela qual os

tecidos se formam durante o desenvolvimento embrionário e pela qual a condrogênese e osteogênese são iniciadas durante seu reparo e/ou regeneração (Hall & Miyake 1995).

Recentemente, Hall & Miyake (2000), descreveram a condensação como a terceira entre quatro fases da esqueletogênese. A primeira é a migração das células para o local da futura esqueletogênese; a segunda é a interação tecidual (epitelial-mesenquimal) que resulta na formação da condensação; e a quarta é diferenciação em condroblastos ou osteoblastos (Hall & Miyake 2000). Segundo Ede & Agerbak (1968), o tamanho da condensação e a interferência no número de células, afetam a esqueletogênese. A condensação reduzida segue um limiar crítico e a esqueletogênese pode não ser iniciada. Porém, um aumento na condensação pode resultar num esqueleto muito grande e/ou deformado. Este tamanho é regulado através da sinalização envolvendo as BMPs (Hall & Miyake, 1995).

Ambos os mecanismos, endocondral e intramembranoso, também são descritos durante a regeneração do tecido, dependendo dos elementos disponíveis no local onde ocorreu a lesão (Shapiro, 2008).

Durante a formação óssea, os osteoblastos sintetizam e depositam colágeno tipo I/III e com o auxílio de proteínas da matriz extracelular, cristais de fosfato de cálcio são depositados sobre as fibras colágenas (Shapiro, 2008).

Significante progresso tem sido realizado nos últimos anos para a compreensão dessa rede de mecanismos moleculares que controlam a diferenciação osteogênica (Deng *et al*, 2008). Estimulantes aspectos no desenvolvimento do esqueleto dos vertebrados têm sido obtidos através de estudos genéticos e moleculares em modelos animais e humanos com desordens inerentes na morfogênese, organogênese e crescimento do esqueleto (Olsen *et al*, 2000, Harada & Rodan 2003; Ralston & Crombrughe, 2006)

Atsawasuwan e colaboradores (2005) estudaram o processo de expressão das isoformas da enzima lisil oxidase, a qual inicia a ligação intermolecular das moléculas colágenas nos resíduos de lisina e hidroxilisina, em células osteoblásticas MC3T3-E1 durante o processo de mineralização das fibras, indicando que sua expressão é altamente regulada durante a diferenciação dos osteoblastos, a qual sugere que a enzima tenha papel durante a estabilização das fibras e sua subsequente mineralização (Atsawasuwan *et al* 2005). Num estudo realizado *in vivo*, Verdelis e colaboradores (2008) atribuíram, em parte, a capacidade da dentina sialoproteína como reguladora da mineralização e remodelação em ossos de camundongos. Genge e colaboradores (2008), baseados em nucleadores de fosfato de cálcio amorfo, estudaram os efeitos da proteína anexina-5 (Anx-5), uma proteína lipídeo dependente para ligar-se ao

cálcio. Neste estudo, os autores demonstraram um aumento na nucleação e formação dos minerais em colágeno tipo II e IX na presença da proteína. No entanto, o processo de mineralização *in vivo* continua não sendo bem compreendido.

1.3 PROTEÍNAS ÓSSEAS

Diversas proteínas fazem parte do tecido ósseo, sendo divididos em proteínas colágenas, como o colágeno do tipo I e III, e as proteínas não colágenas, como sialoproteína óssea, SPARC, osteocalcina, osteopontina, a grande família de proteínas morfogenéticas ósseas (BMPs), entre outras. No entanto, serão descritas somente as proteínas em foco no presente estudo.

1.3.1 COLÁGENO

O colágeno é o tipo mais abundante de proteína no organismo, constituindo uma família de proteínas, as quais exercem diferentes funções, principalmente estruturais. Atualmente, são conhecidos 20 tipos de proteínas colágenas diferentes, podendo ser classificadas em grupos: formadores de fibras longas, como colágeno tipo I, II, III, V e IX; colágenos associados a fibrilas, como tipo IX e XII; colágeno formador de redes, como tipo IV; colágeno de ancoragem, como tipo VII (Junqueira & Carneiro 2004).

O colágeno do tipo I e III, formadores de fibras longas, são os encontrados nos ossos, sendo mais bem abordados no estudo. A molécula colágena contém 3 cadeias polipeptídicas e cada cadeia possui 1000 aminoácidos e uma sequência repetida de aminoácidos Gly-X-Y (na qual, X e Y podem ser qualquer aminoácido ácido, mas geralmente prolina e hidroxiprolina, respectivamente). Essas moléculas são conhecidas como pro-colágeno, os quais se agregam em subunidades para formar fibrilas, arranjadas em três cadeias α -pro, unidas por pontes de hidrogênio e interações hidrofóbicas. Esta molécula pró-colágena é enviada pela célula para o ambiente extracelular por exocitose, sofrendo ação da enzima pró-colágeno peptidase, a qual cliva os peptídeos de registro, para que estas moléculas, agora conhecidas como tropocolágeno, a subunidade colágena básica, possam se agregar formando fibrilas, finas estruturas com 10 a 300nm de diâmetro. Um sistema de ligações covalentes intercadeias estabiliza as fibras (Patino *et al*, 2002), esse sistema é possível devido ao trabalho das enzimas: N-proteinase, a qual removendo a porção N-propeptídeo; pro-colágeno C-proteinase, a qual remove porção C-propeptídeo; e

lisil-oxidase, enzima responsável por iniciar a ligação inter-molecular entre as fibrilas. A biossíntese do colágeno tipo I é o mais estudado até o momento, provavelmente devido a sua alta quantidade no organismo.

O colágeno tem uma gama de propriedades biofísicas e bioquímicas, que fazem desta molécula um importante biomaterial. Estas propriedades incluem solubilidade, resistência, mediação entre interações intercelulares, estabilidade, biodegradabilidade e baixa imunogenicidade (Patino *et al*, 2002).

Com o advento da microscopia eletrônica de transmissão, importantes características em pequena escala sobre o colágeno e a estrutura óssea foram descobertas. O pioneiro a descrever este advento foi Robinson em 1952, observando os componentes inorgânicos dos ossos, os cristais de pequenas formas irregulares e sua relação com a matriz óssea orgânica. Watson & Robinson (1953), notaram a presença de formação óssea através do mesmo modelo de banda observado em colágeno tipo I não mineralizado, porém corado. A explicação para esse fenômeno foi provida por Hodge & Petruska em 1962, que propuseram o modelo de arranjo em bandas do colágeno e estrutura óssea. Neste modelo, os minerais se localizam nos espaços entre as fibrilas. Hoje se sabe que o colágeno forma uma rede tri-dimensional na qual os cristais se depositam e crescem (Weiner & Traub, 1989).

2. REGENERAÇÃO ÓSSEA

Embora os ossos apresentem dureza, estão sujeitos a fraturas, de acordo com o esforço aplicado, sendo estas acompanhadas de hemorragia. As células do periósteo (tecido conjuntivo que reveste os ossos externamente, excluindo cartilagem articular) e endósteo (tecido conjuntivo que reveste os ossos internamente, medula óssea e trabéculas esponjosas) respondem a lesão, ocorrendo rápida proliferação de fibroblastos e células mesenquimais, que envolvem a formação da cartilagem e fibrocartilagem (calo fibrocartilaginoso), e preenchem o local do defeito. Na base do calo, osteoblastos iniciam a formação da matriz óssea, resultando em calo ósseo primário (osso imaturo). Subsequentemente o osso primário é remodelado em osso secundário ou lamelar e os vasos sanguíneos do periósteo e endósteo estão envolvidos na nutrição óssea.

Os ossos têm a capacidade única de se regenerar dependendo do volume danificado, sem o desenvolvimento de cicatriz fibrosa, a qual está presente durante cicatrização de feridas em tecidos

moles. Isto é alcançado através dos estágios complexos e interdependentes do processo de cicatrização, o qual mimetiza o desenvolvimento altamente regulado do esqueleto (Kanczler and Oreffo, 2008; El-Tamer & Reis, 2009).

A remodelação e reorganização do tecido ósseo é resultado de muitos fatores incluindo estímulo mecânico, causas metabólicas (como dieta de cálcio, doenças, idade), alterações hormonais e efeito de drogas.

O tipo de regeneração óssea pode ocorrer por diferentes mecanismos e embora a síntese óssea seja sempre mediada por células mesenquimais ou osteoblastos de superfície, também irá depender do ambiente biofísico, podendo ser: *reparo endocondral*; *reparo ósseo primário*, sem a presença de uma fase cartilaginosa, mediado exclusivamente por osteoblastos provenientes do sistema de Havers intraósseo e osteoclastos; *reparo ósseo direto*, ocorrendo também sem fase cartilaginosa, no entanto, as células derivam da medula óssea; e por *distração osteogênica*, processo biológico de neoformação óssea entre segmentos ósseos que são gradualmente separados por tração controlada. Deve-se ainda considerar o local do defeito, uma vez que o ambiente irá prover os recursos celulares, como agente primário para o reparo (Shapiro, 2008).

No caso de reparo ósseo endocondral, após a fratura, forma-se hematoma contendo rede de fibrina e coágulo, os quais servem de arcabouço para o crescimento celular. Esta primeira etapa é caracterizada por uma fase inflamatória e juntamente com o coágulo sanguíneo são essenciais para o reparo. Células mesenquimais provenientes do periósteo proliferam e outras linhagens osteoprogenitoras se diferenciam e o reparo ósseo tem início a partir da periferia do córtex ósseo. Próximo a fratura, ocorre hipoxia devido ao dano no suprimento sanguíneo, permitindo que células mesenquimais se diferenciem em condroblastos, dando origem a matriz cartilaginosa. Estas massas cartilaginosas e fibrosas formam o calo ósseo, o qual irá estabilizar o local da fratura, sendo posteriormente invadido por vasos sanguíneos e tornando-se mineralizado, formando tecido ósseo. Para o caso de fraturas que exigem fixação, não ocorre a formação de calo e o reparo ósseo ocorre sem a presença de fase cartilaginosa. O tecido ósseo pode ser depositado no córtex e também na cavidade da medula, a partir da diferenciação de células mesenquimais, diretamente em osteoblasto (Shapiro, 2008).

Os defeitos ósseos de grande extensão são um desafio para a cirurgia reconstrutiva. O tratamento mais apropriado é o enxerto autólogo de osso. Porém a quantidade de osso apropriada é limitada e sua retirada é dolorosa, com risco de infecção, hemorragia, inaptidão cosmética, danos nervosos e a perda de função (Damien & Parsons 1991). Uma alternativa para os cirurgiões superarem

este problema é a utilização de biomateriais de origem sintética ou natural, que permite a migração e a proliferação das células ósseas (Langer & Vacanti 1993). Entretanto, o sucesso destes materiais no reparo de defeitos ósseos de grande porte é limitado. Muitas vezes, estes não possuem as características e propriedades osteogênicas e osteoindutiva dos enxertos ósseos.

Boa regeneração óssea foi obtida por arcabouços reabsorvíveis suplementados com células competentes, como por exemplo, células tronco mesenquimais (MSC), “recriando um ambiente embrionário” no tecido adulto danificado, melhorando os resultados clínicos (Petite *et al*, 2000).

3. ENGENHARIA DE TECIDO ÓSSEO

A Engenharia de Tecidos emergiu como uma promissora alternativa buscando soluções para as limitações existentes como os enxertos autólogos e/ou heterólogos para o tratamento de mau funcionamento ou reparo de partes danificadas dos tecidos ou órgãos. A Engenharia de tecidos combina células precursoras provenientes do paciente, matrizes suportes e moléculas bioativas (Langer & Vacanti, 1993), que no tecido ósseo devem induzir a osteogênese. Aplica os princípios e métodos da engenharia e ciências da vida dirigindo a compreensão fundamental da relação estrutura-função nos tecidos normais ou patológicos de mamíferos e no desenvolvimento de substitutos biológicos que restaurem, mantenham ou melhorem as funções teciduais (Nerem, 1992; Langer & Vacanti 1993).

Desde o advento da engenharia de tecido, o tecido ósseo tem despertado particular interesse, uma vez que possui grande capacidade regenerativa (Bessa *et al*, 2008, part II).

O biomaterial utilizado para regeneração óssea deve possuir um gama de características semelhantes ao osso como, preencher ou servir de suporte para o tecido danificado, ser osteoindutor, osteocondutor, biodegradável, permitindo boa osteointegração (Albrektsson & Johansson, 2001; Shishatskaya *et al*, 2006). Segundo Verti (1992) biodegradação refere-se a um agente biológico (enzima, microorganismo ou célula) responsáveis pela degradação e dispersão *in vivo*. A biocompatibilidade pode ser definida como a capacidade do material ter uma resposta apropriada numa aplicação específica, com o mínimo de reações alérgicas, inflamatórias ou tóxicas, quando em contacto com os tecidos vivos ou fluidos orgânicos, compreendendo as interações dos tecidos e fluidos, incluindo sangue, com um material (Williams, 2003).

Osteointegração é um termo definido como o contato direto (em nível microscópico) entre o tecido ósseo vivo e o implante, sem o desenvolvimento de tecido fibroso entre eles. Deve-se ter em mente importantes termos citados em literatura como a osteoindução e osteocondução. Osteoindução é o termo utilizado quando células osteoprogenitoras são estimuladas a se diferenciar, induzindo osteogênese (Wilson-Hench, 1987). Osteocondução é o termo utilizado quando uma determinada superfície tem um ambiente adequado para o crescimento ósseo (Brånemark *et al*, 1977; Albrektsson, *et al*, 1981).

Os arcabouços utilizados na engenharia de tecidos buscam mimetizar as funções da matriz extracelular natural. O primeiro papel dos arcabouços é servir de suporte para adesão celular, facilitando a localização e liberação de células quando estas são implantadas, consequentemente, propiciando um suporte mecânico adequado para o crescimento do novo tecido com suas funções apropriadas (Kim *et al*, 2001; Lanza *et al*, 2000).

Os requerimentos estruturais e mecânicos dos suportes para a engenharia de tecidos e os pré-requisitos para a fabricação dos suportes têm sido avaliados. Ênfase especial é dada ao grupo de técnicas com controle computadorizado, fabricação sólida com forma livre (SFF), também conhecida como prototipagem rápida (RP), técnicas que têm significativa vantagem sobre as convencionais, baseadas em métodos de fabricação manual (Leong, 2003).

Para o sucesso do suportes, estes devem apresentar as seguintes características: uma apropriada macroestrutura para promover a proliferação celular e a produção de matriz específica ao tecido; uma alta porosidade na superfície, com tamanho de poro adequado para permitir o crescimento e regeneração tecidual evitando a oclusão do poro; apropriada morfologia de superfície e propriedades físico-químicas para permitir sinalização intracelular e o recrutamento de células; o material ainda deve apresentar apropriada taxa e degradação, com produtos atóxicos para células (Leong, 2003; Yeong *et al*, 2004).

A sinterização seletiva a laser (SLS) é um tipo de prototipagem rápida com emprego de laser de gás carbônico (CO₂) para sinterização seletiva do pó de polímero ou compósito (polímero/cerâmico, metal multifásico) para formar camadas de material. O feixe de laser é direcionado diretamente na camada de pó através de um sistema de esquadrinhamento preciso a laser. As fusões de camadas do material são fixadas umas sobre as outras criando o objeto tridimensional. Durante a fabricação, o objeto é suportado e encaixado pelo pó circunvizinho não processado e precisa ser extraído das camadas de pó após a fabricação. Devido ao pó ser sujeito a uma baixa força de compactação durante a sua

deposição para formar novas camadas, os objetos fabricados via SLS são usualmente porosos. A porosidade dos dispositivos fabricados via SLS pode ser controlada através do ajuste dos parâmetros do processo SLS (Leong, 2003; Williams, 2005), granulometria do material e projeto mecânico dos poros em três dimensões com uma concepção biomimética (Das, 2003; Williams, 2005).

Matrizes colagênicas tri-dimensionais têm sido outra opção para Engenharia de Tecidos. A partir de uma solução contendo colágeno, aplica-se a técnica de freeze-dryer, obtendo-se uma matriz colagênica tri-dimensional, poros interconectados e de tamanho controlado (O'Brien *et al*, 2004). As propriedades dessas matrizes colagênicas podem ser melhoradas adicionando-se compostos de fosfato de cálcio, como a hidroxiapatita (HA), ambos apresentando capacidade osteocondutivas (Al-Munajjed *et al*, 2009).

Uma variedade de biomateriais tem sido utilizados como suportes osteocondutivos em cirurgias ortopédicas, entre eles, destacam-se os suportes inorgânicos como hidroxiapatita (Kong *et al*, 2007), e os polímeros (Sun *et al*, 2004; Shi *et al*, 2007), sendo estes naturais como colágeno ou sintéticos como PHB, PCL e PLGA entre outros (Lucchesi *et al*, 2009; Qian *et al*, 2009). Para melhorar a qualidade de resposta tecidual e celular, uma série de fatores de crescimento, proteínas osteoindutivas e até mesmo DNA, têm sido utilizados, gerando os compósitos.

3.1 POLÍMEROS

Nos últimos 20 anos, a pesquisa no campo dos polímeros, tem buscado o desenvolvimento de novos métodos para se obterem materiais de origem biológica ou sintéticas, altamente compatíveis, capazes de melhorar a interação com os tecidos (Selfton, 2002; Netti *et al*, 2002). Junto à biocompatibilidade, um material utilizado como prótese, dispositivo biomédico ou suporte para engenharia de tecidos deve possuir um complexo arranjo biofísico, estrutural e propriedades biológicas compatíveis com os tecidos. Além disto, um biomaterial ideal para substituir defeitos no tecido, deve ser capaz de reproduzir suas funções biofísicas e biológicas (Netti *et al*, 2002).

É fundamental que o biomaterial mantenha suas propriedades mecânicas, enquanto o novo tecido se regenera, suportando-o de modo adequado. Se por um lado, o material for pouco resistente, pode se obter substitutos inadequados. Por outro lado, se o material tiver resistência muito superior ao do tecido, pode-se causar tensão nas bordas do implante podendo resultar em fragilidade do osso.

Consequentemente, o biomaterial deve resistir aos procedimentos de esterilização e deve ter uma vida útil longa (Seal *et al*, 2001).

A biointegração ideal tem sido esperada em implantes artificiais. Isto implica no fenômeno que ocorre na interface entre o implante e o novo tecido, não induzindo nenhum efeito deletério como resposta inflamatória intensa ou formação de tecido fibroso. Conseqüentemente, boas propriedades para interação celular na superfície do biomaterial são de grande importância para serem utilizados como implantes.

Isto significa que estes materiais devem possuir propriedades e funções semelhantes as do tecido local (Sodhi, 1996). Como todos os materiais implantados no organismo, os polímeros destinados para a regeneração óssea devem ser biocompatíveis, moldáveis ou polimerizados *in situ* para assegurar um bom ajuste na região do defeito. Como características essenciais, devem suportar a adesão, crescimento e diferenciação celular, promover a rápida difusão de metabólitos, catabólitos e os resíduos gerados (Peter *et al*, 1998).

Polímeros biorreabsorvíveis têm sido amplamente utilizados como suportes em engenharia de tecidos para direcionar o crescimento e diferenciação de células específicas (Patrick *et al.*, 1998). O uso de materiais poliméricos em aplicações na área biomédica é um campo de pesquisa relativamente novo, em contínuo crescimento e para qual os profissionais de diversas áreas de conhecimento unem seus esforços para encontrar soluções de problemas previamente sem resposta (Williams, 1987; Hench, 1998).

No Brasil têm-se verificado um crescimento constante no número de trabalhos envolvendo polímeros com aplicações biomédicas. Vários pesquisadores vêm estudando os polímeros biorreabsorvíveis *in vitro* e sua interação *in vivo* (Lucchesi *et al*, 2009; Messias *et al*, 2009; Lucchesi *et al*, 2007; Silva *et al*, 2006; Pereira *et al*, 2006; Rezende *et al*, 2005; Barbanti *et al*, 2004; Marreco *et al*, 2004; Scapin *et al*, 2003; Luciano *et al*, 2002; Zoppi, *et al*, 2001; Ferreira *et al*, 2001; Duek *et al*, 1999), além dos polímeros de origem natural como as matrizes colagênicas (Goissis *et al*, 2003; Sena *et al*, 2004), quitosana, etc.

3.1.1 POLÍMEROS SINTÉTICOS: PHB, PCL e PLGA.

Os ensaios com emprego de polímeros *in vitro* são estratégias fundamentais para o desenvolvimento no campo da engenharia de tecidos (Tormala *et al*, 1993). Dois campos em particular têm prosperado: a associação de fatores osteo-condutivos com o material implantado e a interação de

células tronco osteogênicas com estes materiais. Em ambos os campos, um entendimento dos fenômenos de adesão celular e em particular, das expressões de proteínas envolvidas na adesão de osteoblastos com os biomateriais é de crucial importância na manutenção da estrutura tecidual, cicatrização, resposta imune, e também na interação do tecido com os biomateriais (Anselme, 2002).

Dentre os polímeros sintéticos, o poli(ácido láctico-co-ácido glicólico) (PLGA) está entre alguns dos aprovados para o uso clínico em seres humanos pelo *Food and Drugs Administration* (FDA). Estes polímeros podem ser facilmente processados dentro da configuração desejada e suas propriedades físicas, químicas, mecânicas e sua degradabilidade, podem ser manipuladas para atender uma necessidade em particular.

O PLGA é conhecido por ser um polímero biocompatível e biorreabsorvível se degradando através de uma simples hidrólise em ácido láctico e ácido glicólico, sendo estes produtos eliminados do corpo através de processos metabólicos normais (Göpferich, 1996). Sua taxa de degradação pode variar desde algumas semanas até anos, simplesmente variando o percentual de copolímeros de ácido láctico para glicólico (Mikos *et al*, 1993), uma vez que estudos demonstraram que o poli(ácido láctico) puro apresenta um longo tempo de degradação *in vitro* (Duek *et al*, 1999). A biocompatibilidade do PLGA também tem sido demonstrada em vários tecidos biológicos (Rezende *et al*, 2005). Porém, apesar de pinos do copolímero poli(ácido láctico-co-ácido glicólico) ter mostrado bons resultados, nas interações células/tecido, estes materiais, não possuem as mesmas propriedades mecânicas que o osso e não podem ser implantados por período de tempo prolongado (Seal *et al*, 2001).

Outro polímero que vêm sendo amplamente usado nos últimos 30 anos para a produção de fios de sutura, sistemas de liberação controlada de fármacos e atualmente está sendo explorado para substituição de enxerto de osso, é a Poli(caprolactona) (PCL) (Heath *et al*, 2002). Este polímero chamou a atenção devido a sua alta biodegradabilidade, biocompatibilidade e boas propriedades mecânicas (Cretu *et al*, 2003). As blendas poliméricas de PCL, assim denominadas por serem obtidas da mistura física de dois ou mais tipos de polímeros, vem sendo investigadas, pois podem melhorar as propriedades físicas e mecânicas quando comparadas aos polímeros puros (Park *et al*, 1992).

Algumas blendas, incluindo polímero sintético e/ou polímero biológico (por exemplo, colágeno), têm sido preparadas com a finalidade de melhorar a biocompatibilidade do primeiro (Giusti *et al*, 1994). Em geral, a preparação de blendas tem sido realizada com o objetivo principal de controlar a velocidade de degradação de polímeros biodegradáveis. O tempo de degradação de um polímero biodegradável para aplicações biomédicas pode variar de meses a anos, dependendo dos balanços

hidrofílico/hidrofóbico e amorfo/cristalino. Estes balanços podem ser controlados em função da quantidade e do tipo de constituintes usados na preparação de uma blenda (Park *et al*, 1992).

Dentre as vantagens dos polímeros biorreabsorvíveis, dependendo dos seus componentes, podem-se obter moldes com rigidez suficiente, oferecendo suporte mecânico por um período de tempo adequado e permitindo a consolidação óssea, até que se inicie o processo de degradação do molde. Dentre os polímeros que apresentam vantagens, como termoplasticidade, biocompatibilidade e biodegradabilidade, destaca-se o poli (3-hidroxibutirato) (PHB) e seus copolímeros, poli(hidroxibutirato-co-hidroxivalerato) (PHBV) (Ferreira, 2001). O PHB e seus copolímeros randômicos contendo unidades do 3-hidroxibutirato e 3-hidroxivalerato (FIG.1) têm sido produzidos a partir de bactérias *Alcaligenes eutrophus* [Hankermeyer & Tjeerdema, 1999].

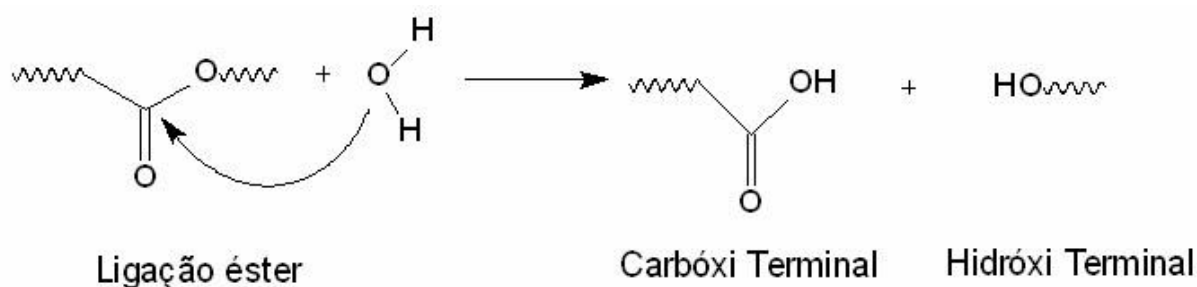


Figura 1 – Na presença de água, as ligações ésteres presentes nestes polímeros, são rompidas, ocasionando a quebra da cadeia, gerando em uma das porções carbóxi terminal e hidróxi terminal.

O PHB é um polímero altamente cristalino, tendo boa biocompatibilidade, porque seus derivados são produtos do metabolismo celular, estando presentes no sangue e tecidos. Entretanto, devido ao fato de ser quebradiço e ter baixa resistência mecânica, sua aplicação é limitada. Embora exista uma diversidade de polímeros conhecidos, poucos têm sido investigados como o copolímero polihidroxibutirato-co-hidroxivalerato (PHBV) (Volova *et al*, 2003).

No entanto os poliésteres biorreabsorvíveis como o PHB e seus derivados, são pobres no que diz respeito à interação célula-matriz. Os PHBs são biomateriais hidrofóbicos (Ferreira *et al*, 2002; Hasirci *et*

al, 2003) apresentando ângulo de contato por volta de 80° , porém a maior parte das células exibem boa adesão celular sobre superfície com ângulo de contato abaixo de 60° (Yamaguchi *et al*, 2004). Ferreira *et al*. (2002), relatam que o PHBV é polímero com características hidrofóbicas, apresentando lenta adesão celular. A adesão celular é quesito essencial para tal finalidade e muitas são as opções disponíveis na engenharia de materiais para incrementar a adesão tais como o recobrimento com polímeros da matriz, enxertos na superfície e o tratamento por plasma (van Kooten, 2004).

3.2 TRATAMENTO DE SUPERFÍCIES

Tem-se demonstrado que modificações químicas na superfície dos materiais têm um importante papel na adesão e crescimento, proliferação e diferenciação celular além de propiciar que células diferenciadas possam depositar e remodelar a matriz extracelular depositada (Dalby *et al*, 2002). Isto indica que se deve dar atenção tanto à estrutura 3D polimérica e também ao tratamento de suas superfícies e/ou incorporar elementos bioativos capazes de influenciar o comportamento celular (Seal *et al*, 2001).

Dentre os tratamentos de superfície, aquele realizado com plasma é particularmente versátil pois a modificação é restrita ao topo da superfície, sem comprometer as propriedades do material como um todo (Chan, 1993). O tratamento por plasma pode ser usado para modificar a superfície do polímero de maneira não específica por mudanças na molhabilidade ou mais especificamente, introduzindo-se uma variedade de grupos funcionais dependendo do tipo de gás utilizado (Keen *et al*, 2006). Além disso, o tratamento por plasma torna a superfície estéril, podendo ser aplicado em dispositivos biomédicos, instrumentos cirúrgicos, engenharia de tecidos e aplicações clínicas. Estudos mostram que o tratamento por plasma pode destruir microorganismos, matando bactérias e vírus, esterilizando o material, representando mais uma vantagem (Chu *et al*, 2002).

Um grande número de pesquisadores tem investigado o tratamento por plasma para a modificação da superfície de polímeros como o PHBV. Kose e colaboradores (2003) modificaram as características químicas e físicas da superfície por plasma de oxigênio e verificaram que esse tratamento aumentou a afinidade das células, as quais cresceram sobre o material, proliferaram e se diferenciaram, dando início a produção de uma matriz. Langowski & Uhrich, (2005), estudaram a modificação do PHBV por plasma de oxigênio, resultando num aumento da hidrofiliabilidade do material. Wan *et al*, (2005), melhoraram a hidrofiliabilidade da superfície do PHBV através da modificação por plasma de oxigênio,

mostrando que o crescimento das células mesenquimais foi melhor na superfície tratada. Keen *et al*, 2006, utilizaram plasma de amônia e aminólise etilenodiamina, observando que o tratamento por plasma acrescentou uma quantidade maior de grupos amina. Lucchesi *et al* (2008) trataram a superfície de membranas de PHBV com plasma dos gases oxigênio e nitrogênio, demonstrando uma alteração no comportamento de adesão de células Vero.

II. OBJETIVOS

- 1) Avaliar o comportamento de osteoblasto-like proveniente de calota craniana de ratos *Wistar* sobre suportes de PCL/PLGA.
- 2) Avaliar o comportamento *in vitro* de osteoblastos-like provenientes de calota craniana de coelhos *New Zealand* sobre arcabouços de PHB tri-dimensionais obtidos por prototipagem rápida e modificados por plasma de nitrogênio e oxigenio.
- 3) Avaliar a interação polímero/célula/tecido *in vivo* após implante de arcabouços de PHB tri-dimensionais obtidos por prototipagem rápida e modificados por plasma de nitrogênio e oxigenio, com o cultivo prévio de osteoblasto-like provenientes de calota craniana de coelhos *New Zealand*.

CAPÍTULO 2

Cell Culture on PCL/PLGA Blends

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ABSTRACT: Bioresorbable polymers have been studied as support for cell culture in the tissue engineering area. Osteoblastic cells were cultivated on poly(ϵ -caprolactone), poly(lactic acid-co-glycolic acid), and (70/30), (50/50), and (30/70) blends. Cytotoxicity and cell adhesion assays and scanning electronic microscopy studies were described. The cells presented significant growth on the blends,

showing no cytotoxic response. Results indicated that these blends are promising as devices for bone tissue applications. © 2009 Wiley Periodicals, Inc. *J Appl Polym Sci* 000: 000–000, 2009

Key words: biomaterials; poly(ϵ -caprolactone); poly(lactic acid-co-glycolic acid); cell adhesion; osteoblast

INTRODUCTION

The treatment of bone defects remains a critical challenge in reconstructive surgery. Currently, autograft bone provides the best available solution for repairing bone defects caused by tumors and serious trauma, but even this approach has limitations. Patients frequently suffer from donor-site morbidity, and there is only limited supply of bone available for grafting. Bone tissue engineering has the potential to solve many of the current clinical challenges, which require large amounts of bone in specific sizes and shapes.¹ Besides, tissue engineering of bone requires cellular components, extracellular matrices, and scaffolds and growth and differentiation factors.

Osteoconductive matrices used as scaffold should satisfy certain requirements. They should be designed to allow diffusion of the nutrients of the transplanted cells and guide cell organization, attachment, and migration.² They are prepared from biodegradable materials of natural origin like collagen, gelatin, hyaluronic acid, and biodegradable polymers such as poly(hydroxybutyric-co-hydroxyvaleric acid) (PHBV) and also from synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic

acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), etc.^{2,3}

As all materials implanted in the organism, the polymers destined for bone regeneration should be biocompatible, molded, or polymerized *in situ* to provide good adjustment to the defect area. As essential characteristics, they should support adhesion, growth, and cellular differentiation, and allow fast diffusion of metabolites and generated residues.⁴

The *in vitro* assays with polymers are fundamental strategies for the development of the tissue engineering field.⁵ In this regard, two fields have progressed: the association of osteoconductive factors with the implanted material and the interaction of sources of osteogenic cells with these materials. In both fields, the understanding of the cellular adhesion phenomena and the expressions of proteins involved in osteoblast adhesion to biomaterials is of crucial importance in the maintenance of tissue structure, cicatrization, immune response, and also in the interaction of tissue and biomaterials.⁶

The cells interaction with biomaterials is an important feature of *in vitro* biocompatibility and cytotoxicity studies. The main parameters of cell-biomaterial interaction are cell adhesion and spreading, and in many studies, a clear distinction is made between materials supporting or hindering adhesion.⁷

Bioresorbable polymers have been receiving special attention as supports for cell culture being a possible alternative for treatment of lesions and tissue losses.⁸

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Among polymers, PLGA is one of the synthetic polymers approved for clinical use in humans beings. This polymer can be processed easily, and their physical properties, chemistry, mechanics, and degradability can be manipulated to assist a specific need.

PLGA has been known as a biocompatible and biodegradable polymer that undergoes degradation through simple hydrolysis into lactic acid and glycolic acid, which are eliminated by the body through normal metabolic processes.⁹ Its degradation rate can vary from some weeks up to years, by simply varying the percentage of copolymers,¹⁰ whereas studies demonstrated that pure PLA presents a long degradation time *in vitro*.¹¹ The biocompatibility of PLGA has also been demonstrated in several biological tissues.¹² Notwithstanding the good results obtained with PLGA copolymer pins in cell/tissue interactions, these materials do not possess the same mechanical properties as bone and should not be implanted for long periods.³

Another polymer that has been thoroughly used in the last 30 years to produce bioresorbable sutures, drug delivery systems, and is now being explored to substitute bone graft, is poly(ϵ -caprolactone) (PCL).¹³ This biodegradable polymer was noticed due to its high biodegradability, biocompatibility, and good mechanical properties.¹⁴

The polymeric blends are obtained by physically mixing two or more types of polymers, which have been previously investigated, and their physical and mechanical properties can be improved when compared with pure polymer.¹⁵ Some blends, which include the mixture of a synthetic polymer and a biological polymer (for instance, collagen), have been prepared with the purpose of improving the biocompatibility of the synthetic polymer.¹⁶ In general, the preparation of blends has been carried out with the main objective of controlling the degradation speed of the biodegradable polymer. The degradation time of material constituted of biodegradable polymer for biomedical applications can vary from months to years, depending on their hydrophilic/hydrophobic amorphous/crystalline balance. This balance can be controlled by the amount and type of polymer used in the preparation of blends.¹⁵

In this study, we have prepared PCL and PLGA blends with the proportions of 70/30, 50/50, and 30/70, respectively, to obtain a support that is compatible with the bone tissue and an acceptable degradation time. This work aimed at evaluating the interaction between osteoblastic cells and membranes by carrying out cytotoxicity and cell adhesion assays and cell-cell and cell-membrane interaction studies by scanning electronic microscopy (SEM).

METHODS

Preparation of blends

Membranes were prepared using poly(*D,L*-lactic acid-co-glycolic acid) (50/50) (PLGA50) (M_w 65,000 g/mol) supplied by Purac (Groningen, The Netherlands) and PCL (M_w 100,000 g/mol) supplied by Sigma (St. Louis, EUA) at PCL/PLGA50 concentrations of 70/30, 50/50, and 30/70 (w/w). Initially, a 10% (w/v) solution of polymer dissolved in methylene chloride (Merck KgaA, Darmstadt, Germany) was used to prepare the samples at room temperature. Then a polymer methylene chloride solution containing 50% (w/v) trisodium citrate ($C_6H_5Na_3O_7 \cdot 5H_2O$) was prepared (Merck, Darmstadt, Germany). Prior to use, the citrate salt was sieved to give particles of 180–250 μ m in diameter. The blends were cast on glass plates and dried for ~24 h in a closed chamber with a constant flow of dry filtered air. After casting, the salt was removed by immersion in distilled water for 24 h followed by rinsing in ethanol for 2 h. The samples were vacuum-dried and stored in a desiccator for 5 days to ensure the total removal of solvent.

Cell isolation, seeding, and culture

Osteoblastic cells were obtained from 10 calvaria of young male adult (20 days old, 150–170 g) Wistar rats (*Rattus norvegicus*), from CEMIB (Centro Multidisciplinar para Investigação Biológica, Campinas, SP, Brazil). Following euthanasia by cervical displacement, the calvaria were aseptically excised, cleaned with a soft tissue, and washed in Dulbecco's modified eagle's medium (DMEM) (Nutricell-Nutrientes Celulares, Campinas, SP, Brazil) containing 0.2M L-glutamine (Sigma Chemical, St. Louis, MO) and 150 μ g/mL gentamicin sulfate (Sigma). This concentration of antibiotics was three times the normal amount used in cell culture and was used as a precautionary measure to avoid contamination during harvest. In sterile atmosphere, the calvaria were fragmented and submitted to enzymatic digestion for cellular isolation in DMEM medium and 1 mg/mL of collagenase type 1A (Sigma Chemical) during 2 h at 37°C. After this procedure, it was submitted to a three-stage centrifugation for 10 min at 240 g force, and subsequently resuspended in DMEM medium containing 10% of fetal bovine serum (FBS) and antibiotic. After centrifugation, the pellet was resuspended in DMEM medium and cellular viability was quantified by staining with Trip-pan Blue vital stain (Sigma) in a Neubauer camera. Osteoblasts were seeded at a density of ~10⁵ cells/mL in culture flasks (TPP-Techno Plastic Products, Trasadingen, Switzerland) containing DMEM medium, supplemented with 10% FBS and 25 μ g/mL

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gentamicin, 10 mM β -glycerol phosphate (Sigma), and 50 mg/mL L-ascorbic acid (Sigma) containing 10 nM dexamethasone (Sigma) to promote cells with osteoblastic phenotype, which were used in experiments after the third subculture. The flasks were incubated in a humidified incubator at 37°C (5% CO₂/balanced air). When cells reached 80% of confluence, cell cultures were considered to have reached full growth and the cells were enzymatically lifted from the flask by using a 625 mg/mL trypsin solution and monitored daily with an inverted microscope, Eclipses TS 100 (Nikon, Tokyo, Japan). Statistical differences ($P < 0.05$) were determined using variance analysis (ANOVA) followed by Tukey's test for multiple comparisons.

Cell adhesion and cytotoxicity assays

Identification of cell adhesion and late cytotoxicity on blends were carried out by performing the MTT assay, a modification of Mosmann¹⁷ method, which was used for both cell adhesion and direct cell cytotoxicity assays.¹⁸ Previously, the sterilized blends ($n = 6$) were placed in a 96-well plate (Corning) with 100 μ L of culture medium and incubated at 37°C for 24 h. After incubation, 2×10^5 cells/mL in 100 μ L DMEN medium supplemented with 10% FBS were added to the wells containing the membranes. The cells were cultured for 2 and 24 h to allow cell adhesion and to conduct direct cell cytotoxicity assays, respectively. After the cells were washed twice with 0.1M phosphate-buffered saline (PBS), pH 7.4, at 37°C and incubated with 100 μ L DMEN medium, a MTT assay mixture [10 μ L per well, containing 5mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Sigma] was added to each well and incubated for 4 h at 37°C. After 4 h, 100 μ L of dimethyl sulfoxide (DMSO, Sigma) and 25 μ L of glycine/Sorensen buffered solution replaced the assay mixture in each well to dissolve the formazan crystals, according to Santos et al.¹⁸ Absorbance was quantified by a spectrophotometer at 540 nm, using a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). MTT is a colorless tetrazolium salt that forms a dark compound when oxidized by mitochondria, which is detected by spectrophotometer. For the cytotoxicity assay, latex membranes were used as positive control, and the culture plate (polystyrene) was used as negative control. For cell adhesion evaluation, Teflon dishes were used as negative control, and the culture plate itself was used as positive control. The membranes were sterilized by UV irradiation for 30 min. Absorbance of all experimental conditions was also read. Comparison of continuous variables for all groups was done with ANOVA.

When a significant difference was found ($P < 0.05$), the groups were compared using Tukey's test.

Scanning electron microscopy

The blends were sterilized by UV irradiation for 30 min and placed in 96-well plates. Approximately 2×10^5 cells/mL osteoblastic cells in DMEN medium supplemented with 10% FBS were seeded in each well containing the membranes and cultured at 37°C. After 6, 24, and 168 h, the samples were fixed in a fixative solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.06% picric acid, 1% tannic acid in 0.1M cacodylate buffer, and the same volume of DMEN medium for 1 h at room temperature (RT), washed in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide in water for 1 h at RT in the dark, washed in water, dehydrated with ethanol, critical point dried (Balzers CDT 030), and coated with gold in a sputter coater (Balzers CDT 050). The coated specimens were observed with a JEOL 5800 SEM.

RESULTS AND DISCUSSION

After enzymatic digestion, the adherence of osteoblastic cells to the culture flask TPP (Techno Plastic Products, Trasadingen, Switzerland) was delayed in ~ 24 h. Initially, they presented round morphology, and after 48 h, the cultivated cells already presented dispersed proliferation and cytoplasmatic projections. When in confluence, the cells acquired a cuboid morphology, which is typical of osteoblastic cells in culture.⁶

Cytotoxicity analyses of blends

The results expressed in absorbance obtained after the samples were read in the microplate reader (540 nm) are presented in Figure 1. The variance analysis (ANOVA) showed significant differences between the positive, negative controls, and the samples studied ($P < 0.05$). Through Tukey's test, it was shown that the blend composition 70/30 presented a better result when compared with the blend 50/50 ($P < 0.05$). All blends assayed showed absorbance indexes higher than the negative control ($P < 0.05$), demonstrating that polymeric materials or their 70/30, 50/50, and 30/70 compositions do not present cytotoxicity indexes. All the tests developed are in agreement with standardization norms of ISO and evaluation of biomedical devices.^{19,20}

The yellow stain of the reagent MTT is converted inside the mitochondria into a dark blue stain, formazan (translated by the largest absorption of MTT), which demonstrates that cellular mitochondrial activity is present. Although toxic substances do not

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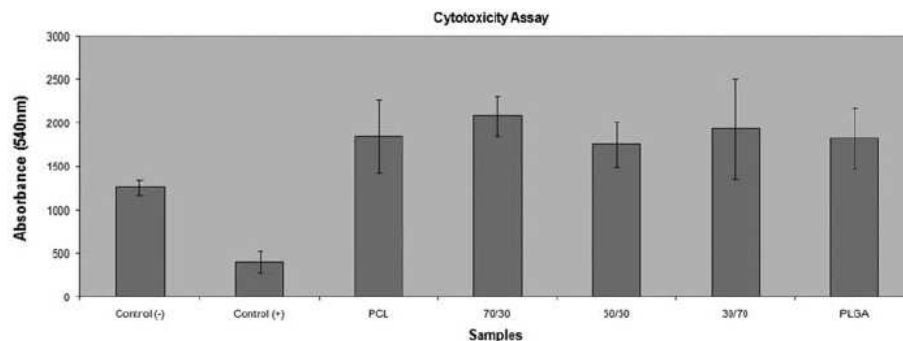


Figure 1 Cytotoxicity assay results obtained after 24 h of cell culture (negative control = culture plate; positive control = latex membranes embedded in phenol; $n = 6$).

act in a specific cellular level, they can affect their functions,²¹ thus early determination of mitochondrial activity in terms of cellular sensitivity, in our opinion, was enough to evaluate cellular viability with respect to subsequent proliferation. Absence of cytotoxicity does not check on the material biocompatibility; yet the determination of cytotoxic potential is an important issue in standard tests such as ISO.¹⁹

Our results are in agreement with the literature. Rezende et al.¹² used PLGA membranes as skin curative in Wistar rats and did not observe cytotoxic effects on tissues. However, the rate of PLGA degradation should be evaluated on the short and long terms, once this copolymer possesses as characteristic a high degradation rate depending on its composition.²² The degradation products of α -hydroxy acids alter the pH and could cause cytotoxic effects, in which degradation is greater than "rebalance" of local pH.²³ According to Seal et al.,³ this degradable material serves as support for cell proliferation and secretion of the extracellular matrix. Currently, other products have been studied for specific applications in skin reconstruction, including fibroblasts and keratinocytes culture in PLGA microspheres and other materials. Kweon et al.²⁴ reported that PCL also allowed the growth of osteoblastic cells on samples. In this report, they have shown that the cells were capable of proliferating on different PCL scaffolds. Other reports show that PCL membranes and blends of PCL-Nafion do not produce lethal effects to osteoblastic human cells.²⁵ The compositions assessed in our study are physical mixtures of PLGA and PCL, and it was already anticipated that the blends would not result in toxicity. The cytotoxicity assays of these polymers indicate good tolerance of osteoblastic cells and absence of toxicity. However, confirming that a material is not toxic to cells does not mean that it will be a good substratum. The interaction of the biomaterial with the cells through cellular adhesion is also necessary.

The first step for the selection of a material to be used as implant in humans is to evaluate the toxicity and damages that it might cause to tissues. The great majority of biomaterial compositions developed is rejected in this stage because of their toxic effects that can lead to cell death or promote strong tissue reaction, such as inflammatory processes or even tissue necrosis. Thus, toxicity evaluation of materials is a problem to be solved.

Cell adhesion analysis of blends

The results obtained through cell adhesion assays are shown in Figure 2. The statistical analysis of the materials showed significantly superior indexes to the negative control (Teflon disks) ($P < 0.05$). The blend composition 30/70 presented indexes similar to the positive control (polystyrene plate) ($P < 0.05$). There were differences between the 70/30 and 30/70 compositions, and the blend 30/70 presented a better absorbance index than 70/30 composition ($P < 0.05$).

All the evaluated membranes, independent of their composition, presented adhesion capacity; however, the ultrastructural differences must be emphasized. According to Vogler and Bussian,²⁶ the rate of initial adhesion in plastic substratum is a good parameter to characterize the compatibility of materials. Despite the slow cell adhesion in synthetic materials described in the literature,²⁷ in our studies, the polymers showed satisfactory results in the analysis of cell adhesion independently of the composition used. It is also described in the literature that the surface of polyesters is hydrophobic²³ and that most of the cells adhere more easily to hydrophilic surfaces.²⁸ Nevertheless, van Kooten et al.²⁹ mentioned that only wettability is not a decisive factor to improve adhesion and cellular spread. In our studies, cell adhesion was not apparently affected by surface hydrophobicity, but other factors might have interfered in this process, once the adhesion between

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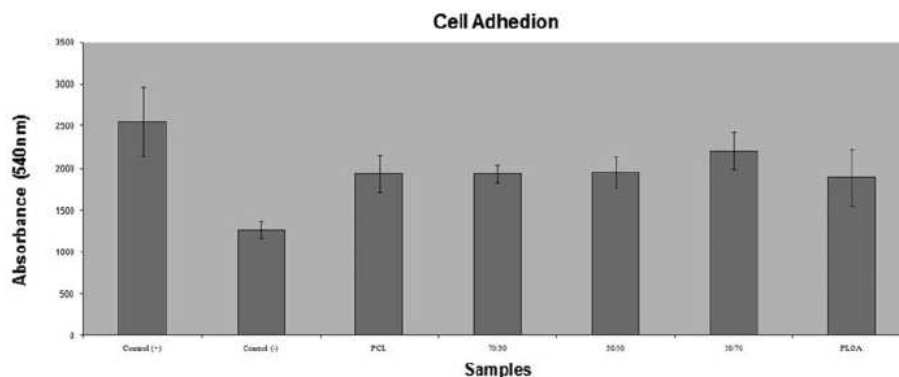


Figure 2 Cell adhesion assay results obtained after 2 h of cell culture (positive controls = culture plate; negative control = teflon dishes; $n = 6$).

osteoblasts and the surface of synthetic materials depends on the adhesion of molecules, specific membrane receptors, besides factors such as interaction of electric charges, hydrogen bonds, electrostatic and characteristic topographical forces of the polymer,³⁰ and the properties of the surface of the material that have a crucial role in cell adhesion behavior. Similar results were found by other researchers who have cultivated osteoblastic human cells in PLLA and PLGA membranes and reported that osteoblasts have a quite slow pattern of adhesion on PLLA substrate, but PLGA was shown to be a more adhesive substratum to this cellular type.⁸ PCL was also demonstrated to be a polymer that is capable of allowing not only the cell adhesion but also its proliferation.²⁵

Tang and Hunt³¹ carried out studies with PCL and PLGA in the compositions 90/10, 80/20, and 70/30. The blends showed a rough and porous morphology after degradation in culture medium mainly due to PLGA degradation, and the 70/30 blend presented the most porous structures. This study is in agreement with our results that reported that the 30/70 blend containing more PLGA is more porous than the other blends, which is probably the reason it provides better structures to the adherence of osteoblasts.

Ultrastructural analysis of cell adhesion

Through SEM, it was observed that cells adhered to all the samples studied, allowing osteoblasts to establish on surfaces and start cell polarization for the extension of cytoplasmatic projections; however, morphologic alterations were observed depending on the composition of blends [Fig. 3(A,B)]. These results are in agreement with the literature that describes that once the adhesion on substratum is established, the cells start to respond to it. Usually, in response to the "signs" originated by the substratum, cells alter their morphology, growth and proliferation, differentiation pattern, and behavior or even several of the parameters mentioned herein, simultaneously.²⁷

SEM analysis allowed visual confirmation of the good polymer/osteoblastic cell interaction. After 6 h of culture, the osteoblastic cells adhered to the substrates presented prolonged morphology with ramifications of cytoplasmatic projections in most of the samples, and the presence of cells with round morphology on 100/0 and compositions with higher amounts of PCL (70/30) was observed. It was not possible to observe significant differences in the number of cells on the different membranes; however, the blend 0/100 showed the greatest amount of particulate material on cellular surfaces.

After 24 h of culture, it was observed that osteoblastic cells cultured on PLGA blends and its compositions, 30/70 and 50/50 [Fig. 3(C-E)], presented more prolonged morphology, whereas on PCL blends and composition 70/30 [Fig. 3(A,B)], osteoblastic cells were shown to be more dispersed with greater amounts of fine filopodes, besides large lamellipodes occupying a larger area of the samples in relation to the other compositions. In all blends, the presence of structures similar to microvillousities was noticed. The presence of microvillousities in cellular surfaces evidences that the cell maintained its biosynthetic capacity. Moreover, cell adhesion to the substratum not only stimulates proliferation but also its biosynthetic activity.³² A greater amount of cells was observed in 70/30 blends and pure PCL. These cells presented cytoplasmatic projections extended from other osteoblastic cells showing a sensitive change in its form, the cells assumed a flat morphology, some times prolonged other times round on the substratum. In fact, if cells are very flat, it is an indication that a great cellular interaction with the substratum has occurred.

It was possible to observe that the osteoblastic cells present in all samples showed metabolic

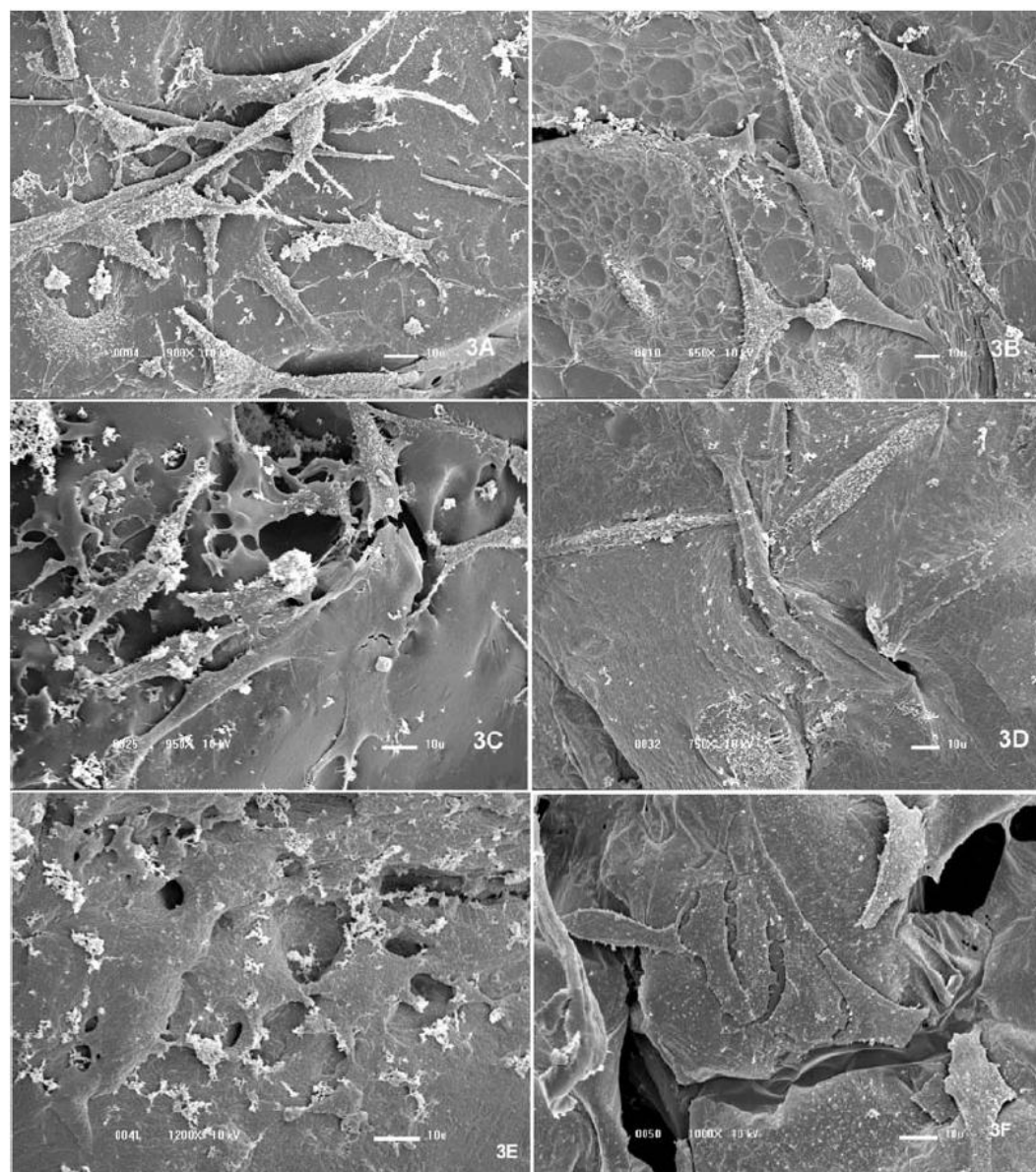


Figure 3 Scanning electronic microscopy of osteoblastic cells cultivated on the different substrates. (A) After 24 h of culture: (A) 100/0 blend; (B) 70/30 blend; (C) 50/50 blend; (D) 30/70 blend; (E) 0/100 blend; (F) Note: The organic material on cell surfaces and confluence was observed in all compositions after 168 h in culture.

activity because of the emergence of vesicles, which suggests deposit of organic material on the polymeric substrates. The interaction of cells with porous PCL and PLGA and their compositions is high, given that they are capable of growing and proliferating on those blends. Ciapetti et al.³³ studied osteoblastic cells growing on PCL macro and micro-porous supports with the addition of hydroxyapatite and noticed that in both supports the cells were interlinked by cytoplasmic prolongations. In addition, they observed signs of bone differentiation such as the production of alkaline phosphatase and

deposition of calcium salts on the substrate after 4 weeks of culture.

After 168 h of culture, a greater density of cells on the blends was noticed [Fig. 3(F)], i.e., the cells covered the samples in monolayer and presented cuboid morphology which is characteristic of osteoblastic cells when *in vitro* confluence.⁶ These cells were also observed to grow very close to each other suggesting an intimate connection among them [Fig. 3(A–D)]. In this condition, in some areas, the delimitation of the cells and their respective cellular limits is difficult to be noticed [Fig. 3(B,C)]. The presence

of organic material on cell surfaces was shown to be evident in membranes, except on PCL blends and 70/30 composition, whereas the amount of organic material seemed to be greater on PLGA blends [Fig. 3(E)]. Briefly, PCL and 70/30 blend with a higher proportion of PCL presented initially higher amounts of organic material, which was inverted after 168 h of culture, whereas larger amount of organic material was observed on PLGA blends and on 30/70 composition in the same period of culture. This is probably due to the fact that cells are in different maturation stages depending on the polymeric composition studied.

The cells also seemed to have preference for macro and micropores formed when they were formed with sodium citrate lixiviation. They adhered to the innerside of macropores and also cast out cytoplasmatic projections over them. Whenever cells adhered inside micropores, a similar behavior was noticed; however, there were instances they were avoided. According to Berry et al.,³⁴ the cells are sensitive to topography alterations, which influences cellular mobility and possibly cell proliferation. In studies in which the PCL surface was modified by the alteration of its nanotopography, an increase in the adhesion rate of osteoblastic cells was shown.³² However, according to Anselme,⁶ through the melting method employed to obtain pores with sodium citrate salt, the interconnection that is necessary for cell-cell contact among the pores cannot be preserved.

Our results are in agreement with similar studies with PCL and PLGA blends developed by Tang et al.,³⁵ in which they reported that osteoblasts adhered and spread throughout all blends that presented polygonal shaped cells.

SEM results indicated that PCL, PLGA polymers, or their blends are promising for the development of devices for bone tissue engineering, as they bring together other features, for instance, mechanical properties.

CONCLUSION

The results obtained demonstrated that all blends presented characteristics that are desirable in devices for bone tissue applications, such as adhesion, growth and cellular proliferation, and absence of cytotoxic effects.

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CAPÍTULO 3

RAPID PROTOTYPING OF *POLY(HYDROXYBUTYRATE)* SCAFFOLDS TREATED BY PLASMA TO SUPPORT OSTEOBLASTIC CELLS FROM RABBIT CALVARIA.

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ABSTRACT

The complexity of bone into three dimensional (3D) in vitro models remains one of the most important challenges in the field of the tissue engineering. The models should faithfully mimic these tissues, resembling their organization, their mechanical properties and their physiological response to different stimuli. Indeed, the development and the optimization of novel culture systems may be necessary to face the next questions of bone physiology. For the new therapeutic strategy, it is indispensable to provide cells with a local environment that enhances and regulates their proliferation and differentiation for cell-based tissue regeneration. Biomaterial technology plays an important role in the creation of this cell environment. In this work three dimensional PHB scaffolds were obtained by rapid prototyping. Cells obtained from rabbit calvaria were culture on the scaffold 3D PHB scaffolds. The cells response were evaluated by MTT test, Sirius Red and SEM. The PHB showed good cells viability with no cytotoxic effects with or without treatment. The physical-chemical modifications on the samples caused by nitrogen plasma treatment improve cell attachment. The samples allowed improve the synthesis of type I collagen as one of the characteristics of osteoblast cell. The polymer PHB meets the requirements to act as scaffold for Tissue Engineering by being biocompatible, support growth and cell organization and by having its degradation products non-toxics to the cells. The selective laser sintering has been a good technical to obtain 3D scaffolds for Tissue Engineering.

Key-words: *Poly(hydroxybutyrate) (PHB), Tissue engineering, Osteoblast.*

INTRODUCTION

Bone fracture treatment is a widespread clinical practice and about 50% of these treatments need surgical intervention (PRAEMER, 1992). Along with the surgery, problems for the restoration of the mechanical integrity of the tissue and the establishment of the continuity of skeleton may appear (MA, 2001). Tissue engineering, however, appears as the most viable alternative for the restoration of the skeleton continuity and the bone fixation (FLEMING, 2000).

The concept of Tissue Engineering is based on a cell/polymer system in which a bioresorbable polymer acts, for a period of time, as a substrate or scaffold to promote tissue formation (PETER, 1998). Most of bioresorbable implants are of the class of aliphatic polyesters derived from the α -hydroxy acids family (NEBE, 2001). Other family that is widely studied is that of polyhydroxyalkanoates, which are polyesters accumulated by bacteria as a store of carbon and energy (REDDY, 2003).

Poly(3-polyhidrxxybutyrate), PHB, is an example of polyhydroxyalkanoates polyesters family, being produced by bacterial fermentation. When deposited in the environment this biomaterial suffers the action of aerobic microorganisms, being completely degraded into CO₂ and H₂O. PHB is a bioresorbable polymer, therefore it is degraded by hydrolyses, generating products that are absorbed by the organism (KOSE *et al.*, 2003). It was first discovered in *Bacillus megaterium*, in 1925, by Lemoigne at the Institute Pasteur of Paris. Since then, investigations have revealed its occurrence in a large number of bacteria and algae (BARHAM, 1990).

Due the properties as biodegradability, biocompatibility, optical activity, piezoelectricity and non-toxicity, PHB has been studied for a variety of medical applications such as controlled drug-release system (POUTON & AKHTAR, 1996), surgical sutures, wound healing, in orthopedics (HOCKING, 1994) and as pericardium substitute (LANGER & VACANTI, 1995).

In last years it has been studied in Tissue Engineering in the form of porous substrate (DOYLE *et al.*, 1996; HAZARI *et al.*, 1999). The polymer PHB meets the requirements to act as scaffold for Tissue Engineering by being biocompatible, support growth and cell organization and by having its degradation products non-toxics to the cells (ZHIJIANG, 2006).

There is an advantage to use this polymer, it is coming from the Brazilian industry, PHB Industrial, which produces the polymer to about U\$ 5.00/Kg in installation of 50t/year. That cost is significantly lower than the cost of the material from the international competitor, because there is technology in the production of derived substances from sugar-cane, raw material abundant in Brazil. Moreover, the cost of the polymer is also lower when compared to other bioresorbable polymers with

applications in the biomedical field, such as poly(L-lactic acid), poly(glycolic acid) and poly(*p*-dioxanone).

Shishatskaya and Volova (2004) studied the cytotoxic effects of PHB and PHBV films *in vitro*. The authors showed that the materials studied have no cytotoxic effects on fibroblastic NIH 3T3 cells. The polymer was also studied *in vivo*. Other similar work developed by the group, surgical sutures made of PHB and PHBV were implanted in the muscles of *Wistar* rats, obtaining inflammatory responses similar to the one obtained with silk yarn and lower than the response obtained with catgut suture (SHISHATSKAYA *et al.*, 2004). Malm *et al.* (1992) used PHB to correct defects of the atrial septum in calf. Twelve months after the implant, no polymer material was identified, but by polarized light microscopy, small particles of the polymer with a persistent foreign body reaction could be observed.

Most studies involving the PHAs as biomedical devices give attention to the copolymer PHBV. Due to PHB high level of cristalinity, there are difficulties in the process of obtaining devices with this polymer. However, by the technique employed in this work, the rapid prototyping by Selecting Laser Sintering (SLS) showed better results with PHB than with its copolymer poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), which led us to use the PHB in our studies.

The characteristics of Tissue Engineering scaffolds are major concerns in the quest to fabricate ideal scaffolds. The solid freeform (SFF), also known as rapid prototyping, technologies which are fast becoming the techniques of choice for scaffold fabrication with the potential to overcome the limitations of conventional manual-based fabrication techniques. SFF-fabricated scaffolds have been found to be able to address most, if not all the macro- and microarchitectural requirements for TE applications as pore size, porosity and pore interconnectivity (Leong *et al.*, 2003).

Another issue is to establish of a stable interface between a biomaterial and the host environment is governed by both the material surface properties and the type and state of the biological tissue. The surface properties can be altered to suit a specific biomedical application, once the most of the polymers have hydrophobic surfaces, and most of the cells prefer to attach on hydrophilic surfaces (Yamaguchi *et al.*, 2004).

Plasma treatment can be used to modify a polymer surface in a nonspecific manner by changing the wettability or in a more specific manner by introducing a variety of functional groups depending on the type of gas used.

In the present study, we treated tridimensional PHB scaffolds, obtaining by rapid prototyping, by oxygen and nitrogen plasma with the aim to evaluate the behavior of cell adhesion, proliferation and differentiation of osteoblasts cells from rabbit calvaria.

MATERIAL AND METHODS

Scaffolds Preparation

Three-dimensional PHB (Biocycle[®], PHB Industrial S/A, Brazil) scaffolds were designed using a 3-D solid modeling software, exported to a Sinterstation 2000TM machine (3D Systems, Valencia, CA), and constructed by Selective Laser Sintering processing (SLS). This PHB has a particle size distribution in the 10-100 μm range. The circular scaffolds were produced with 6mm of diameter, 2mm of thickness and 200-500 μm porous size. The samples were storage under vacuum until plasma treatment.

Plasma treatment

The plasma treatment was performed using a radio frequency Anatech LTD Ashing plasma apparatus. The plasma conditions employed in the treatment was PHB untreated; PHB treated by N₂, 50W, 150mTorr and 600s; PHB treated by O₂, 100W, 300mTorr and 600s. The values of power, pressure, and time were chosen based on preliminary studies with PHBV (Lucchesi *et al.*, 2008).

Cell isolation, seeding and culture

Osteoblastic cells were obtained from 10 calvaria of young male adult (15 days old, 100–150 g) *New Zealand* rabbits, from university's central animal house (PUC/SP-CCMB, Centro de Ciências Médicas e Biológicas, Sorocaba, SP, Brazil). Following euthanasia by cervical displacement, the calvaria were aseptically excised, cleaned with a soft tissue, and washed in Dulbecco's modified Eagle's medium (DMEM) (Nutricell - Nutrientes Celulares, Campinas, SP, Brazil) containing 125 $\mu\text{g/mL}$ gentamicin sulfate and amphotericin B (Sigma). This concentration of antibiotics was 5 times the normal amount used in cell culture and was used as a precautionary measure to avoid contamination during harvest. In sterile atmosphere, the calvaria were fragmented and submitted to enzymatic digestion for cellular isolation with 1 mg/mL collagenase type 1A in DMEM (Sigma Chemical, St. Louis, MO) during 2 hours at 37°C. After this procedure, it was submitted to a three-stage centrifugation for 10 minutes at 1200 rpm (200 $\times g$), and subsequently resuspended in DMEM containing 10% of fetal bovine serum (FBS) and antibiotic. After centrifugation, the pellet was resuspended in DMEM and cellular viability was quantified by staining with Tripán Blue vital stain (Sigma) in a Neubauer camera. Osteoblasts were seeded at a density of approximately 10^5 cells/mL in culture flasks (TPP – Techno Plastic Products, Trasadingen, Switzerland)

containing DMEM, supplemented with 10% SFB (both Nutricell), 25 µg/mL gentamicin, 2.5 µg/mL amphotericin B, 10 mM β-glycerol phosphate, 50 mg/mL L-ascorbic acid (Sigma) and 10 nM dexamethasone (all Sigma) to promote cells with osteoblastic phenotype, which were used in experiments after the third subculture. The flasks were incubated in a humidified incubator at 37°C (5% CO₂/95% balanced air). When cells reached 80% of confluence, cell cultures were considered to have reached full growth and the cells were enzymatically lifted from the flask by using a 0.25% trypsin/EDTA solution and monitored daily with an inverted microscope, Eclipses TS 100 (Nikon, Tokyo, Japan). The culture medium was replaced three times for week.

Cell Adhesion and Viability Assays

Identification of cell adhesion and viability on blends were carried out by performing the MTT assay, a modification of Mosmann's (Mosmann, 1993) method, which was used for both cell adhesion and direct cell cytotoxicity assays (Santos Jr *et al*, 2001) Previously, the sterilized scaffolds were placed in a 96-well plate (Corning) with 100 µL of culture medium and incubated at 37°C for 24 h, according to Santos Jr., *et al*. (2005). After incubation, 2x10⁵ cells/mL in 100 µL DMEM supplemented with 10% FBS were added to the wells containing the membranes. The cells were cultured for 2 h and 24 h to allow cell adhesion and to conduct cell viability assays, respectively. After the cells were washed twice with 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 37°C and incubated with 100 µL DMEM, an MTT assay mixture [10 µL per well, containing 5mg/mL of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide - MTT, Sigma] was added to each well and incubated for 4 h at 37°C. After 4h, 100 µL of Dimethyl Sulphoxide (DMSO, Sigma) and 25 µL of Glycine/Sorensen buffered solution replaced the assay mixture in each well to dissolve the formazan crystals. Absorbance was quantified by a spectrophotometer at 570 nm, using a Elx-800-UV, Bio-Tek Instruments, USA. MTT is a colorless tetrazolium salt that forms a dark compound when oxidized by mitochondria, which is detected by spectrophotometer. For the viability assay, latex membranes embed in 1% phenol were used as positive control, and the culture plate (polystyrene) was used as negative control. For cell adhesion evaluation, Teflon dishes were used as negative control and the culture plate itself was used as positive control. The membranes were sterilized by UV irradiation for 30 min. Absorbance of all experimental conditions was also read. Comparison of continuous variables for all groups was done with ANOVA. When a significant difference was found ($p < 0.05$), the groups were compared using Tukey's test.

Colorimetric assay by Sirius Red

Previously, the sterilized scaffolds were placed in a 96-well plate (Corning) with 100 μ L of culture medium and incubated at 37°C for 7, 14 and 28 days. Sirius Red was purchased from Sigma (Sigma Chemical, St Louis, MO, US). The dye was dissolved in saturated aqueous picric acid at a concentration of 1 mg/mL. Bouin's fluid were used to fix the samples and it was prepared by mixing 15 mL saturated aqueous picric acid with 5 mL 35% formaldehyde and 1 mL glacial acetic acid. Although both reagents are stable for months, but we used freshly prepared dye reagent for each experiment and all chemicals were of analytical grade. Staining of extracellular matrix (ECM) standards and cell layers, and quantification of the dye reaction. Solutions of the different ECM materials were dissolved in PBS at 0.1% (1 mg/mL), pipetted into 24-well plates, and air dried overnight in a sterile bench before fixation with 1 mL/well Bouin's fluid. The cells layers were extensively washed with PBS prior and after fixation. Then, the cells were stained by Sirius Red for 1 h under mild shaking on a microplate shaker. Thereafter, the dye solution was removed by suction and the stained cell layers washed with 0.01 N hydrochloric acid to remove all non-bound dye. The stained material was dissolved in 0.1 N sodium hydroxide using a microplate shaker for 30 min at room temperature. The dye solution was transferred to the plate reader and the optical density (OD) measured with a Biorad microplate reader 355 at 550 nm and the 0.1 N sodium hydroxide was used as a blank (Tullberg & Jundt, 1999).

Scanning Electron Microscopy (SEM)

The scaffolds were sterilized by UV irradiation for 30 min and placed in 96-well plates. Approximately 2×10^5 cells/mL osteoblastic cells in DMEM supplemented with 10% FBS were seeded in each well containing the membranes and cultured at 37°C. After 6h, 1d, 2d, 7d, 14d and 21 days, the samples were fixed in a fixative solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.03% picric acid, 1% tannic acid in 0.1M cacodylate buffer and the same volume of DMEM for 1h at room temperature (RT), washed in 0.1M cacodylate buffer, post-fixed in 1% osmium tetroxide in water for 1h at RT in the dark, washed in water, dehydrated with ethanol, critical point dried [Balzers CDT 030] and coated with gold in a sputter coater [Balzers CDT 050]. The coated specimens were observed with a JEOL 5800 scanning electron microscope.

RESULTS AND DISCUSSION

In this study we used osteoblastic cells from the calvaria of *New Zealand* rabbits, 15 days old, to evaluate the biocompatibility of the 3D PHB scaffolds, and to examine the possibility of its use in bone fractures recuperation processes.

Cell Viability Assay

The statistical analyze of the viability assay showed difference among the samples and the controls, in which the positive control (phenol) presented a lower absorbance than the negative control (polystyrene) and all the samples in study presented a higher level of absorbance than the controls, positive and/or negative ($p < 0.05$). There was no significant difference among the plasma treated and untreated samples ($p > 0.05$) (Figure1).

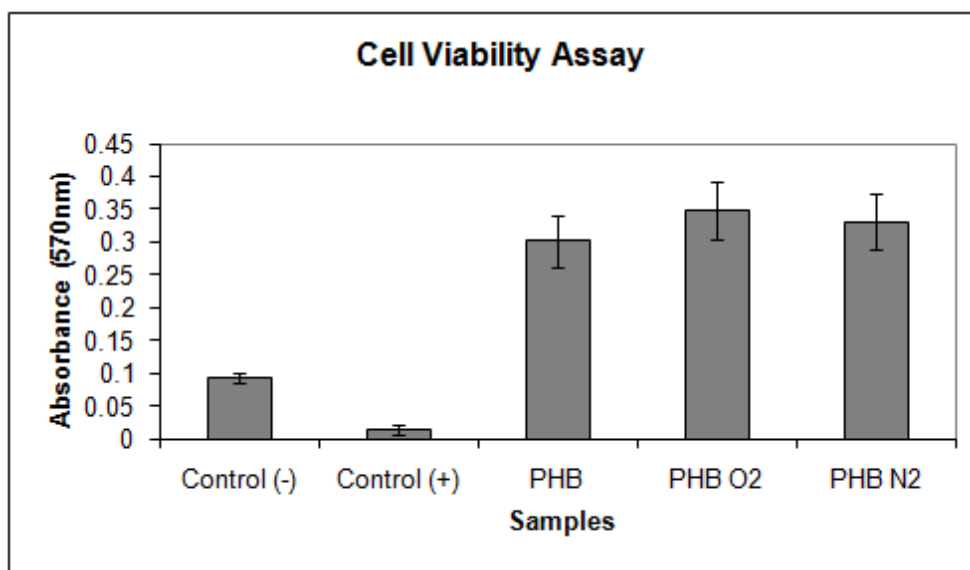


Figure 1 – Evaluation of cellular viability on three-dimensional devices of PHB. Quantification by MTT.

The PHB scaffolds, treated or not, showed to be less cytotoxic to the cells. There was no significant difference among the samples ($p > 0.05$). Since the polystyrene has good biocompatibility, our results indicate the good cytocompatibility of the material.

A variety of polyesters has been used as biomedical device, with excellent results as its biocompatibility (SHIVE, 1997). PHB is among those polyesters with good results *in vivo* (SHISHATSKAYA *et al.*, 2004) and *in vitro* (SHISHATSKAYA & VOLOVA, 2004).

Shamsuria *et al.* (2004) studied the cytotoxicity effects of hydroxyapatite (HA), of natural coral (NC) and of polyhydroxybutarate (PHB) with human osteoblastic cells CRL-1543. After 72h hours of incubation, PHB samples obtained the higher percentage of viable cells. Suwantong *et al.* (2007) studied the biocompatibility of mouse fibroblasts (L929) and Schwann cells (RT4-D6P2T) seeded on fiber mats of PHB and PHBV, not observing any negative response about the cytotoxicity of the materials. Our results are found in agreement with the literature, where PHB does not present any rate of toxicity. The plasma treatment in this study, independently of the gas used, did not alter the cytocompatibility of the material.

The cytocompatibility of the material is not the only requisite to consider it biocompatible, but it's an important characteristic. MTT is reagent metabolized in the mitochondria, which allows us to say, that in the intracellular levels, the cells presented viables (MARQUES *et al.*, 2005), which is also an important factor of the biocompatibility of the material.

Cell adhesion

In this study the statistical analyze of cell adhesion after 2 hours of incubation showed significant differences among the positive (polystyrene plates) and negative controls (Teflon) and the samples, where the positive control (+) showed superior absorbance over the negative control (-) and all the samples in study presented higher rates of absorbance than both controls ($p < 0.05$). The untreated PHB scaffolds presented significant difference compared to the nitrogen plasma treated PHB, which presented the highest absorbance rate ($p < 0.05$) (Figure 2).

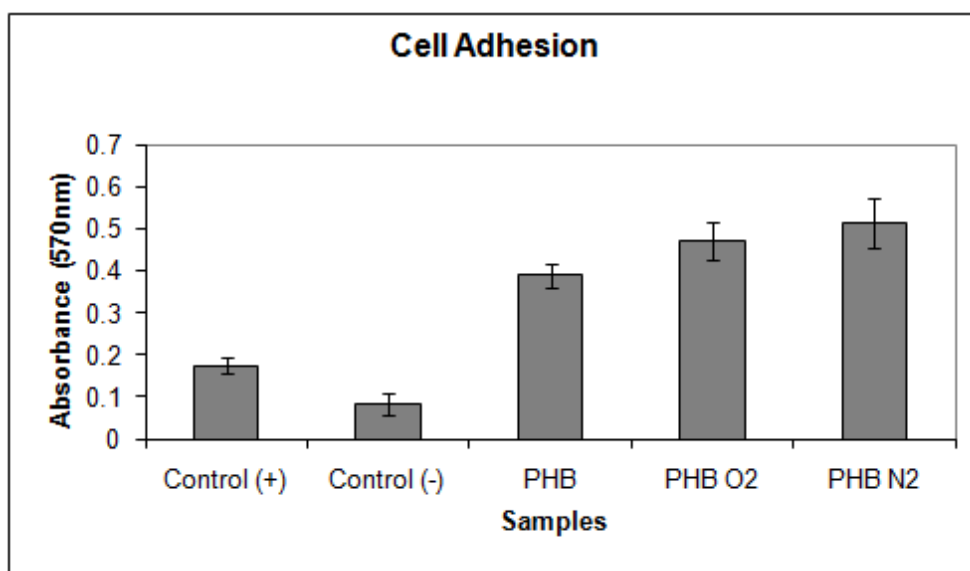


Figure 2 – Evaluation of cellular adhesion on three-dimensional devices of PHB. Quantification by MTT

The three-dimensional PHB scaffolds, treated or not, showed to allow the cell adhesion of osteoblasts even higher than the positive control. When we compare our samples, we observe that the nitrogen plasma treated samples presented better results and it was efficient to improve the cell adhesion of the material.

The cell adhesion phenomenon is of extreme importance to the biomaterials science. Once adhered on the material the cells can migrate and/or proliferate and exercise its physiological activities, such as the production of extracellular matrix. The adhesion is a consequence of the interaction of the proteins adsorbed on the substrate. These interactions involve electric charge, bridges of hydrogen and electrostatic forces. Other factors that modulate cell adhesion are the functional chemical groups present on the polymer surface, which is related to the hydrophilicity/hydrophobicity of the material surface (CHU *et al.*, 2002). Surface rugosity is other important factor that can affect cell adhesion.

Cell adhesion to polymeric substrates is one of the main points of tissue engineering, because the adhesive interactions control the cell physiology and also determines cell morphology (NEBE, 2001). According to some authors the hydrophilicity is a factor very important to the biomaterial cytocompatibility studies. Cell adhesion and growth on biomaterials surfaces are strongly influenced by the hydrophilicity/hydrophobicity balance, or wettability (TEZCANER *et al*, 2003; MA *et al*, 2003).

The literature has also reported that there is no obvious correlation between hydrophilicity and cell behavior, as cell attachment; others demonstrated that the cells prefer to anchor on hydrophilic surfaces

(WAN *et al.*, 2003). Another studies showed that the cells adhere, spread and grow more easily on moderate hydrophilic substrate than on hydrophobic or very hydrophilic substrate (TEZCANER *et al.*, 2003; MA *et al.*, 2003; WAN *et al.*, 2003).

Although no topographical analyze of the materials used in this study was made, its widely described in the literature that the plasma treatment modifies physico-chemically the materials surface. Wan and collaborators could observe that the PLLA, when treated by ammonia plasma, had its hydrophilicity and energy surface level was improved . And such improvement provided a better quality of cell adhesion (WAN *et al.*, 2003). Wang *et al.* (2005) cultivate osteoblasts (from bone marrow of rabbits and fibroblasts (L929) on PHB and PLA, having better results of cell adhesion for PHB. Lucchesi and collaborators (2007) studied Vero cells on PHBV samples treated by N₂ and O₂ plasma and showed that the plasma treated improved the cell attachment on the scaffolds.

Collagen quantification assay by Sirius Red

The biochemistry analyze of the quantification of collagen after 7 days of culture revealed that there was significant difference between the control (polystyrene plate), the untreated samples and the samples treated by nitrogen plasma ($p < 0.05$). There was no statistically difference between untreated PHB and PHB treated by nitrogen. The PHB treated by oxygen showed similar absorbance compare to control ($p > 0.05$). After 14 days of culture the analyses revealed that there was significant difference among the control and scaffolds ($p < 0.05$). But, there was no difference between the PHB treated or untreated samples. After 28 days there was no significant difference among the samples ($p > 0.05$) (Figure 3).

Besides the samples with cells, we also dyed samples of PHB without cell, treated and untreated, and we discount the value of their absorbance in the value of the other samples (with cells). It was made because of the possibility of the dye react with the biomaterial.

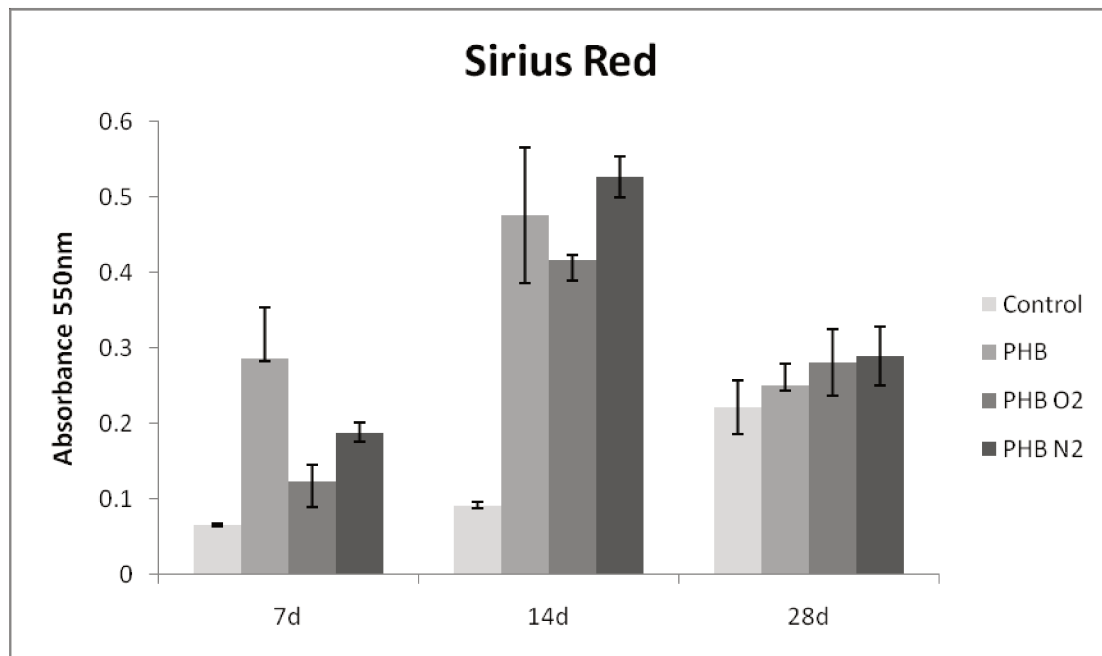


Figure 3: Quantification of collagen on three-dimensional devices of PHB after 7, 14 and 28 days of cell culture through the absorbance of *Sirius red*.

As we could see by the graphics, the PHB scaffolds treated by plasma of O₂ and N₂, obtained a value of absorbance below the value obtained by the untreated PHB after the first 7 days of culture. With 14 days of culture, the treated samples showed a significant improvement in the amount of collagen produced, obtaining values of absorbance closer to the ones obtained by the untreated PHB.

Among the advantages of the method of coloring by *Sirius red* stands out the fact that the dye is highly specific for collagen proteins type I and III, in this case. The method is based on the selective connection of the *Sirius red* (acid dye) with collagen proteins (basic amino acids) and it has been applied to quantify collagen, not reacting with other components of the extracellular matrix, like fibronectin for example. This is important because fibronectin can be expressed in large quantities by pre-osteoblastic cell with poor differentiation and fast proliferation (TULLBERG-REITEN & JUNDT, 1999).

Mineralization substances such as dexametasone, ascorbic acid and β -glicerophosphate, present in the culture medium used in this study, can alter collagen synthesis after prolonged periods. Tullberg-Reiten & Jundt (1999) verified that the combination of ascorbic acid and β -glicerophosphate stimulate the synthesis of collagen, and that dexametasone reduce the synthesis after 14 days of culture. The author also confirms that the ascorbic acid is considered an important inductor of synthesis of collagen, and that the ascorbic acid well as the β -glicerophosphate is essential to the maturation of the bone mineralization.

In the present study the control samples received the same mineralization medium that the PHB samples received, however, the higher rate of absorbance obtained by the PHB samples demonstrated that the polymer not only allowed the osteoblasts to synthesize collagen type I and III, but also stimulate its synthesis. It was not possible to do an evaluation about the influence of each mineralization substance in the synthesis of collagen in our study, once the culture medium used had three substances together. Such fact, however, do not affect our work, once the amount of collagen produced by the cells cultured on PHB was superior than the amount produced by the ones cultured on the polystyrene plate as control.

Collagen is the major component of the extracellular matrix and it represents about 90% of the organic substances present in the bone tissue. According to Tullberg-Reiten & Jundt (1999), places with large collagen deposition can represent places of synthesis of mature matrix before mineralization begins. Due to the material be three-dimensional and opaque, the visualization of these deposits was not possible by optical microscopy, but the quantitative analyze shows us evidence of its presence as both of its synthesis.

Yet, collagen type I is the major extracellular component of the osteoblasts phenotype characterization (TULLBERG-REINERT & JUNDT, 1999). The high absorbance of the Sirius red evidences the phenotype of the osteoblastic cells in this study. The use of this method for the characterization of the cell phenotype has been described in the literature (SARUWATARI *et al.*, 2005).

For the statistical analyses we applied variance analyze (ANOVA) and Tukey's test when the difference was significant ($p < 0.05$) to compare the different treatment.

Ultrastructural study

Through the scanning electron microscopy we could observe the PHB scaffold with cells and its different treatments, which allowed cell growth on all samples (Fig. 4-9). The scaffolds showed to be unstable during the analyses, which made the image obtaining harder. According to previous results obtained by the group, the PHB scaffolds showed rough structure suggesting higher porosity and low mechanical properties (Oliveira et al, 2007).

After 6 hours of culture, the cells adhered on PHB scaffolds treated and untreated by plasma, apparently similarly, with rounded morphology and not differing in quantity (Fig. 4). These aspects did not differ from the ones obtained after 24 hours of cell culture, although a huge amount of fibrilar material was observed on cells surface, that could suggested some matrix synthesis (Fig. 5). We could observe that after 48 hours of culture the presence of vesicles on cell surfaces was more frequent (Fig. 6). From day 7, there are evidences that the cells recover the entire scaffold, presenting structures similar to

microvilosities, being possibly, secretion of extracellular matrix (Fig. 7), specially at the treated samples by plasma (Fig. 7B-7C). The samples after 14 days of culture, presented a high quantity of fibril material, seeing cell emitting cytoplasmatic projections. The fibril material on cell surfaces presented itself in fibrous form (Fig. 8). After 21 days of culture, cells recovered the polymeric devices, presenting themselves close to one another and emitting cytoplasmatic projections to cellular groups more distant and granules that could suggesting minerals depositions since we used osteogenic medium (Fig. 9).

In general, the cells presented a rounded morphology, elongated or polygonal, with cytoplasmatic projections and particulate material on cell surface. The samples, independently of the treatment employed, presented themselves extremely irregular, which made the comparisons very difficult. However, it's widely described in the literature that the plasma treatment, modify the surface properties of the material (KEEN *et al.*, 2006) and that these surface properties influence the events of cell/substrate interaction. This is clear in many studies that show that these properties determine the type of biological molecule adsorbed (BOYAN *et al.*, 1996). Other factors that influence in the material biocompatibility are surface rugosity, surface chemical groups and surface hydrophilicity. Lucchesi *et al.* (2008) observed that Vero cells, when cultured on PHBV membranes treated by oxygen or nitrogen plasma presented different behavior. Besides that, we should take into consideration the fact that different cellular types respond in different ways to different surfaces (COCHRAN *et al.*, 1994). According to Bowers *et al.* (1992), cells similar to osteoblast, "osteoblast-like", present a higher percentage of cell adhesion on substrates with rough surface, while fibroblasts adhere much better on flat surfaces. However, the PHB samples behaved themselves unstably to the electron beam, impairing not only the images analyses, but also its obtaining. By this technique, it was not possible to compare the plasma treated and untreated scaffolds.

The selective laser sintering has been a good technical to obtain 3D scaffolds for Tissue Engineering. For biomedical field it might be a great tool with high precision to mimetize bone architecture besides of the micro-fabrication.

CONCLUSION

The PHB showed good cells viability with no cytotoxic effects with or without treatment. The physical-chemical modifications on the samples caused by nitrogen plasma treatment improve cell attachment. The samples allowed improve the synthesis of type I collagen as one of the characteristics of osteoblast cell. The selective laser sintering has been a good technical to obtain 3D scaffolds for Tissue Engineering.

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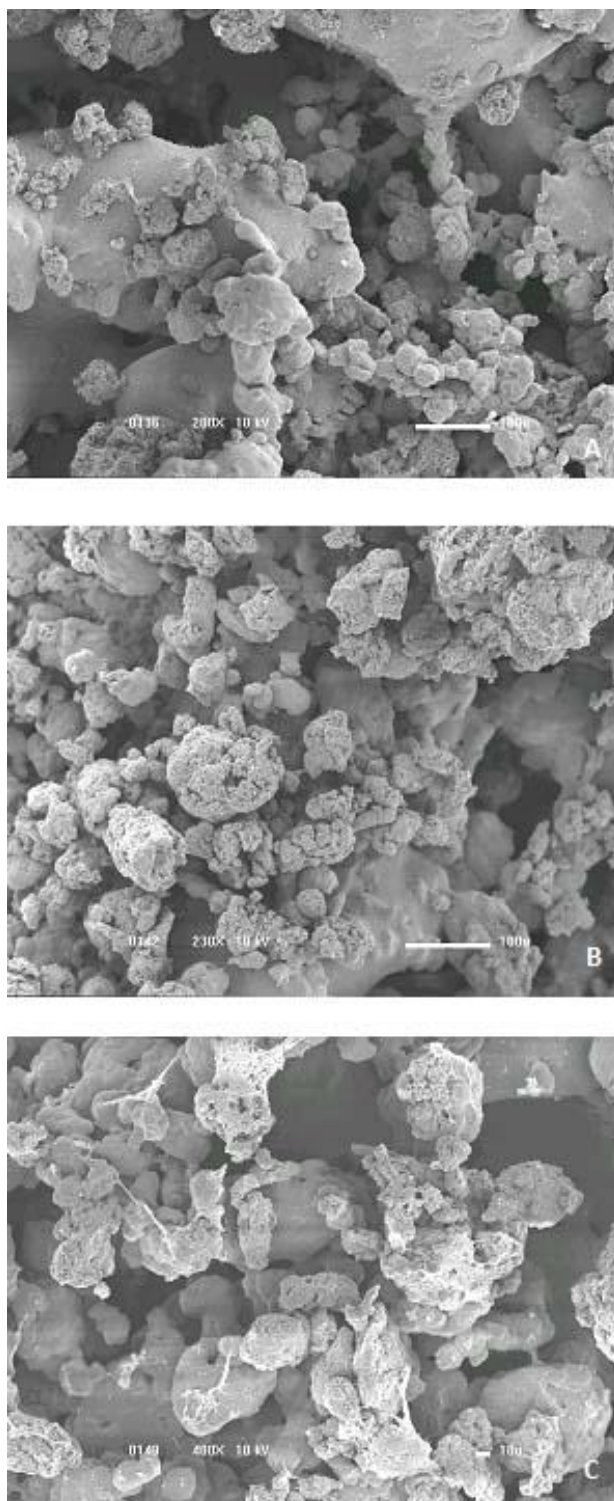


Figure 4: Scanning Electron Microscopy of PHB scaffold after 6 hours of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

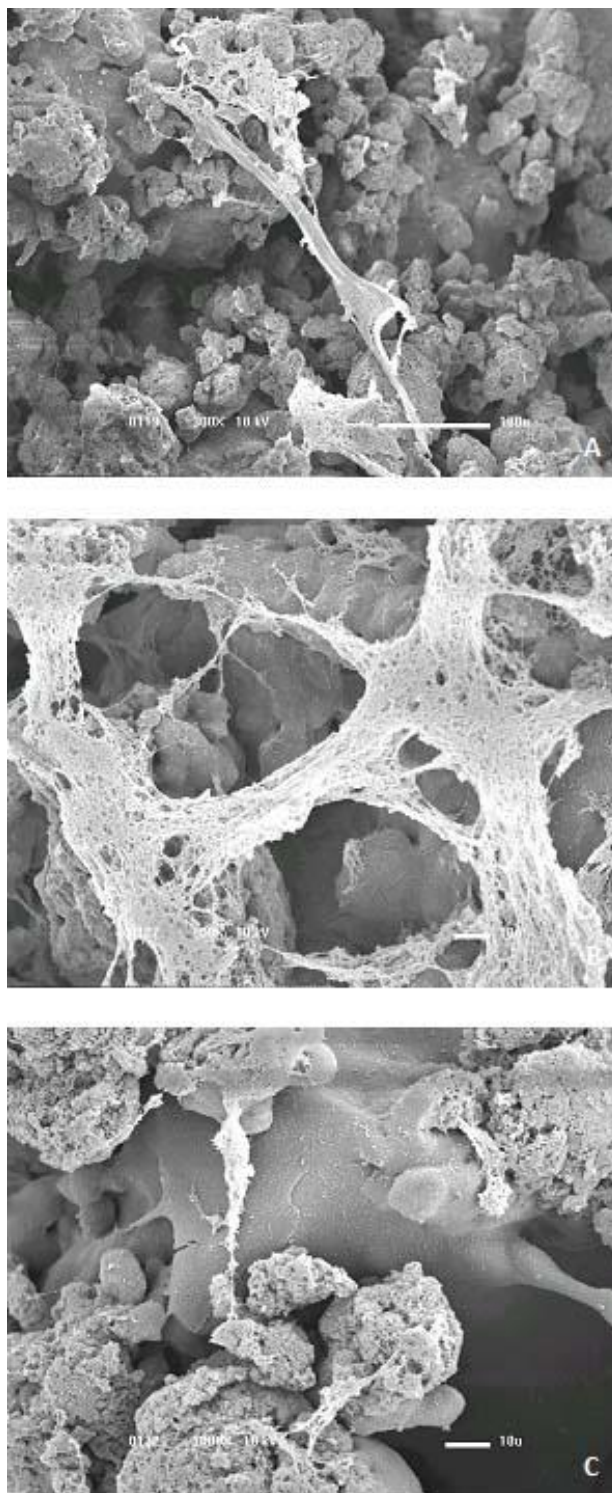


Figure 5: Scanning Electron Microscopy of PHB scaffold after 24 hours of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

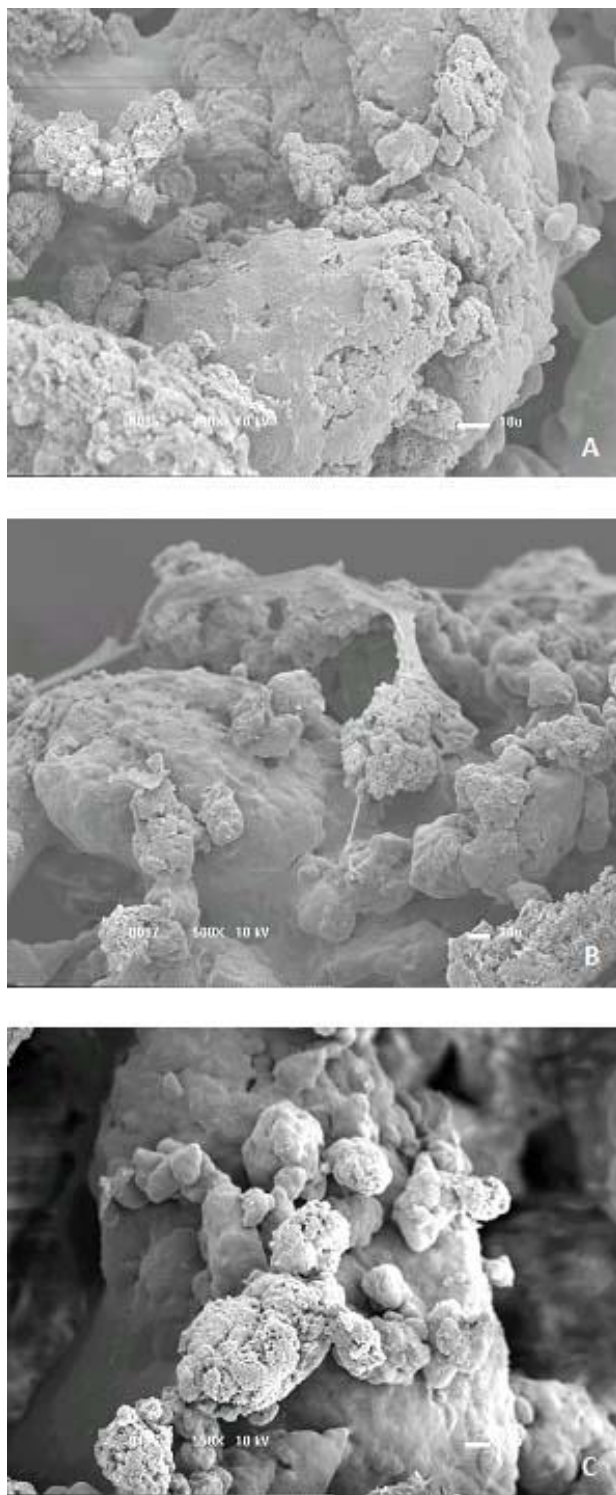


Figure 6: Scanning Electron Microscopy of PHB scaffold after 48 hours of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

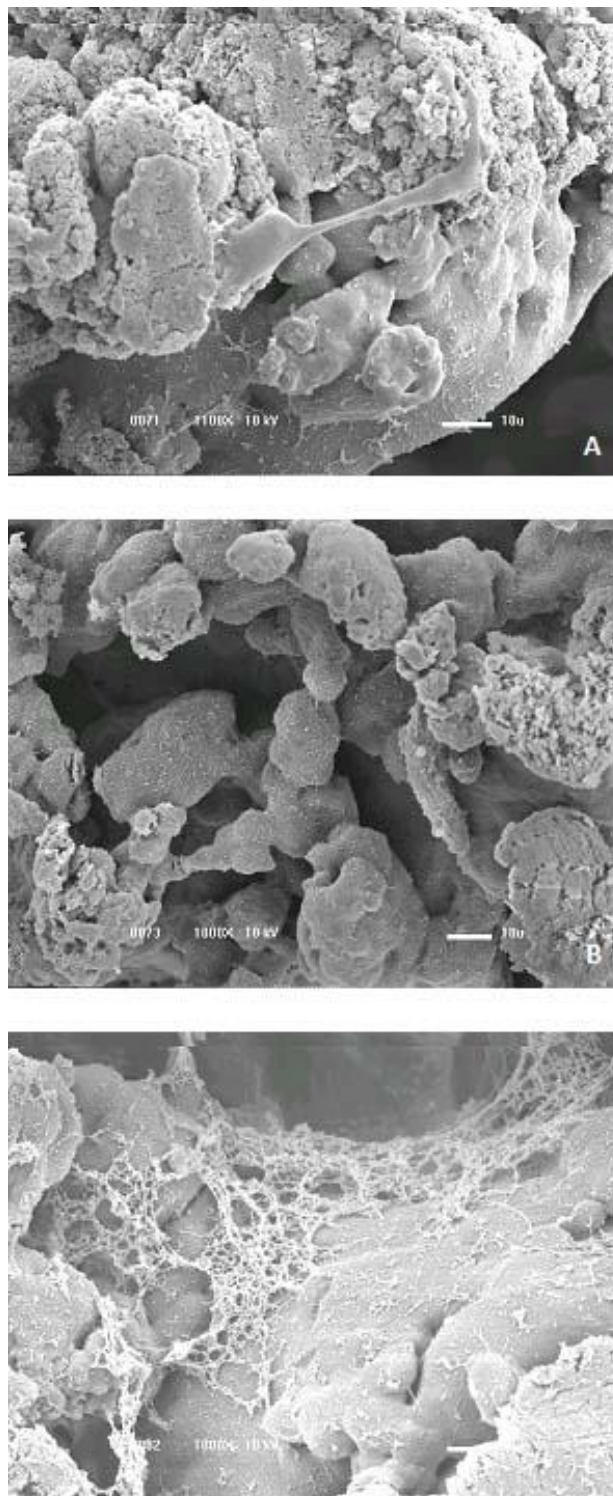


Figure 7: Scanning Electron Microscopy of PHB scaffold after 7days of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

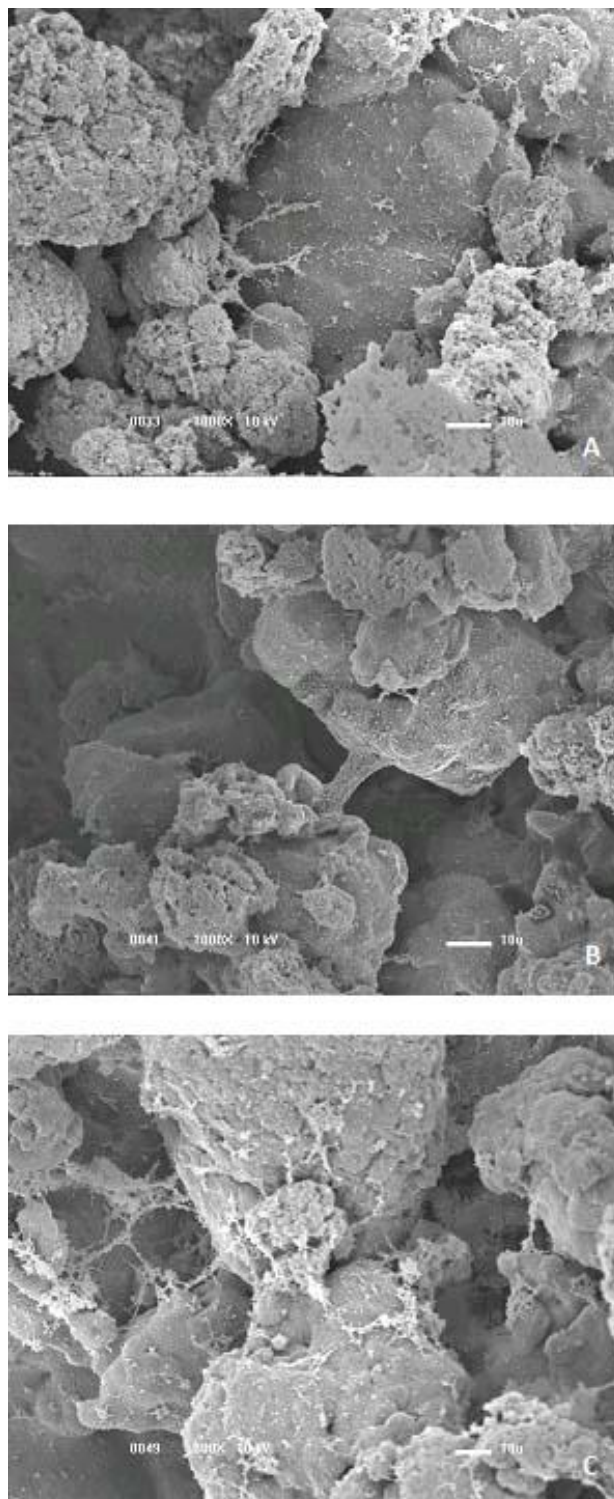


Figure 8: Scanning Electron Microscopy of PHB scaffold after 14 days of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

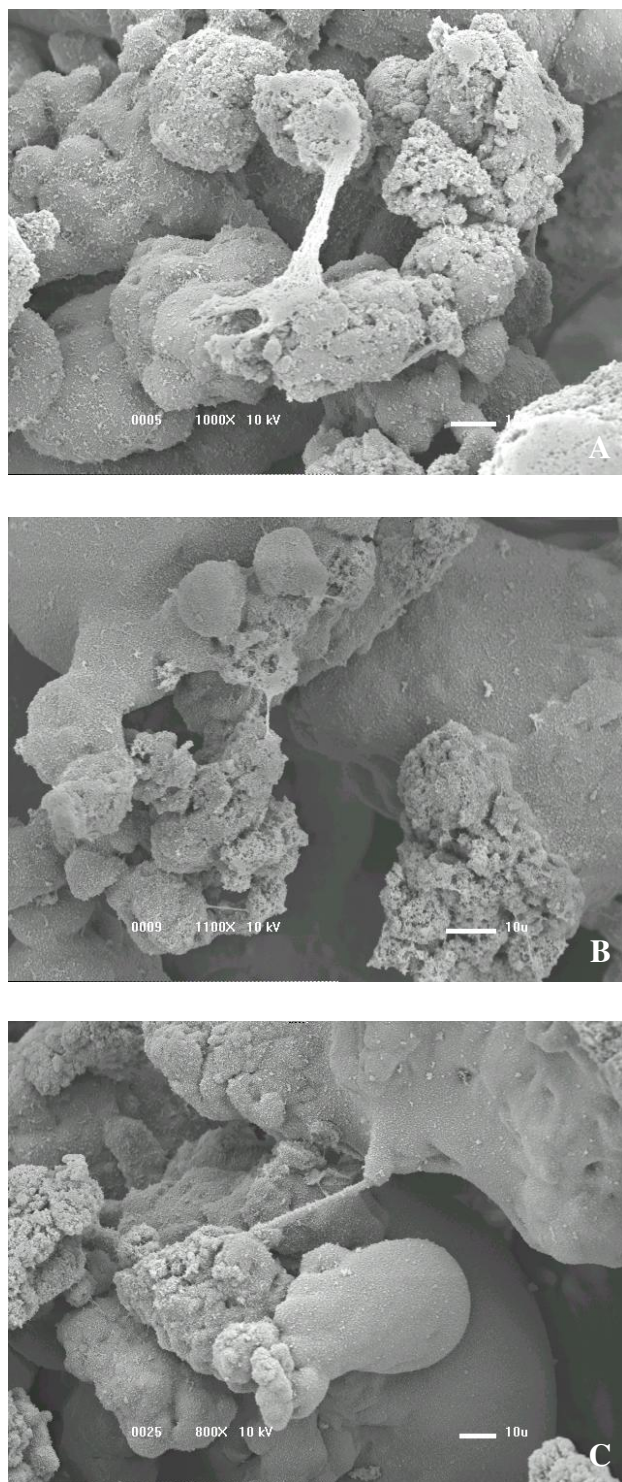


Figure 9: Scanning Electron Microscopy of PHB scaffold after 21 days of cell culture. A) PHB; B) PHB O₂; C) PHB N₂.

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CAPÍTULO 4

OSTEOBLAST CELLS ON 3D PHB OBTAINED BY RAPID PROTOTYPING: *IN VIVO* STUDY

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ABSTRACT

Controlling the differentiation of bone mesenchymal cells and providing tissue functions in engineered constructs before implantation are major challenges. Poly(hydroxybutyrate) (PHB) has good properties as biodegradability, biocompatibility, optical activity, piezoelectricity, non-toxicity and also it has been studied for a variety of medical applications. Here we evaluate the tissue interaction of 3D PHB scaffolds implant in rabbit calvaria. The scaffolds were obtained by rapid prototyping and some of the scaffolds were treated by nitrogen plasma. The PHB scaffolds had osteoblast cells culture prior implant in rabbit calvaria model at 7, 15, 30, 60 and 90days. Histological evaluation were used to investigated the response of the tissue to the three-dimensional environment provided by polymer and cells. The PHB porous scaffolds had higher amount of new bone area compared to scaffolds without cell culture and defect control area, suggesting that scaffold material and design combinations could be tailored to facilitate filling of bony defects.

Key-words: Poly(hydroxybutyrate) (PHB); Bone; Calvaria; *In vivo*; Rapid Prototyping.

INTRODUCTION

The reconstruction of calvarial and maxillofacial bone defects caused by trauma, diseases, or congenital anomalies needs reliable reconstruction tools. The repair of three dimensional structurally complicated bone defects poses a challenge to implant design and manufacturing. The combination of computer tomography and rapid prototyping technology has offered solutions to this challenge (Chiarini *et al*, 2004; Choi & Cheung, 2005; Tuusa *et al*, 2008).

The development and modification of orthopedic implants has taken place for many years in an effort to create an optimal interaction between the body and the implanted material. The goal of achieving an optimal bone-implant interface has been approached by the alteration of implant surface topography,

chemistry, energy and charge as well as bulk material composition. Schmidt *et al.* (2001) defines an ideal bone implant material as having a biocompatible chemical composition to avoid adverse tissue reaction, excellent corrosion resistance in the physiologic milieu, acceptable strength, a high resistance to wear and a modulus of elasticity similar to that of bone to minimise bone resorption around the implant (Pearce *et al.*, 2007).

In order to determine whether a new material conforms to the requirements of biocompatibility and mechanical stability prior to clinical use, it must undergo rigorous testing under both initial *in vitro* and then *in vivo* conditions. However, *in vitro* characterization is not able to demonstrate the tissue response to materials, instead being confined to the response of individual cell lines or primary cells taken from animals. No *in vitro* cell culture system is able to produce loading that simulates the *in vivo* situation and currently very few *ex vivo* systems are able to approach such physiological loading (and usually only with small tissue samples (Davies *et al.*, 2006). For these reasons animal models are essential for evaluating biocompatibility, tissue response and mechanical function of an orthopaedic material prior to clinical use in the human (Pearce *et al.*, 2007).

Poly(hydroxybutyrate) (PHB) is known as a very rigid, highly crystalline, and hence slow degrading, but thermoplastic polymer (Gogolewski *et al.*, 1993). It is thought to be useful for bone tissue-engineering approaches due to the negligible acidification of surrounding tissue during degradation compared to other polyesters (Chen & Wang, 2002). Porous scaffolds of PHB and co-polymers have mainly been revealed with good biocompatibility and they have been used for many medical applications (Zheng *et al.*, 2003).

Another crucial characteristic is the geometry of a scaffold, such as the pore size, porosity, and pore interconnectivity, which can affect the diffusion of the nutrients, cell attachment, migration, and tissue ingrowths that are necessary for the bone formation process (Kim *et al.*, 2008_a). Others studies aim to evaluate the effect of implant surface modification on alteration of the bone-implant interaction (Kim *et al.*, 2008_b). In order to draw accurate conclusions regarding the effects of implant modification, we implant 3D PHB scaffolds treated by N₂ plasma with osteoblast cells as a purpose to filling out bone defects.

METHODS

Scaffolds Preparation

Three-dimensional PHB (Biocycle[®], PHB Industrial S/A, Brazil) scaffolds were designed using a 3-D solid modeling software, exported to a Sinterstation 2000TM machine (3D Systems, Valencia, CA), and constructed by Selective Laser Sintering processing (SLS). This PHB has a particle size distribution at the 10-100 µm range. The scaffolds were produced with 6mm of diameter, 2mm of thickness and 200-500 µm porous size. The samples were storage under vacuum until plasma treatment.

Plasma treatment

The plasma treatment was performed using a radio frequency Anatech LTD Ashing plasma apparatus. The plasma conditions employed in the treatment was PHB untreated; PHB treated by N₂, 50 W, 150mTorr and 600s; PHB treated by O₂, 100 W, 300mTorr and 600 s The values of power, pressure, and time were chosen based on preliminary studies with PHBV (Lucchesi *et al.*, 2008).

Scanning Electron Microscopy (SEM)

The blends were sterilized by UV irradiation for 30 min and placed in 96-well plates. Approximately 2×10^5 cells/mL in DMEM medium supplemented with 10% FBS were seeded in each well containing the PHB scaffolds and cultured at 37° C. After 24 hours, the samples were fixed in a fixative solution prepared by dissolving 2.5% paraformaldehyde, 2.5% glutaraldehyde, 0.03% picric acid, 1% tannic acid in 0.1M cacodylate buffer and the same volume of DMEN medium for 1h at room temperature (RT), washed in 0.1M cacodylate buffer, post-fixed in 1% osmium tetroxide in water for 1h at RT in the dark, washed in water, dehydrated with ethanol, critical point dried (Balzers CDT 030, Balzers Inc., Elgin, IL, USA) and coated with gold in a sputter coater (Balzers CDT 050, Balzers Inc., Elgin, IL, USA). The coated specimens were observed with a JEOL 5800 scanning electron microscope.

Cell isolation, culture and seeding

Osteoblastic cells were obtained from 10 calvaria of young male adult (15 days old, 230–280 g) *New Zealand* rabbits, from university's central animal house (PUC/SP-CCMB, Centro de Ciências Médicas e Biológicas, Sorocaba, SP, Brazil). Following euthanasia by cervical displacement, the calvaria were aseptically excised, cleaned with a soft tissue, and washed in Dulbecco's modified eagle's medium (DMEM) (Nutricell - Nutrientes Celulares, Campinas, SP, Brazil) containing 150 µg/ml gentamicin sulfate (Sigma Chemical, St. Louis, MO) and 15µg/ml Amphotericin B (Sigma). This concentration of antibiotic was 3 times the normal amount used in cell culture and was used to prevent contamination during harvest. In sterile atmosphere, the calvaria were fragmented and submitted to enzymatic digestion for cellular isolation in DMEM medium and 1mg/mL of collagenase type II (GIBCO, Invitrogen Corporation, Carlsbad, CA) during 2 hours at 37°C. After this procedure, it was submitted to a three-stage centrifugation for 10 minutes at 240 g force, and subsequently resuspended in DMEM medium containing 10% of fetal bovine serum (FBS) and antibiotic. After centrifugation, the pellet was resuspended in DMEM medium and cellular viability was quantified by staining with Tripán Blue vital stain (Sigma) in a Neubauer camera. Osteoblasts were seeded at a density of approximately 10^5 cells/ml in culture flasks (TPP-Techno Plastic Products, Trasadingen, Switzerland) containing DMEM medium, supplemented with 10% FBS and 50 µg/mL gentamicin, 10 mM β-glycerol phosphate (Sigma), and 50 µg/mL L-ascorbic acid (Sigma) containing 100 nM dexamethasone (Sigma) to promote cells with osteoblastic phenotype, which were used in experiments at the third subculture. The flasks were incubated in a humidified incubator at 37°C (5% CO₂/balanced air). When cells reached 80% of confluence, cell cultures were considered to have reached full growth and the cells were enzymatically lifted from the flask by using a 625 mg/mL trypsin/EDTA (Nutricell) solution and monitored daily with an inverted microscope, Eclipse TS 100 (Nikon, Tokyo, Japan). At the third subculture 2×10^5 cells/ml were seeded in the PHB scaffold treated and untreated by plasma and it was kept for 24 hours at the same conditions for the implant procedures.

Animal model

The animal model used in this study was *New Zealand* rabbit (3 months age) with 2.5-3.0 Kg. The animals were obtained from University's Central Animal House at Pontificia Universidade Católica de

São Paulo, in Centro de Ciências Médicas e Biológicas de Sorocaba (PUC-SP/CCMB). The animals were kept in cages separately and received commercial food and water “ad libitum”. Rabbits are the most commonly used animals for medical research, being used in approximately 35% of musculoskeletal research studies (Neyt *et al.*, 1998). International standards established regarding the species suitable for testing implantation of materials in bone, state that dogs, sheep, goats, pigs or rabbits are suitable. Although rabbit have a different microstructure from humans histologically (Wang *et al.*, 1998), there are ethical implications of using companion animals for medical research. Rabbits are commonly used for screening implant materials prior to testing in a larger animal model (Pearce *et al.*, 2007).

The experimental protocol used in this work is in agreement with both the standards of The American Society for Testing and Materials (ASTM F-981-93) and the Ethical Principles for Animal Experimentation adopted by the Brazilian Animal Experimentation Board (COBEA). It was also approved by the Research in Ethics Commission from Vale do Paraíba University according to Ethics and Principles from National and International Guidelines of research involving animals under protocol number A38/CEP/2008.

Surgical procedure

The rabbits were acclimated for 1 week before operation and monitored for general appearance, activity, excretion, and weight. Fifteen rabbits were used and were randomly shared in 5 groups, equivalent at the different times of implant of the scaffold (7, 15, 30, 60 and 90 days), and 3 rabbits were used for each time. They were implanted PHB scaffolds with different treatments in the same animal: PHB with cells, PHB without cells and PHB with cells and treated by Nitrogen plasma. Cylindrical implants are not recommended to be larger than 2mm in diameter and 6mm in length for rabbits (International Standard ISO 10993-6, 1994; Pearce 2007).

The animals were anesthetized with ketamine (50 mg/kg IM) and xylazine (5 mg/kg IM). After shaved and disinfecting the operative site, the periosteum was resected, and 4 defects site with 6 mm diameter each were provoked carefully by dental bar cooled (Beltec) with sterile saline solution. The craniotomy segment was removed and the damage to the underlying dura and superior sagittal sinus vein were avoided. The defect was rinsed with sterile saline thoroughly and bone fragments were washed out.

Three defects were filling out with the scaffolds and one of them was kept without scaffold as a defect control. The design of the scaffolds implants follow above: Group 1 – Defect control, it was not filling out (G1); Group 2 – Defect filling out by PHB without cells (G2); Group 3 – Defect filling out by PHB with cells (G3). Group 4 - Defect filling out by PHB treated by nitrogen plasma with cells (G4). (Fig.1).

The calvaria segments were removed carefully without disturbing the underlying dura-mater. The animals were harvested under pre-anesthesia by administration of Ketamine (50 mg/kg IM) and Xylazine (5 mg/kg IM), and euthanized with Sodium Pentobarbital (200mg/Kg IP). After that, the soft tissues were removed, and the skull was resected. The samples were kept in 4% formaldeide solution for histological procedures.

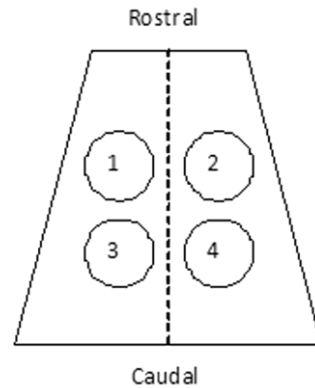


Figure 1: Design of the surgery at the rabbits. 1 - Control defect site; 2 - PHB implanted without cells; 3 - PHB implanted with cells; 4 - PHB treated by nitrogen plasma with cells.

Histology

The calvaria segments for histological examination were fixed in 4% buffered formalin (pH 7.4). The samples were decalcified in 10% EDTA solution and then they were dehydrated in a graded series of ethanol, and half of the samples were embedded in resin methylmethacrylate (Kit #14702231731, Historesin, Leica) and another half in paraffin (Histoseck, Merck). After polymerization, 3 μm and 5 μm sections respectively, they were cut from the center of the implants using a microtome RM2245 (Leica Reichert Jung, Nußloch, Germany). Light microscopic sections were stained with Toluidine Blue and Hematoxylin-Eosin, respectively. The samples were observed and evaluated by optical light microscope (Nikon, Eclipse E800, Tokyo, Japan).

RESULTS

Gross Analyses

All 15 rabbits tolerated the anesthesia and the surgical procedures well and experienced no complications during the experimental period. Upon sacrifice, gross examination of the dissected specimens of the calvaria showed no difference between the PHB groups and the control group defects in terms of signs of inflammation and integrity of the healing wound.

Ultrastructural analyses

Through the scanning electron microscopy it was possible visualize the ustrastructural morphology of PHB scaffolds (Figure 2) and with cells attached after 24h (Figure 3). We can observe

clusters of cells attached in the PHB treated and untreated by plasma after 24 hours of culture in osteogenic medium. The cells adhered to the scaffolds and interact themselves showing cells agglomerate (Figure 3A-B). We could note some products on cells and scaffolds surface.

Histological Analyses

The healing of the defects in the control group was mainly by scar formation. There were a few bony islands scattered along the defect margins, which might have resulted from bone debris produced by drilling through bone. The healing of the PHB group produced a tissue regenerate with many blood vessels and cellular marrow spaces.

At 7 days post-surgery all the PHB samples with and without cells showed fibrin network, mesenchymal cells, blood vessels (Figure 4), edema, cell proliferation with no significant inflammatory reaction, and at PHB N₂ some cells showed osteoblast's phenotype (Figure 4D). The defect control was noted large hemorrhage area, bone fragments, and some bone formation close to the edge of the defect (Figure 4E).

At 15 days post-surgery it was observed at all the samples with PHB, fibrous connective tissue, fibroblasts, blood vessels, and mesenchymal cells (Figure 5). At the defect control, it was observed fibrous connective tissue without bone formation (Figure 5A). At the PHB treated by N₂, it was noted osteoblasts cells secreting bone matrix, collagen fibers and a slight inflammatory process (Figure 5D).

At 30 days post-surgery, it was observed fibrous connective tissue around all of the samples with PHB as fibroblasts, blood vessels and mesenchymal cells. It was presented osteoblasts around of the polymer and of the edges of the defect. Large amount of new bone formation almost cover the defect (Figure 6). The PHB without cells, it was showed osteoids and osteoblasts around of the polymer (Figure 6C). PHB treated by N₂, there was new bone tissue growing up from the edges of the defect toward to the polymer with the fibrous connective tissue, osteoids and osteoblasts (Figure 6D). The control defect area there was growth bone tissue near of the edges, and a thick layer of fibrous connective tissue, with no significant new bone formation (Figure 6A).

After 60 days post-surgery, the connective tissue around all of the PHB samples was slight. Blood vessels and mesenchymal cells condensed were presented with bone tissue growing up from the edges and absent inflammatory process. The mainly difference between the samples were that PHB with cells showed more of osteoids and osteoblast around the polymer than the other samples with new bone formation (Figure 7). The defect control area has shown bone tissue growing up from the edges, some osteoids and larger amount of connective tissue than PHB samples (Figure 7A-D).

The 90 days PHB samples with cells tended to have more mature, trabecular bone whereas the PHB without cells samples had more bone tissue and less trabeculated chunks of bone. Although, statistics analyses were not made it to quantified. It was observed connective tissue, blood vessels, mesenchymal cells aggregated, osteoblasts-like and growth of bone tissue from the edges of the defect in all of the polymers. PHB treated by N₂ and PHB with cells, we could observe osteocytes in the bone matrix and osteoblasts between bone fragments. The bone matrix showed some organizations and large amount of osteoblasts were observed (Figure 8B and 8C). The defect control was almost filling out by immature bone tissue (Figure 8A).

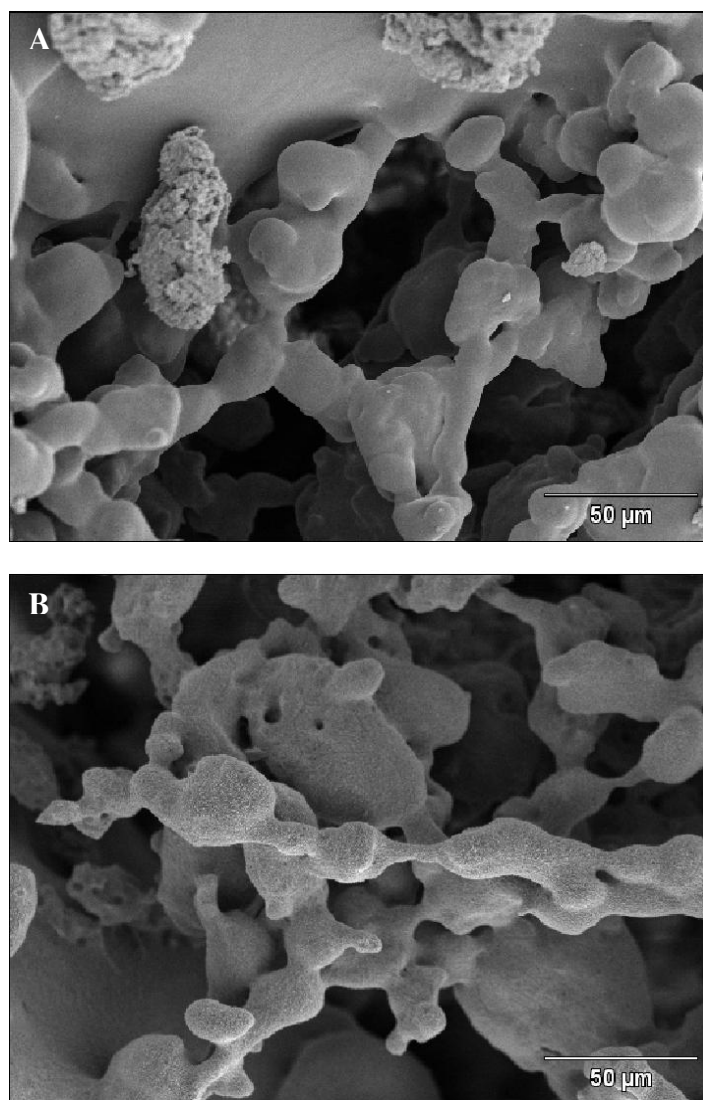


Figure 2: Scanning electron microscopy of PHB scaffolds without cell culture. A) PHB without treatment; B) PHB treated by nitrogen plasma.

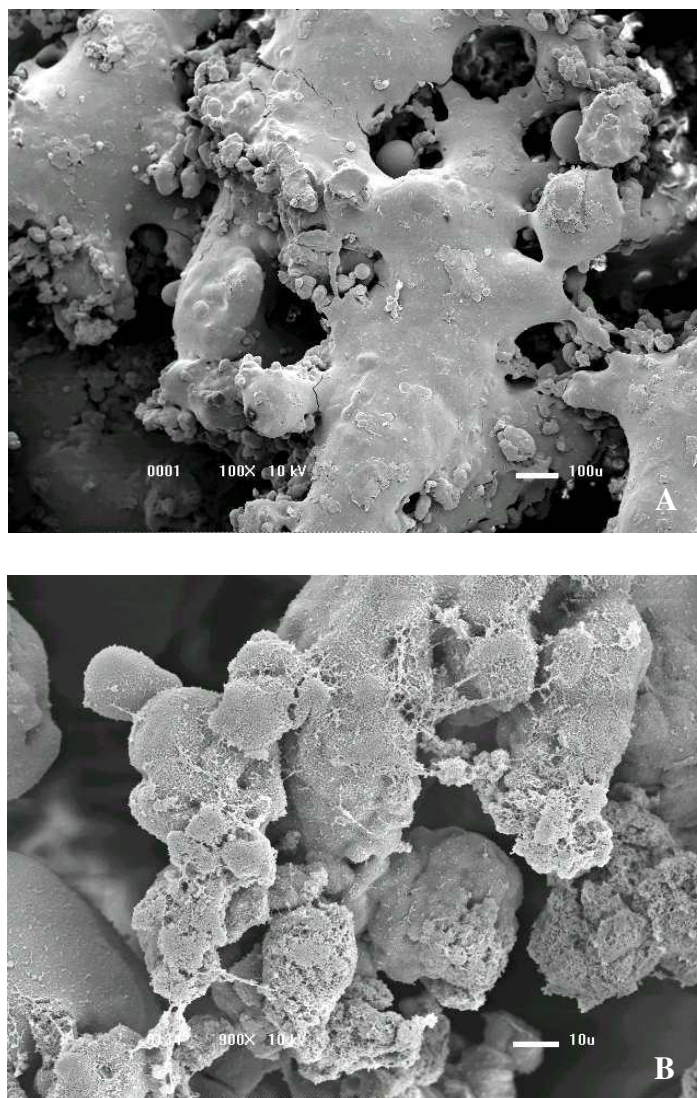


Figure 3: Scanning electron microscopy of PHB scaffolds. A) PHB without treatment; B) PHB treated by nitrogen plasma.

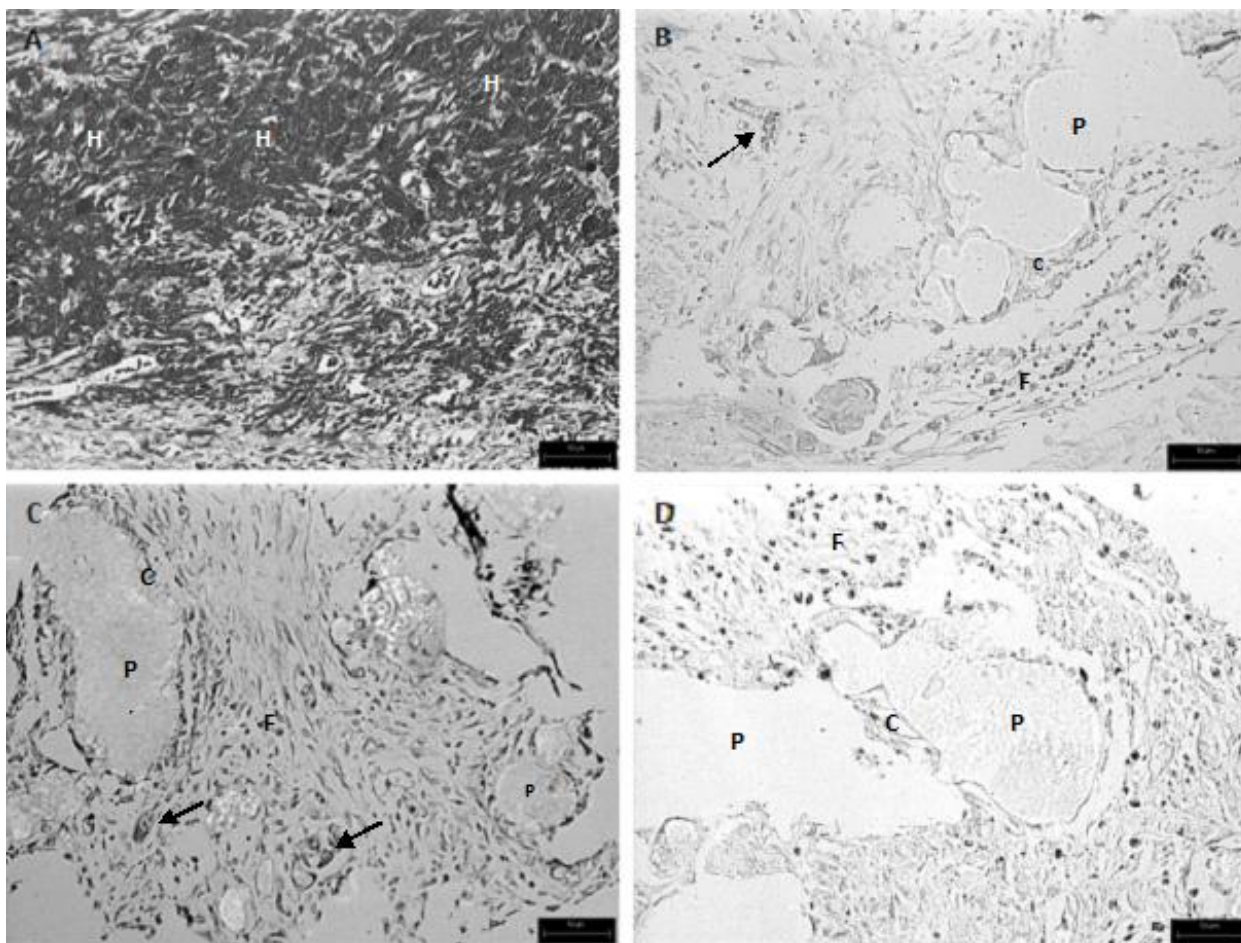


Figure 4: Sections after 7 days of implant. All the PHB samples (P) showed mesenchymal cells (C), blood vessels (arrow), fibrin network (F) and edema. A) Control defect showing with hemorrhage area (H); B) PHB without cells; C) PHB with cells; D) PHB N₂ with cells. Bar = 50μm.

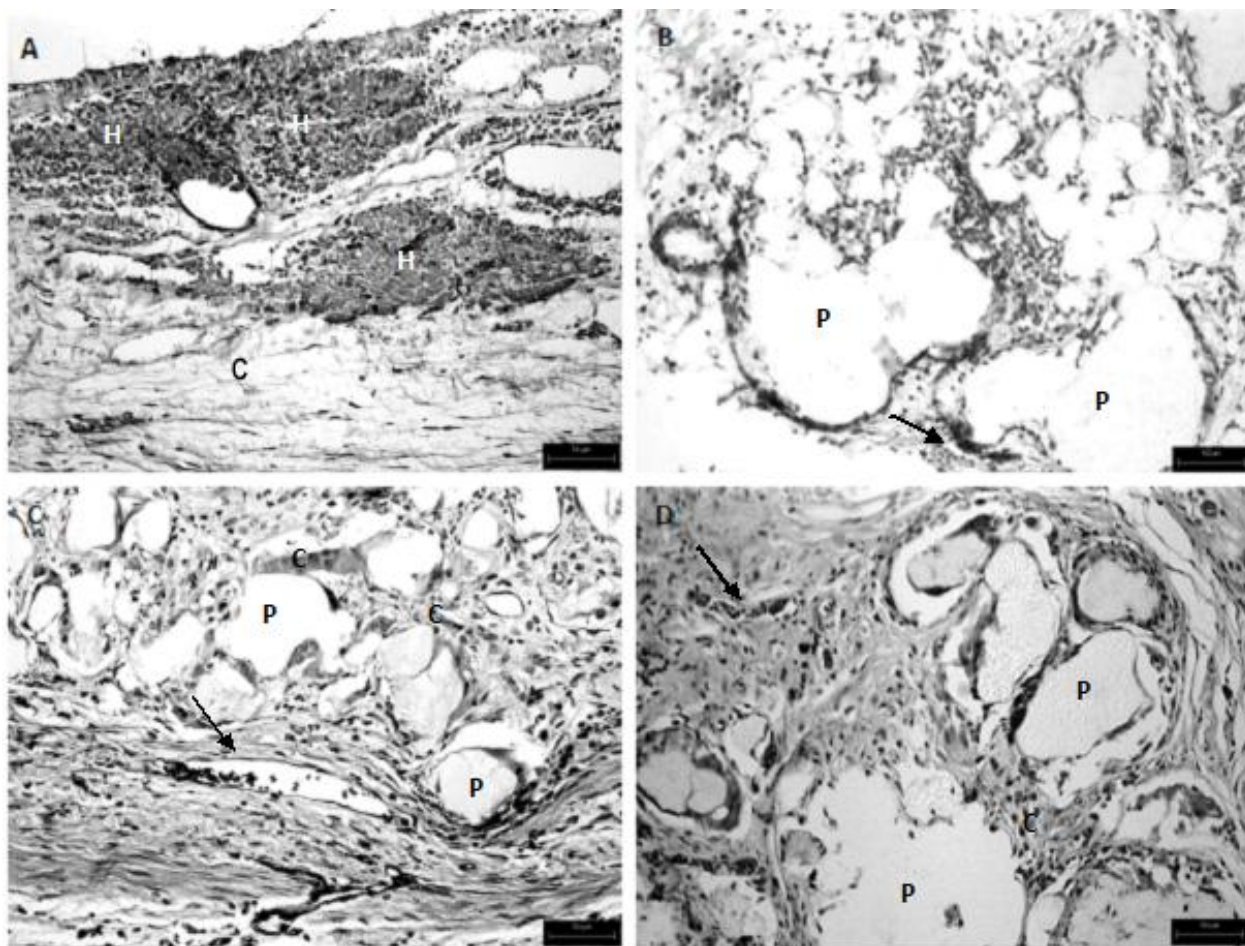


Figure 5: Sections after 15 days of implant. The PHB samples with cells culture prior implant showed cells (C) in differentiation process around the polymer (P), blood vessels (arrow). A) Control defect showing with hemorrhage area (H) and edema; B) PHB without cells showed more cells of inflammatory process than the samples with cells culture prior implant; C) PHB with cells; D) PHB N₂ with cells. Bar = 50μm.

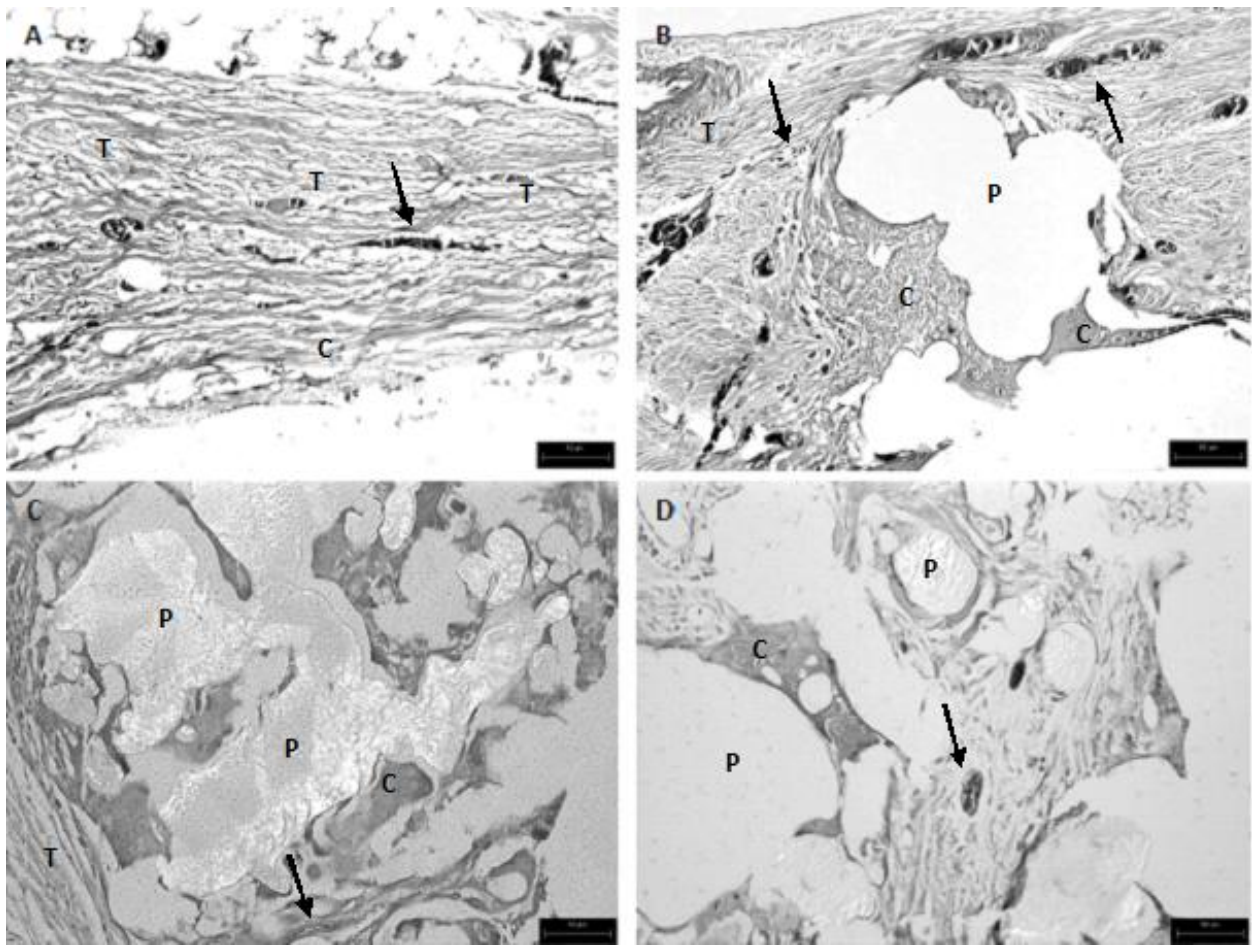


Figure 6: Sections after 30 days of implant. All of the samples with PHB (P) showed fibroblasts, blood vessels (arrow), fibrous tissue and cells (C). A) Control defect; B) PHB without cells showed a larger amount of connective tissue (T) than the other PHB samples; C) PHB with cells; D) PHB N₂ with cells. Bar = 50µm.

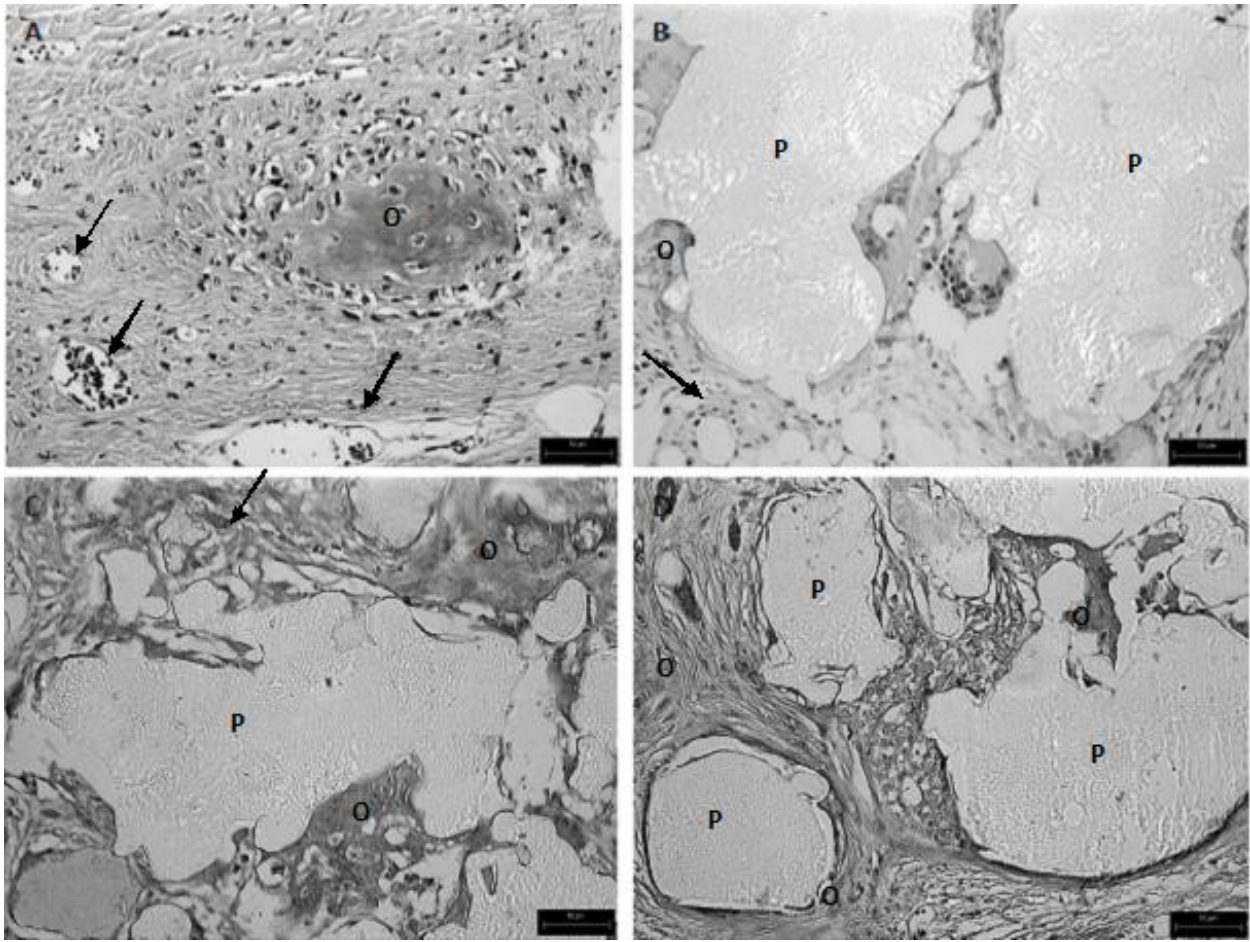


Figure 7: Sections after 60 days of implant. The connective tissue around all of the PHB samples was slight. Blood vessels (arrow) and mesenchymal cells condensed (C) were presented with some osteoids (O), mainly in the samples with cell cultures prior implant. A) Control defect; B) PHB without cells; C) PHB with cells; D) PHB N₂ with cells. Bar = 50µm.

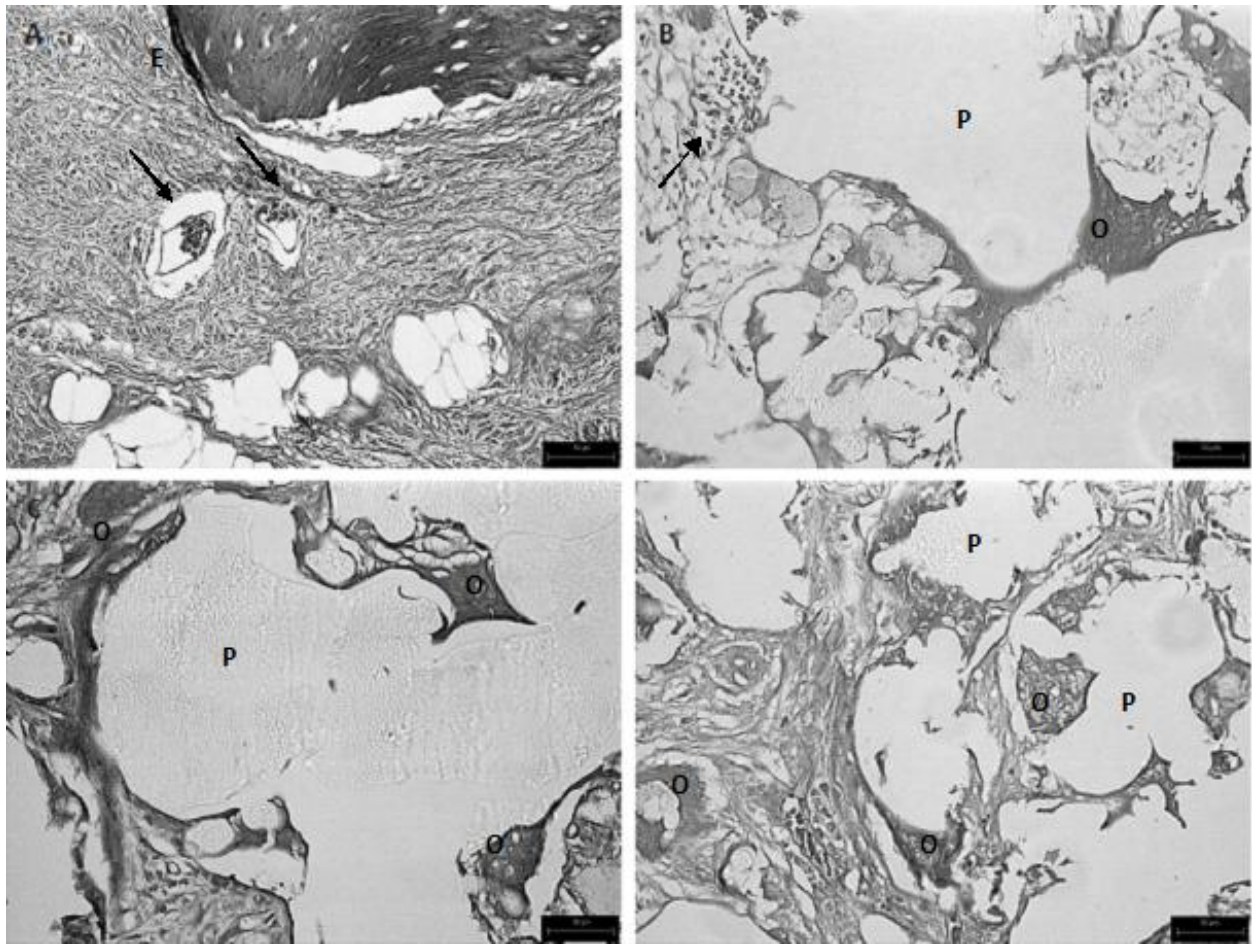


Figure 8: Sections after 90 days of implant. A) Control defect with bone repair from the edges (E); B) PHB without cells; C) PHB with cells; D) PHB N₂ with cells. Note the samples with cell culture prior implant there were more osteoids (O) than PHB without cells or the defect control area. All the samples (P) showed blood vessels (arrow), mesenchymal cells, osteoblast cells and connective tissue. Bar = 50µm.

DISCUSSION

Bone defects are a significant clinical problem. These defects can be caused by inflammation, trauma, congenital malformation, diseases or oncological surgery (Mai *et al.*, 2006). The available literature data suggest that both nonresorbable and resorbable polymers can be used for the preparation of the scaffolds for the treatment of bone defects (Hoffman *et al.*, 2009; Jiya *et al.*, 2009).

The bioresorbable polymers should satisfied several biological requirements as the polymer do not induce foreign body reaction, was not be carcinogenic, mutagenesis or teratogenesis, did not cause allergies, hypersensitivity ou toxicity, even stimulate the complement system, it is a relevant importance (Orefice *et al.*, 2006). However, is has been described in literature that ever material introduce in the body it will have some kind of reaction, in a large or smaller proportion. At this point all of the PHB samples used in this research were appropriated according to biological and technical requirements, which are in agreement with Gogolewski *et al.*, 2004. The scanning electron microscopy (SEM) analyses showed that even with 24 hours the cells were capable to produce some extracellular matrix protein, because it was possible to observe some molecule on the cells and scaffolds surfaces.

After implantation at the rabbit calvarial defects, all the scaffold groups were observed to be compatible with the soft and hard tissues, without showing any significant adverse reactions. No inflammatory cells were found in any of the specimens. Compared to the control group, wherein new bone formation most of the time it was limited only to the defect margin (Figure 6A), all of the scaffold groups showed a large amount of new bone formation, with the ingrowth of bony tissues into the pore channels (Figure 5 and 6). The same process was noted in our samples and defect control.

Short time post-implantation, there was some edema, which it has been related as a normal physiological process of bone repair (Köse *et al.*, 2004). In the implants or control defect, it was noted hemorrhage, as blood vessels, matrix destruction and probably death of bone cells around. During the healing, it is normal that the clot, wastes cells, and matrix are removed by macrophages, and the periosteum and endosteum near of the implant site response with an intense cell proliferation (Davies, 2000).

After one or two months the PHB samples shown more osteoids and new bone formation than the defect control, mainly the samples with cells (Figure 4 and 5). It was observed lots of agglomerated cells (Figure 5B-D). These are a common phenomenon described of cell condensations by large number of authors (Fell, 1925; Hall & Miyake 1992; Olsen et al, 2000; Shapiro 2008) that should happen before cell differentiation. It was unclear in this research those agglomerated cells are cells from the body and those cells migrated for the local lesion or if those cells were in the scaffold. We believe that both hypotheses could happen because even the PHB without cells it was observed some cells condensation, in a smaller proportion though.

For long term post-implantation, we believed that the retard in the bone growth, in the group with PHB compared to the defect control should be due to the slow degradation of the polymer, which acts as a physical barrier avoiding the bone growth. On the other hand, for human being could be really useful if we consider the fact that human bone take at least 4 months to regenerate (Davies, 2005). And we should consider that apparently the same amount of bone was observed at the control defect. One of the major problems encountered by surgeons who deal with large bone defects is the difficulty of maintaining

viability within bone-grafted tissue in order to ensure graft survival and eventual restoration of the defect (Jan *et al*, 2006). In this study, the PHB had being shown suitable as a long term implant, and besides, the first step is designated a good material that it will be able to be use as bone in the future.

It is known bone tissue has a great regenerative potential with the capacity to restore its structure and originals functions. There are situations when the bone defect is not to be able to regenerate by itself. In that case, could be necessary a bone graft to suitable treatment and good prognostic (Nair *et al*, 2007).

A critical-sized defect is by definition the smallest full thickness osseous wound that will not heal spontaneously during the lifetime of an animal (Schmitz JP & Hollinger, 1986; Jan *et al*, 2006; Prado *et al*, 2006). According to Hollinger 1990, it was found experimentally that the CSD size for rabbits is 15 mm or more. Although there are some authors using different sizes as a defect control. Kim *et al*. (2008_b) investigate the *in vivo* performance of the engineered bioceramic scaffolds using a 10mm rabbit calvarial defect model. Hokugo *et al*. (2007) investigated the enhancement of biological function of platelet growth factors after integration of PRP into biomaterials, using a 5mm defect as a control in rabbit calvaria.

The rabbit calvarial defect model is analogous in many ways to clinical maxillofacial reconstruction. There is an osseous defect with a periosteal blood supply and there is a membranous pattern of bone repair and healing. One difference, however, is the presence of a pulsatile dural layer in the base of the rabbit calvarial model, which is not present in extracranial maxillofacial wounds (Ahmed *et al*, 2006). However, at the present study the animal model was suitable for our purpose.

A great number of biomaterials have been tested in the last decades for bone tissue engineering, but few have been demonstrated good results. Among the polymers, the PHB have been used as medical device and it has been shown good results *in vivo* and *in vitro* (Shishatskaya *et al*, 2004; Shishatskaya & Volova, 2004). A large amount of requirements are needed for a suitable material, and some of those are theirs architecture, and surface properties. With the aim to improve these property of the materials for tissue engineering, scaffolds has been developing and the rapid prototyping appear as a great tool. Therefore, exploiting a scaffold with high porosity and interconnectivity in conjunction with high mechanical strength has been one of the important issues in the production of artificial bone grafts (Kim *et al*, 2008_b).

In this study, we used a specially designed three-dimensional PHB as scaffold to the osteoblast for filling out bone defects and improve bone augmentation. Bone is a complex and structured three-dimensional tissue. PHB scaffold developed with macrochanneled pores (200-500µm) shows ongoing increases in the cell proliferation. Although there are few *in vivo* studies reporting the effect of porosity using the PHB scaffolds, some limited number of works has been carried out using other types of materials. Roy *et al*. (2003) reported improved tissue ingrowth and new bone formation on the composite scaffolds (PLGA/tricalcium phosphate) by increasing the porosity, demonstrating that channel size, porosity, and pore size can be controlled and used to influence new bone formation and calvarial defect healing. Hu *et al* 2009, suggested that the NF scaffolds resembling a native collagen fibrillar matrix, with appropriate pore sizes, and a high-porosity, effectively support osteogenesis of hMSCs showing mineralized bone formation. Tian *et al*, 2009 investigate *in vivo* biocompatibility and osteogenesis as well as degradability of the porous strontium-doped calcium polyphosphate (SCPP) scaffolds enhanced osteogenesis and degradability. According to Karageorgiou & Kaplan (2005), the porosity and pore size of biomaterial scaffolds play a critical role in bone formation. In vivo, higher porosity and pore size result in greater bone ingrowth.

To regenerate new bone tissues, the appropriate biological properties are required for cell scaffolds including good cell attachment/proliferation, differentiation function, and support of bone formation in three dimensions prior to vast degradation of the scaffold (Tanaka *et al*, 2008). Concerning the degradation, as seen in Figure 5B-D, the scaffolds implanted between calvaria bone and periosteum had maintained their three-dimensional structures at least 2 months. Importantly, new bone was found in the PHB scaffold.

According to our results all PHB scaffolds did not showed severe inflammatory response and allowed bone regeneration compare to defect control. PHB obtained by rapid prototyping, treatment by plasma and seeded cells onto scaffolds prior implant have been shown good techniques to obtain biocompatible, 3D scaffolds which it will improve bone regeneration, being promises to Tissue Engineering.

CONCLUSION

We conclude that the PHB scaffold with osteogenic cells could be useful as graft on flat bone tissue for the purpose of bone thickness augmentation improving regeneration.

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CAPÍTULO 5

CONCLUSÕES

As blendas de PCL/PLGA apresentaram características desejáveis como dispositivos para aplicações em Engenharia de Tecido Ósseo, como adesão, proliferação celular e ausência de efeitos citotóxicos.

Os arcabouços de PHB apresentaram boa viabilidade celular, não apresentam qualquer efeito citotóxico nos arcabouços independentes do tratamento por plasma. As modificações físico-químicas nos arcabouços por plasma de nitrogênio melhoraram a adesão celular. Os arcabouços permitiram a síntese de colágeno tipo I, sendo esta uma importante característica de células osteoblásticas. O processo de obtenção dos arcabouços de PHB por prototipagem rápida apresentou-se uma ótima técnica para o desenvolvimento de dispositivos tri-dimensionais aplicado a Engenharia de Tecidos.

A interação célula/PHB/tecido mostrou-se promissora como suporte para o propósito de acelerar o reparo ósseo. Os arcabouços de PHB com células previamente cultivadas apresentaram maior formação óssea, sugerindo que a combinação do arcabouço e pré-cultivo celular facilitam a regeneração óssea, podendo ser utilizados para o preenchimento de defeitos ósseos.

Os polímeros PCL, PLGA e PHB apresentaram boa interação com células osteoblásticas possibilitando a continuidade de seus estudos com aplicação à Engenharia de Tecidos Ósseos.

CAPÍTULO 6

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **“Comportamento de células osteoblásticas sobre biomateriais poliméricos”**.

() não se enquadra no Artigo 1º, § 3º da Informação CCPG 01/2008, referente a bioética e biossegurança.


() está inserido no Projeto CIBio (Protocolo nº _____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Certificado nº A53/CEP/2009 - Univap).

() tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº _____).



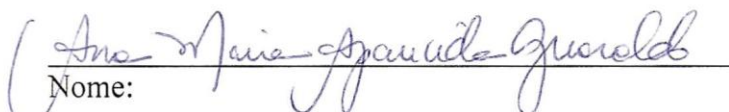
Aluna: Carolina Lucchesi



Orientador: Prof. Dr. Paulo Pinto Joazeiro

Para uso da Comissão ou Comitê pertinente:

(X) Deferido () Indeferido



Nome:

Função:


Profa. Dra. ANAMARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEAI/IB - UNICAMP

COMITÊ DE ÉTICA EM PESQUISA
UNIVERSIDADE DO VALE DO PARAÍBA
UNIVAP
CERTIFICADO

Certificamos que o Protocolo n.º A053/CEP/2009, sobre *“Estudo in vitro e in vivo das interações de células osteoblásticas com matrizes de poli (3-polihidroxibutirato)-PHB obtidas por sinterização seletiva a laser”*, sob a responsabilidade da **Profa. Dra. Eliana A.R. Duek**, foi **aprovado** por esta Comissão de Ética em Pesquisa por estar de acordo com os Princípios Éticos seguindo as Diretrizes Nacionais e Internacionais da pesquisa envolvendo animais.

Informamos que o pesquisador responsável por este Protocolo de Pesquisa deverá apresentar a este Comitê de Ética um relatório das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação.

São José dos Campos, 20 de novembro de 2009.



PROFA. DRA. CRISTINA PACHECO SOARES

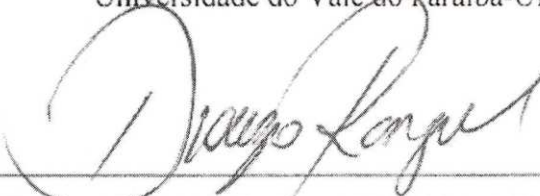
Presidente do Comitê de Ética em Pesquisa
Universidade do Vale do Paraíba-UNIVAP



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PROF. DR. DRAUZIO EDUARDO NARETTO RANGEL

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