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ARNALDO RODRIGUES DOS SANTOS JÚNIOR

Cultura de Células Vero sobre polímeros bioabsorvíveis a base de poli(L-ácido láctico)"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

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ABREVIAÇÕES UTILIZADAS

BMP – proteína morfogenética óssea.

PDS – poli(*p*-dioxano).

PGA – poli (ácido glicólico).

PHB – poli(hidroxibutirato).

PHBV – poli(hidroxibutirato-co-hidroxivalerato).

PLA – poli(ácido láctico).

PLGA – poli(ácido láctico)/poli(ácido glicólico).

PLLA – poli(L-ácido láctico).

PLLA/ PHBV – poli(L-ácido láctico)/poli(hidroxibutirato-co-hidroxivalerato).

rhBMP-2 – proteína morfogenética óssea humana tipo 2 recombinante.

TB – Azul de toluidina.

XP – Xylidine ponceau.

ARTIGOS QUE COMPÕEM ESTA TESE

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TRABALHOS PARALELOS DESENVOLVIDOS DURANTE O DOUTORADO

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Resumo

Os biomateriais poliméricos são compostos desenvolvidos para serem utilizados como substitutos de tecidos danificados. Uma classe de biomateriais poliméricos utilizados são os bioabsorvíveis, que são compostos que se decompõe tanto *in vitro* quanto *in vivo*, e são utilizados em tecidos que necessitam de um suporte temporário até que a recomposição tecidual se concretize. Dentre os vários polímeros bioabsorvíveis, se destacam os dispositivos a base do poli(L-ácido láctico) [PLLA] por apresentar boa biocompatibilidade e os produtos de sua decomposição serem eliminados do corpo por vias metabólicas.

Membranas densas de PLLA ou com poros de diferentes diâmetros (> que 45μm, entre 180-250μm e entre 250-350μm) foram avaliadas quanto a adesão, crescimento e diferenciação de células fibroblásticas em cultura. Observamos que as células aderem lentamente às membranas de PLLA. Uma vez aderidas, as células apresentaram variações em seus padrões morfológicos de acordo com as características estruturais do substrato no qual elas cresceram. Em todos os substratos de PLLA estudados, as células não apenas foram capazes de proliferar sobre a superfície dos mesmos como também foram hábeis em produzir uma matriz extracelular rica em colágeno tipo IV e fibronectina.

Avaliamos também o comportamento das células fibroblásticas sobre blendas de PLLA com poli(hidroxibutirato-co-hidroxivalerato) [PHBV] em diferentes proporções (100/0, 60/40, 50/50, 40/60, 0/100). Nas blendas de PLLA/PHBV, também observamos a adesão celular lenta e uma morfologia variável, variando de células arredondadas à completamente achatadas e unidas por prolongamentos, de acordo com as características físicas e topográficas do substrato. Também observamos que os substratos foram capazes de estimular a proliferação celular e a produção de um matriz extracelular rica em colágeno tipo IV e fibronectina.

A análise conjunta dos resultados mostra que os diferentes substratos a base de PLLA estudados não apresentam toxicidade, uma vez que as células são capazes de crescer e proliferar sobre eles. Sobretudo, as células foram capazes de se diferenciarem sobre os diferentes polímeros, uma vez que passam a produzir uma matriz extracelular rica em colágeno IV, comportamento incomum a células fibroblástica.

Abstract

Polymeric biomaterials are developed compounds used as substitutes for damaged tissue. A class of biomaterials is the bioabsorbable ones, which are polymers that degrade *in vitro* as well as *in vivo* and are used in tissues that need a temporary support until its tissue regeneration happens. Among the several bioabsorbable polymers, the poly (L-acid lactic) [PLLA] based devices are eminent because their decomposition products are eliminated from the body by metabolic ways and due to their good biocompatibility.

PLLA dense membranes or with different diameter pores (smaller than 45 μm, between 180-250μm and 250-350μm) were evaluated by their ability of stimulating adhesion, growth and differentiation of fibroblastic cells in culture. We observed that the cells adhere slowly to the dense and porous PLLA membranes. Once adhered, they presented a differentiated morphological pattern in accordance with the substrate characteristics that they had grown. In all the PLLA substrates studied, the cells were not only capable of proliferating over the substrates but also producing an extracellular matrix, rich in type IV collagen and fibronectin.

We also evaluated the fibroblastic cells behavior over the different proportions blends of PLLA with poly(hydroxybutirate-co-hydroxyvalerate) [PHBV] (100/0, 60/40, 50/50, 40/60, 0/100). At the PLLA/PHBV blends, we also noticed a slowly cellular adhesion and a variable morphology according to the physical and surface substrate characteristics. We also observed that the substrate were capable of stimulating cell proliferation and the production of an extracellular matrix rich in type IV collagen and fibronectin.

The results analysis show that the different PLLA based substrates studied, do not present toxicity, once the cells could differentiate over the different polymers, which was demonstrated by the production of an extracellular matrix rich in type IV collagen, an uncommon behavior to fibroblastic cells.

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I. INTRODUÇÃO

Durante séculos, grandes lesões teciduais, originadas normalmente de traumas mecânicos ou de doenças degenerativas, trouxeram problemas em função dos poucos recursos terapêuticos disponíveis. A remoção da porção lesada era a prática mais comumente utilizada, o que trazia uma série de limitações aos portadores daquela enfermidade. A extração de grandes porções de tecidos levava a um significativo decréscimo da qualidade de vida do paciente e, assim, a substituição de regiões corpóreas danificadas tornou-se um objetivo desde tempos remotos. Com o aumento da expectativa de vida do ser humano, obtida com o descobrimento dos antibióticos e dos quimioterápicos, além da melhoria das condições sanitárias e de higiene nas populações humanas, a busca por metodologias para a substituição de tecidos lesados tornou-se uma necessidade (HENCH, 1998).

Existem dois procedimentos que visam suprir a falta de alternativas clínicas à substituição dos tecidos e órgãos danificados ou comprometidos: os *transplantes* e os *implantes*. Em relação aos transplantes, os tecidos ou órgãos podem ser obtidos de doadores vivos, como no caso do coração ou rins, ou de cadáveres, como no caso de ossos liofilizados e congelados. Em ambos os casos, para a utilização dos mesmos, torna-se necessário à utilização de drogas imunossupressoras, com o intuito de evitar a rejeição dos órgãos, e de outros medicamentos que neutralizem a possível contaminação microbiana (HENCH, 1998). Além disso, os transplantes têm a desvantagem de trazer uma série de questões éticas e até mesmo religiosas. Por outro lado, dispositivos confeccionados com biomateriais desenvolvidos para servirem como implantes, além de não apresentarem vários dos problemas referidos acima, são desenvolvidos para atuarem na interface com os tecidos receptores no organismo, interagindo com eles. Estes dispositivos concebidos para

serem implantados são atualmente conhecidos como *biomateriais* (HUBBELL, 1995; HENCH, 1998).

A ciência dos biomateriais é uma área multidisciplinar. Seu objetivo geral é não apenas o desenvolvimento de compostos a serem utilizados como substitutos de tecidos danificados, mas também o entendimento das interações destes com o organismo receptor. Isso tem levado diferentes pesquisadores, entre eles médicos, engenheiros, químicos e biólogos, à criação e ao aperfeiçoamento de dispositivos que sejam biocompatíveis e funcionais que, quando implantados cirurgicamente, possibilitem uma melhoria na qualidade de vida dos pacientes que os utilizem.

Os avanços tecnológicos têm possibilitado o desenvolvimento de materiais de alta performance, termoplásticos, elastômeros, super ligas, cerâmicas bioativas e compostos bioabsorvíveis. Por outro lado, tendo-se em vista a necessidade de avaliação biomédica destes materiais, têm sido propostas simulações biomecânicas, testes *in vitro* para a avaliação de citotoxicidade e da biofuncionabilidade destes compostos (ISO 10993-5, 1992(E); KIRKPATRICK, 1992; KIRKPATRICK *et al.*, 1998). Concomitantemente, implantes destes biomateriais em animais de experimentação têm sido feitos com o objetivo de se avaliar sua biocompatibilidade e biofuncionabilidade.

Por definição estabelecida no *Consensus Conference of the European Society for Biomaterials*, "biomaterial é todo material não vivo usado em dispositivo médico, ou biomédico, objetivando a interação com o sistema biológico" (WILLIANS, 1987). Embora não sejam a maioria dos biomateriais empregados, uma classe de compostos amplamente utilizada experimentalmente são *polímeros*, isso é, uma macromolécula relativamente grandes cuja estrutura é constituída de unidades repetitivas covalentemente ligadas

conhecidas como *meros* (ou unidade química repetitiva ou resíduo de monômero). Os polímeros podem ser classificada com um *homopolímero*, quando o é formada apenas um tipo de mero, ou um *copolímero*, quando a cadeia apresenta dois ou mais meros diferentes, ou como uma *blenda*, que é a mistura física de dois polímeros sem que haja uma ligação química entre eles (MANO, 1994).

Além disso, os biomateriais podem ser classificados como *bioestáveis* ou *permanentes* e *bioabsorvíveis ou temporários* (TÖRMÄLÄ *et al.*, 1998). Os materiais poliméricos bioestáveis ou permanentes são compostos utilizados com o objetivo de substituir um tecido lesado por tempo indeterminado. Desta forma, são produzidos de modo a reter as suas características mecânicas e físico-químicas por longos períodos (TÖRMÄLÄ *et al.*, 1998). Estes tipos de dispositivos são comumente empregados experimentalmente como próteses substituindo articulações danificadas, válvulas cardíacas, lentes intra-oculares e outras utilizações. Por outro lado, existem tecidos que necessitam de um suporte que preencha apenas temporariamente a região lesada, até que a recomposição tecidual se concretize, ou ainda que direcione o processo regenerativo. Nestas situações, uma alternativa são os biomateriais temporários. Os materiais poliméricos bioabsorvíveis são compostos que são degradados tanto *in vitro* quanto *in vivo* e por isso são utilizados em dispositivos temporários (TÖRMÄLÄ *et al.*, 1998). Para ambos os tipos de materiais citados, uma vez que o objetivo geral é a restauração da funcionabilidade ou substituição dos tecidos danificados, é de extrema importância a compreensão das interações células-biomateriais, bem como a influência destes polímeros no padrão de adesão, crescimento e diferenciação celular (HENCH & ETHRIDGE, 1982).

Inicialmente, os biomateriais foram desenvolvidos para que permanecessem inertes no organismo. Sendo assim, os estudos voltavam-se para a forma de se prevenir ou minimizar as reações teciduais indesejáveis. Embora tais abordagens continuem sendo de grande importância, atualmente os novos polímeros são concebidos para que haja uma efetiva interação com os tecidos, provocando respostas fisiológicas como crescimento e/ou diferenciação celular no sítio de implantação (HUBBELL, 1995). Nas últimas décadas, avanços significativos foram obtidos no entendimento dos mecanismos de interação das células animais com o seu ambiente natural, a matriz extracelular (HAY, 1982; PIEZ & REDDI, 1984; ALBERTS *et al.*, 1994), bem como a influência desta no crescimento e diferenciação celular (SANTOS Jr & WADA, 2001). Esse conhecimento vem sendo utilizado para o desenvolvimento de polímeros que possam mimetizar as características da matriz extracelular, exercendo assim um papel ativo na restauração tecidual.

Após a produção de um biomaterial, uma das principais etapas para a avaliação deste composto, seja ele material polimérico ou não, é testá-lo como substrato para o crescimento de células em cultura. Desta forma é possível aferir de maneira rápida e satisfatória e com alta reprodutibilidade a possível toxicidade do dispositivo em questão (ISO 10993-5, 1992(E); MALMONGE *et al.*, 1999). Uma vez que o material seja classificado como não citotóxico, ele passa a ser avaliado quanto a sua capacidade de interagir com células em cultura ou com tecidos em animais de experimentação. Normalmente, para que ocorra uma boa interação polímero-célula é necessário que se estabeleça a adesão celular ao substrato. Embora o substrato não necessite obrigatoriamente apresentar características semelhantes às da matriz extracelular para que a adesão celular ocorra, a similaridade físico-química é desejada quando o objetivo é a promoção da

diferenciação celular ou para que um determinado polímero tenha uma interação mais efetiva no sítio de implantação (LANGER & VACANTI, 1993; HUBBELL, 1995). Desta forma, atualmente busca-se a produção de polímeros que apresentem características físico-químicas e mecânicas – tais como hidrofilicidade/hidrofobicidade, disposição de cargas elétricas, dureza, elasticidade, resistencia – o mais próximas possíveis aos tecidos nos quais serão implantados. A boa integração do biomaterial com células ou tecidos depende ainda da própria estrutura dos dispositivos produzidos. Alguns trabalhos mostram que materiais porosos promovem o crescimento celular, bem como induzem as células a produzir componentes de matriz extracelular. A distribuição uniforme e as interconexões dos poros são importantes para facilitar a formação de tecidos na forma de uma rede organizada, tendo grande aplicação na reconstrução tecidual (WALD *et al.*, 1993; van SLIEDREGT *et al.*, 1994; ZOPPI *et al.*, 1999). Para isso, é necessário em primeiro lugar, que o composto poroso seja capaz de estimular não apenas o crescimento das células, mas também sua proliferação. A porosidade e interconexão dos poros são essenciais para a proliferação de vasos, facilitando a nutrição do tecido ao redor do implante.

A cultura de células sobre diferentes biomateriais permite ainda que se possa realizar o implante de enxertos em tecidos lesados. Este tido de dispositivo é denominado *auto-enxerto* ou *implante autólogo*[1] (HENCH, 1998). Esta técnica é realizada com a utilização de células sadias provenientes do próprio paciente onde o polímero será implantado. O transplante autólogo apresenta algumas vantagens sobre o transplante de

[1] Os enxertos teciduais e transplantes podem ser classificados geralmente como *autólogos* (feito com tecidos do próprio indivíduo), *isólogos* (entre indivíduos diferentes porém geneticamente iguais e de mesma espécie), *alólogos* (entre indivíduos diferentes, mas da mesma espécie) e *xenólogos* (entre indivíduos diferentes e de espécies diferentes).

órgãos. Pelo fato da população de células isoladas serem expandidas *in vitro* por meio de técnicas de cultura celular, somente um pequeno número de células do doador são necessárias para se preparar o implante. O uso de células autólogas permite ainda evitar problemas imunológicos como rejeições ou processos alérgicos (MIKOS *et al.*, 1994; van SLIEDREGT *et al.*, 1994; BARBANTI *et al.*, 1999; TEMENOFF & MIKOS, 2000).

Grandes variedades de dispositivos temporários vêm sendo utilizadas em sistemas biológicos, sendo que os mais empregados são os poliésteres derivados de α–hidroxi ácidos como o poli(L-ácido láctico) [PLLA] e o poli(ácido glicólico) [PGA] (TÖRMÄLÄ *et al.*, 1998). Estes materiais apresentam boa biofuncionabilidade. Em seu processo de degradação, o polímero é quebrado em unidades menores por hidrólise simples e os produtos de sua decomposição podem ser eliminados do corpo por vias metabólicas, como a via do ciclo do ácido cítrico, ou diretamente por excreção renal (HOLLINGER & BATTISTONE, 1986; AN *et al.*, 2000). A taxa de degradação do polímero depende de vários fatores, tais como o tamanho do implante, o tipo e o massa molar do material do qual é constituído, a morfologia do material (cristalina ou amorfa), a presença de aditivos ou impurezas em sua composição, o mecanismo de degradação (clivagem enzimática ou hidrólise), o sítio de implantação do polímero e até mesmo a idade do indivíduo (BARBANTI *et al.*, 1999; AN *et al.*, 2000).

Embora a degradação de polímeros bioabsorvíveis seja efetuada predominantemente por hidrólise simples, há relatos na literatura que a decomposição do PGA e do PLLA ao menos em parte também seja estimulada por enzimas (WILLIANS & MORT, 1977; LI *et al.*, 1990). Esquematicamente, a degradação por hidrólise dos α–hidroxi ácidos, como o

PLLA, pode ser vista da Figura 1. As formas de eliminação dos produtos de degradação de alguns poliésteres podem ser vistas na Figura 2.

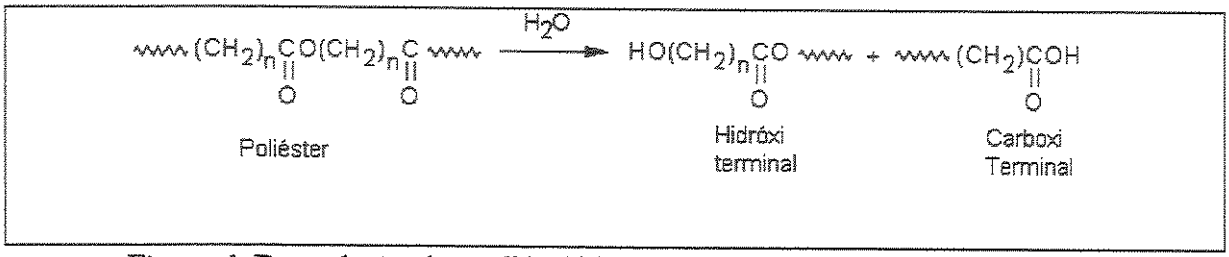

Figura 1. Degradação dos poli(α-hidróxi ácidos) pelo processo de hidrólise.

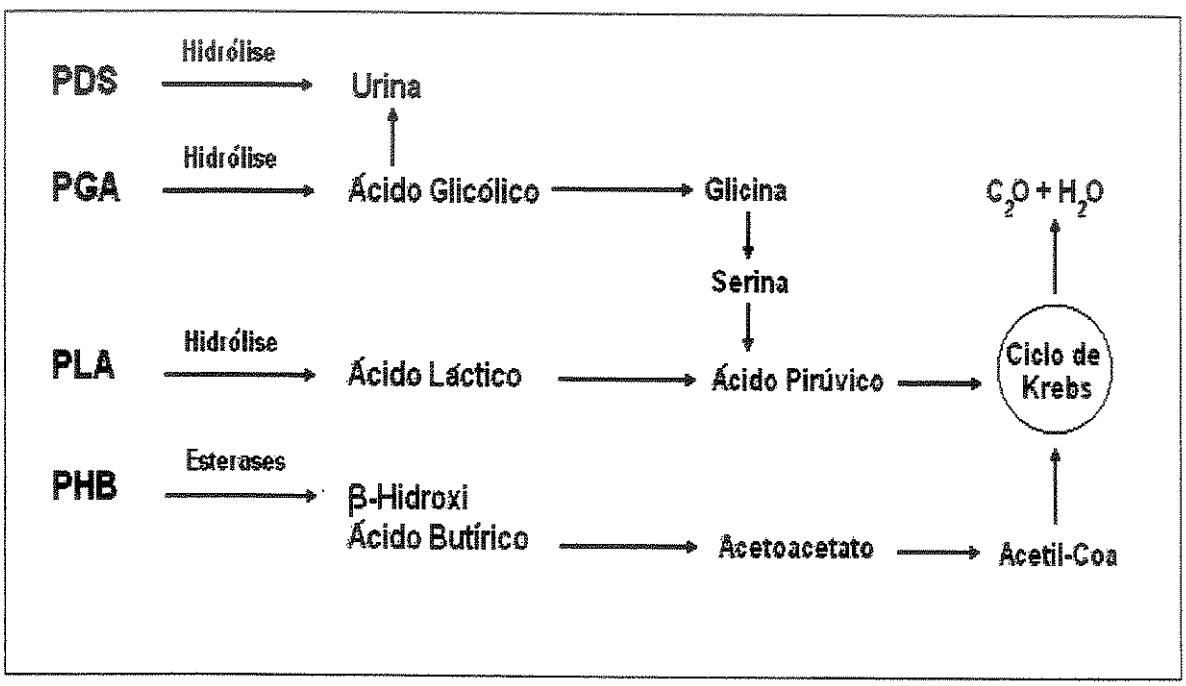

Figura 2. Via de degradação e excreção de alguns poliésteres: poli(*p*-dioxano) [PDS], poli(ácido glicólico) [PGA], poli(ácido láctico) [PLA] e poli(hidroxibutirato) [PHB] (Extraído e modificado de AN *et al.*, 2000).

MILLER e colaboradores (1977) acompanharam a taxa de degradação de blendas de poli(ácido láctico)/poli(ácido glicólico) [PLGA] em implantes orais, na parede abdominal e em ossos de ratos. Esses autores não encontraram variações na taxa de degradação desses polímeros em relação aos locais de implante. Foi mostrado, porém, que a degradação desses materiais pode variar de acordo com a proporção de PLLA em relação ao PGA. A degradação do PLLA é também comumente mais lenta (THOMSON *et al.*, 1995).

I.1. Dispositivos de PLLA para o tecido cartilaginoso

A utilização de materiais bioabsorvíveis no auxílio ao reparo da cartilagem articular vem sendo pesquisada nos últimos anos. O tecido cartilaginoso, uma vez danificado, apresenta pouca ou nenhuma capacidade regenerativa e determinadas lesões podem evoluir para alterações degenerativas bastante graves nas articulações (JUNQUEIRA & CARNEIRO, 1999; TEMENOFF & MIKOS, 2000). Além de não se conhecer ao certo os mecanismos de formação da cartilagem articular, existem poucos procedimentos clínicos alternativos à substituição da articulação por próteses que possam preencher pequenos defeitos, surgidos em decorrência de traumas ou doenças degenerativas. Desta forma, busca-se intensamente materiais que possam mimetizar o comportamento biomecânico da cartilagem articular objetivando a restauração das articulações (TEMENOFF & MIKOS, 2000). Dentre os diferentes compostos estudados para tal aplicação destacam-se alguns materiais poliméricos, tanto temporários como permanentes. Dentre os materiais bioabsorvíveis que vem sendo estudados para utilização como matriz cartilaginosa

temporária destacam-se os polímeros de PLLA e PGA bem como seus copolímeros e blendas.

FREED e colaboradores (1993) estudaram condrócitos cultivando-os sobre uma matriz fibrosa de PGA e membranas porosas de PLLA. Os resultados mostraram a neoformação de tecido cartilaginoso comparável a condrócitos cultivados em substratos de colágeno obtido de cartilagem articular. Nesta situação, os condrócitos foram capazes de crescer sobre esses polímeros por até seis meses, mantendo a forma do dispositivo original e resultando na formação de um tecido com características semelhantes às da cartilagem, inclusive com a formação de glicosaminoglicanos e colágeno tipos I e II (FREED *et al.*, 1993). Por outro lado, GRANDE *et al.* (1997) relatou que células cartilaginosas cultivadas em poliésteres como PLLA e PGA tendem a ter um aumento na síntese de proteoglicanos e de colágeno quando comparadas com células cultivadas em matriz colagênica. PUELACHER *et al.* (1994) estudaram o crescimento de condrócitos *in vitro* e *in vivo* em moldes compostos de PGA e PLLA que simulavam a morfologia da cartilagem nasal humana. Estes autores observaram que nestas estruturas houve a formação de um tecido que simulava as características da cartilagem hialina. Os resultados experimentais destas técnicas de reconstrução tecidual, uma vez aperfeiçoada, apresentam aplicações potenciais em ortopedia, cirurgia plástica reconstrutiva e cirurgia craniomaxilofacial. Além disso, foi demonstrada por a formação de uma estrutura com características teciduais semelhantes à cartilagem hialina após seis semanas, quando células pericondriais foram cultivadas sobre membranas de PLLA e implantadas na região condilar femural de coelhos (FREED *et al.* 1994; CHU *et al.*, 1995).

Células obtidas de cartilagem articular humana, mantidas em cultura sobre dispositivos constituídos por diferentes poliésteres bioabsorvíveis mostraram que o processo de adesão era proporcional a hidrofilicidade dos polímeros. Apesar disso, não foram observadas variações no espalhamento das células sobre os diferentes biomateriais. Embora as células estudadas tivessem uma menor adesividade às membranas de PLLA que ao PLGA, sobre o PLLA as células mostraram uma melhor capacidade proliferativa (ISHAUG-RILEY *et al.*, 1999). Também foi observado que condrócitos humanos cultivados em membranas de PLLA mostraram menor capacidade de produzir matriz cartilaginosa e colágeno tipo II em relação a membranas de PLGA. Por outro lado, sobre o PLLA, as células apresentaram maior capacidade de sintetizar colágeno tipo I (ROTTER *et al.*, 1998). Outros resultados interessantes foram relatados com suportes de PLLA utilizados para a reconstituição de lesões em meniscos. Foi observado que os implantes porosos puderam guiar o crescimento vascular para dentro da região lesada (KLOMPMAKER *et al.*, 1991). Mais recentemente, foi relatado a reconstrução de menisco canino utilizando copolímeros de ácido láctico/ε-caprolactona (de GROOT *et al.*, 1997). Estes resultados demonstram que os princípios da engenharia de tecidos com a utilização de materiais bioabsorvíveis compõem uma área de trabalho bastante promissora e certamente trará resultados bastante significativos em um futuro próximo.

Embora experimentalmente tenhamos várias informações sobre o crescimento e diferenciação de células crescendo sobre os diferentes biomateriais, raros são os trabalhos que se dispõem a avaliar o comportamento estrutural e biomecânico dos polímeros uma vez implantados em tecidos articulares animais (MALMONGE *et al.*, 2000). Desta forma a

busca por um material polimérico que melhor mimetize funcionalmente a cartilagem articular ainda persiste.

I.2. Dispositivos de PLLA para o tecido ósseo

Com o desenvolvimento metodológico aplicado a engenharia de tecidos, novos procedimentos passaram a ser utilizados na restauração óssea. O processo de regeneração óssea ideal requer quatro fatores principais: um *sinal morfogenético*, *células* que respondam a esse sinal, um *carreador* ou *veículo* que possa liberar esses sinais em regiões específicas e que possa servir como suporte para o crescimento celular e finalmente, se possível, uma boa *vascularização* na região de neoformação óssea (BURG *et al.*, 2000). Materiais poliméricos podem servir como suporte para o crescimento celular, permitindo a penetração de vasos sangüíneos e em alguns casos, até mesmo exercem atividade morfogenética. No caso de materiais bioabsorvíveis, eles são muitas vezes enriquecidos com hidroxiapatita, fatores de crescimento, proteínas morfogenéticas ósseas (BMPs) além de outros elementos ósseos, tornando-os muito eficientes no estímulo a neoformação óssea em regiões lesadas (AN *et al.*, 2000).

O transplante de diferentes tipos de células isoladas e cultivadas em substratos de PLLA e PGA vem sendo investigado como uma forma de substituição temporária de porções teciduais danificadas (LANGER & VACANTI, 1993). Foi observado que em copolímeros de PLGA quando implantados em ossos, concomitantemente a biodegradação do material ocorre a neoformação de tecido ósseo no local do implante. Além disso, o PLGA apresenta a vantagem adicional de sua degradação completa ser bastante variável,

podendo ocorrer em semanas ou em anos, dependendo da razão dos poliésteres presentes nos copolímeros (REED & GILDING, 1981).

Células osteoblásticas cultivadas em filmes de PLLA, PGA e PLGA mostraram um padrão de adesão e espalhamento celular bastante satisfatório, além de apresentarem a capacidade de crescer e proliferar sobre o substrato. Além disso, as células sobre estes polímeros demonstraram um aumento na atividade da enzima fosfatase alcalina, um marcador de diferenciação e atividade óssea, e na síntese de colágeno I (ISHAUG *et al.*, 1994). Resultados similares foram obtidos cultivando-se osteoblastos em dispositivos tridimensionais de PLGA. Neste caso, foi observado ainda a mineralização da matriz óssea produzida (ISHAUG *et al.*, 1997; ISHAUG-RILEY *et al.*, 1998). Interessante ressaltar que, mesmo células obtidas de medula óssea quando cultivadas em membranas porosas de PLGA e implantadas no interior de mesentério de ratos foram capazes de iniciar a formação ectópica de tecido ósseo (ISHAUG-RILEY *et al.*, 1997).

Outra abordagem bastante interessante é a adsorção aos polímeros de fatores que estimulem as células a se diferenciarem. Há relatos onde foi adicionada a forma recombinante da proteína morfogenética óssea humana tipo 2 (rhBMP-2) em membranas bioabsorvíveis. Nesse caso foi observado que, nos osteoblastos cultivados nos substratos com rhBMP-2, houve uma maior produção de matriz óssea em relação aos controles (LEE *et al.*, 1994; WHANG *et al.*, 1998). Experimento semelhante foi realizado por HOLLINGER *et al.* (1998) onde a rhBMP-2 foi adsorvida a uma matriz de colágeno I. Quando esta matriz colagênica foi implantada em porções fraturadas de ossos, houve a neoformação de tecido ósseo bem como a integração do implante com o osso lesado.

Embora os resultados obtidos sejam bastante animadores, esta abordagem terapêutica parece ser limitada pelo tamanho da fratura produzida (LEE *et al.*, 1994).

I.3. Vantagens do PLLA

As vantagens da utilização de compostos bioabsorvíveis são inúmeras em relação a outros procedimentos cirúrgicos mais tradicionais. Por exemplo, dispositivos de fixação interna, utilizados em cirurgias ortopédicas, perdem sua função de manter os tecidos unidos quando a recomposição estrutural se conclui. Implantes bioabsorvíveis para fixação interna apresentam a vantagem de eliminar uma segunda intervenção cirúrgica para sua remoção. Eliminam-se também os riscos com implantes metálicos tais como corrosão ou atrito com o osso (BÖSTMAN & PIHLAJAMÄKI, 2000). O PLLA apresenta ainda a vantagem de induzir uma resposta inflamatória significativamente menor que o PGA. Em alguns relatos, reações teciduais adversas não foram sequer descritas (BÖSTMAN & PIHLAJAMÄKI, 2000). A estrutura do PLLA é mostrada na Figura 3.

Apesar de em algumas situações a utilização dos polímeros bioabsorvíveis ser vantajosa, os benefícios de seu emprego tornam-se insignificantes se a biocompatibilidade dos materiais é questionada. Na maioria dos casos, os efeitos adversos induzidos por polímeros bioabsorvíveis aos tecidos são causados por seus produtos de degradação. Nesse sentido, o PLLA é diferente e vantajoso em relação ao PGA por apresentar uma degradação mais lenta, o que acarreta uma liberação de produtos de degradação em menor quantidade. Além disso, ele é mais hidrofóbico por apresentar vários grupos metil, enquanto que o PGA é mais hidrofílico, mais ácido causando uma diminuição no pH tecidual no local do

implante, o que pode contribuir com as reações teciduais encontradas (BÖSTMAN & PIHLAJAMÄKI, 2000).

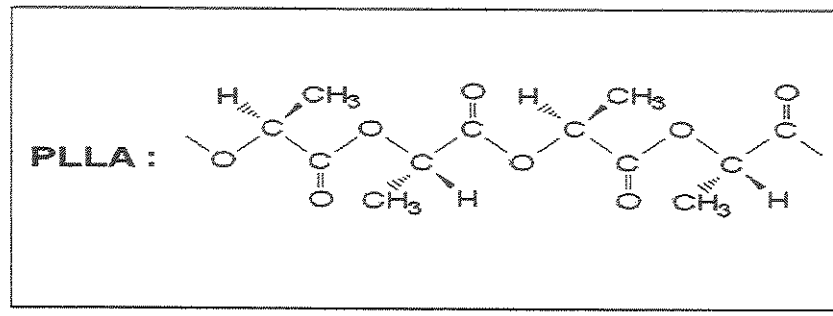

Figura 3. Estrutura molecular do poli(L-ácido láctico).

Tem sido postulado que materiais porosos implantados *in vivo* apresentam uma melhor integração com o tecido receptor. O diâmetro ideal dos poros para o crescimento tecidual ainda apresenta algumas discordâncias. Alguns autores falam em variações de diâmetro entre 300-400μm, outros dão intervalos ainda mais amplos na ordem de 200-400μm, ao passo que existem bons resultados onde os poros apresentam variações mínimas na ordem de 5-15μm (BURG *et al.*, 2000 em revisão). O diâmetro ideal dos poros é portanto algo a ser elucidado. O poli(hidroxibutirato-co-hidroxivalerato) [PHBV] também é um poliéster bioabsorvível, produzido por microorganismo, que vem sendo investigado recentemente. As blendas, misturas físicas de dois polímeros, podem ainda trazer algumas vantagens em relação aos polímeros puros pois podem apresentar algumas características mecânicas desejáveis que combinam propriedades de diferentes polímeros. O uso de blendas de PHBV com o PLLA é uma proposta nova na literatura e a avaliação biológica deste composto pode ser importante e de grande relevância no contexto da engenharia de tecidos.

II. OBJETIVOS DESTE TRABALHO

A cultura de células constitui-se uma ferramenta extremamente útil para a avaliação biológica dos biomateriais, seja para determinar sua possível toxicidade, ou a influência destes no processo de adesão, crescimento e diferenciação celular, sempre com o objetivo de melhorar a performance destes compostos. Tendo em vista que os testes *in vivo*, embora indispensáveis, apresentam custo elevado e demanda grande tempo, o teste *in vitro* é recomendado principalmente quando se tem um grande número de amostras que necessitam ser analisadas sob condições estritamente controladas. Isso permite que se faça uma pré-seleção rápida de materiais os quais serão posteriormente avaliados em animais de experimentação. Estamos inseridos em uma área de pesquisa multidisciplinar onde vários laboratórios trabalham em conjunto com o objetivo comum de desenvolver, selecionar e aperfeiçoar biomateriais poliméricos que possa ser utilizados futuramente como uma próteses ou para guiar/estimular o processo de regeneração de diferentes tecidos. Nossa contribuição consiste na avaliação *in vitro* de dispositivos a base do poliéster poli(L-ácido láctico).

Os objetivos específicos deste trabalho são:

1. Avaliar a adesão e a morfologia das células fibroblásticas cultivadas em membranas de poli(L-ácido láctico) densas e com poros de diferentes diâmetros (menor que 45μm, entre 180-250μm e entre 250-350μm).

2. Avaliar o padrão de crescimento, proliferação e diferenciação de células fibroblásticas para as membranas de poli(L-ácido láctico) densas e com poros de diferentes diâmetros (menor que 45μm, entre 180-250μm e entre 250-350μm).

3. Avaliar o comportamento de células fibroblásticas no que diz respeito ao padrão de adesão, morfologia e crescimento, sobre blendas de PLLA/PHBV em diferentes proporções (100/0, 60/40, 50/50,40/60, 0/100).

4. Avaliar se a adição gradual de PHBV às blendas com o PLLA é capaz de alterar o padrão de proliferação e diferenciação de células fibroblásticas cultivadas sobre eles.

III. ARTIGOS CIENTÍFICOS

III.1.

Vero Cell Growth and Differentiation on Poly(L-Lactic Acid) Membranes of Different Pore Diameter

Artificial Organs
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Vero Cell Growth and Differentiation on Poly(L-Lactic Acid) Membranes of Different Pore Diameters

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Abstract: In the last few years, the demand has increased for research on polymeric materials, which can be used as substitutes for injured tissues and organs or to improve their regeneration. In this work, we studied poly(L-lactic acid) (PLLA) membranes, a resorbable biomaterial, which were either dense or had different pore diameters (less than 45 μm, between 180 and 250 μm, and between 250 and 350 μm), in relation to stimulation of cell adhesion, growth, and differentiation in vitro. We used Vero cells, a fibroblastic cell line, as the biological model of investigation. We found that cells attached slowly to all PLLA membranes studied. On the other hand, once the adhesion occurs, the cells are able to grow and differentiate on the different polymers. The cells grew to form a confluent monolayer and were capable of producing collagen Type IV and fibronectin on different PLLA membranes. This behavior indicates that cells try to create a better environment to stimulate their growth. This also indicates that Vero cells alter their differentiation pattern once they are producing extracellular matrix molecules related to epithelial differentiation. **Key Words:** Biomaterials—Cell culture—Cell adhesion—Cell growth—Cell differentiation—Poly(L-lactic acid).

Recently, the use of biomaterials for the restoration of damaged human body parts has increased. This has stimulated the development of new research technologies for polymeric materials that can be used as substitutes for injured tissues and organs or to improve the regeneration of the original tissues or organs. The devices that can be used in implants can be classified as permanent or temporary material. Permanent materials include prostheses which are used to substitute the damaged parts, such as the knee, hip, shoulder, elbow, fist, and so on, for an unspecified period of time (1). Temporary materials are components that are degraded in vitro and in vivo (1). These are used in the restoration of injured tissues until the wound has healed. In both cases, for the utilization of biomaterials, it is very important to know the pattern of cell growth and differentiation in relation to these biomaterials because this behavior could be important for the development of biopolymers designed to stimulate the regeneration of damaged tissues (1,2).

Poly(L-lactic acid) (PLLA) is a temporary biomaterial whose degradation in vitro or in vivo occurs by hydrolysis (2). PLLA devices are frequently used as supports for cell culture (3) or experimental treatment of some damaged animal tissues, such as cartilage (4) or bone (5,6), mainly due to their very good biocompatibility (7). In this work, we evaluate the PLLA membranes with different pore diameters as a support for cell culture. In a biomaterial device, a porous and mechanically stable structure is important and desirable for improving cell growth. Uniformly distributed and interconnected pores in a polymer should permit cell distribution throughout the device and organize a tissue-like structure.

Cell culture is a very important methodology for biomaterials research because it permits a fast evaluation of the biological performance of the biopoly-

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mers. Thus, it is possible to search quickly for the best samples to be used in experimental animals. Our aim is the biological evaluation of PLLA membranes, either dense or with different pore diameters, to stimulate cell adhesion, growth, and differentiation in vitro. It is important to evaluate the cell–biomaterial interaction for the prediction of possible reactions to these polymers in vivo when used as substitute body parts or to stimulate the regeneration of damaged tissues.

MATERIALS AND METHODS

Preparation of PLLA membranes

Dense membranes

PLLA membranes were prepared by dissolving PLLA (molecular weight, 300,000) (Medisorb Technologies International L.P., Cincinnati, OH, U.S.A.) at room temperature in 10% chloroform solutions (wt/vol) (Merck KGaA, Darmstadt, Germany). After polymer dissolving, the solution was transferred to petri dishes. The drying procedure was made by casting in a closed chamber connected by compressed dry air flow (0.01 nm^3/h) for 24 h.

Porous membranes

The porous PLLA membranes were made by dissolving PLLA in 10% chloroform solution (wt/vol) at room temperature. Additionally, 40 g of sodium citrate (Fluka Cheme, Buchs, Switzerland) with the salt diameter previously sieved (grain diameter < 45 μm, 180–250 μm, and 250–350 μm) was added in portions of 10 g to 70 ml (PLLA-chloroform) solution to achieve a film having a pore volume of approximately 80%. The drying procedure was the same as described for the nonporous film. After drying, the films were washed in demineralized water for 24 h to remove the salt and subsequently washed for 6 h in ethanol. In both cases, the polymers were then dried and maintained in vacuum for 5 days to guarantee the total withdrawal of the solvent.

For cell culture utilization in all experiments, the dense or porous PLLA membranes were sterilized with 70% ethanol overnight. The sterility of samples was then tested. The samples were washed 3 times in Ham's F-10 medium without fetal calf serum (FCS) and then incubated in the same culture medium for 24 h at 37°C before cell inoculation.

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*) obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were used. These cells were cultured in Ham's F-10 medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 10% FCS (Nutricell Nutrients Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are a lineage that is recommended for studies of cytotoxicity and for cell–substratum interactions with biomaterials research (8).

Cell adhesion assay

A modification of Mosmann's method (9) was used for cell adhesion assay. Briefly, the sterilized PLLA membranes, both dense or with different pore diameters, were inoculated in a 96 well plate (Corning/Costar Corp., Cambridge, MA, U.S.A.) in culture medium for 24 h at 37°C. After this incubation time, 100 μl of cell suspension (1.0×10^5 cell/ml) in Ham's F-10 medium (Sigma) with 10% FCS (Nutricell) was inoculated in the wells with different PLLA membranes. Cell-free wells with the same culture conditions were used as control reactions. The cells were cultured for 2 h in Ham's F-10 with 10% FCS at 37°C, washed twice with 0.1 *M* phosphate-buffered saline (PBS) in pH 7.4 at 37°C, and then received 100 μl of medium without FCS and with 10 μl of 3-(4,5-dimetiltiazol-2-il)-2,5 difenil bromete tetrazolium (MTT) (Sigma). After 4 h, 100 μl of isopropanol acid (Isofar Ind. Produtos Químicos, Jacaré, RJ, Brazil) was added. The wells were read in Multiskan Bichromatic Version 1.06 microplate reader with a 540 nm wave length. As a positive control, the culture plate itself (polypropilene) was used while silicone membranes served as a negative control. We also read the absorbance of all experimental conditions (dense or porous PLLA, negative or positive controls) without cells for control of MTT reaction. All experiments were done in triplicate.

Scanning electron microscopy

After detachment with trypsin-EDTA (Nutricell), 1.0×10^5 cells/ml were inoculated on the dense or porous PLLA, previously sterilized in Ham's F-10 medium (Sigma) with 10% FCS (Nutricell). As a control, we used cells cultured on glass coverslips in the same culture conditions. After 24 h of incubation, the samples were fixed in 3% glutaraldehyde (Sigma) in phosphate buffer 0.1 *M* at pH 7.2 for 45 min at 4°C, and postfixed with 1% OsO_4 (Sigma) for 2 h at 4°C. The specimens were then dehydrated through an ethanol series, critical point dried, and coated with gold sputturing. The coated specimens were viewed and photographed with a JEOL 300 scanning electron microscope (SEM). All experiments were done in triplicate.

Immunocytochemical analysis

After detachment with trypsin-EDTA (Nutricell), 1.0×10^5 cells/ml were cultured on the dense or po-

rous PLLA membranes in Ham's F-10 medium (Sigma) with 10% FCS (Nutricell). After 120 or 240 h of culture, the samples were fixed in Karnovsky fixative (paraformaldehyde 4% [Reagen Quimibras Ind. Química, Rio de Janeiro, RJ, Brazil]/glutaraldehyde 2.5% [Vetec Química Fina Ltda, Rocha, RJ, Brazil] in phosphate buffer 0.1 *M* at pH 7.2), dehydrated with an ethanol series, cleared with xylene, and included in paraplast. Seven-micrometer sections were obtained. The sections were stained with toluidine blue, pH 7.0 (Sigma), for morphological evaluation of samples. We also used monoclonal primary anticollagen Type IV (Sigma, clone Col-94, dilution 1:500) and anticellular fibronectin antibodies (Sigma, clone FN- 3E3, dilution 1:400), both obtained from mouse ascitic fluid. Secondary rabbit antimouse IgG FITC conjugate antibodies (Sigma, dilution 1:200) also were used. The material was washed in 0.1 *M* PBS, in pH 7.4, at 37°C and incubated for 1 h with bovine serum albumin 1% (BSA) (Sigma) in PBS to block nonspecific staining. The preparations were incubated overnight in a moist chamber at 4°C with monoclonal anticellular fibronectin or anticollagen Type IV. After washing in PBS at 37°C, the antigenic sites were observed using indirect marking with antimouse IgG FITC conjugated antibody treatment. The samples were observed with an inverted microscope (Olympus IX50) equipped for fluorescence analysis. Control experiments were performed with the omission of primary antibodies. All experiments were done in triplicate.

RESULTS

Cell adhesion assay

After incubation in culture conditions, we found that, in all samples, the PLLA was less capable of stimulating cell adhesion than the positive controls (polypropylene) used. Actually, the results of cellular adhesion on PLLA membranes were very similar to the negative control (silicone) used. For statistical evaluation of the results, a one-way ANOVA was used. The result ($F = 20.93972$) was significant ($p = 0.000015$). Statistical differences among groups were detected by Tukey's *t* test at a 5% level of significance. Positive control mean is statistically different from all samples studied. The negative control mean and the different PLLA membranes means were considered not significantly different. The results are shown in Table 1 and in Fig. 1.

Scanning electron microscopy

With the SEM observations made, we could see cells growing on the glass coverslip forming a nonconfluent cell layer. These cells showed an irregular morphology, sometimes with an elongated form and some with microvilli and/or cell prolongations on their surface (Fig. 2a). The cells cultured on dense PLLA membranes showed a less elongated morphology and a great number of microvilli and/or vesicles on their cell surface. We also found that cell processes were more frequent (Figs. 2b and c). On the 45 μm pore PLLA membranes, we saw flattened cells with the largest number of microvilli found on their surface (Fig. 2d). We did not find cell prolongations linking one cell with another. On the 180 to 250 μm pore PLLA membranes, we found cells with an irregular morphology and many prolongations between the cells. Often, these prolongations formed a thin fibrillar, reticulated material on the cell surface (Fig. 2e). The cells cultured on the 250 to 350 μm pore PLLA membranes were very flattened with

TABLE 1. *Absorbance of the different samples studied in adhesion assay*

Samples	Substrate	Substrate + cells	Cells	SD
Positive control	0.1236	0.3910	0.2674	0.0164
Negative control	0.1603	0.2143	0.0540	0.0094
Dense PLLA	0.3436	0.4070	0.0634	0.0117
PLLA pd <45 μm	0.8573	0.8610	0.0370	0.0217
PLLA pd 180–250 μm	0.7913	0.8500	0.0587	0.0519
PLLA pd 250–350 μm	0.7286	0.8040	0.0754	0.0634

For statistical evaluation of the results, one-way ANOVA was used ($F = 20.93972$, $p = 0.000015$). Statistical differences among groups were detected by Tukey's test at a 5% level of significance. The positive control mean is statistically different from all samples studied. The negative control mean and the different PLLA membrane means were not significantly different.

PLLA: poly(L-lactic acid), pd: pore diameter.

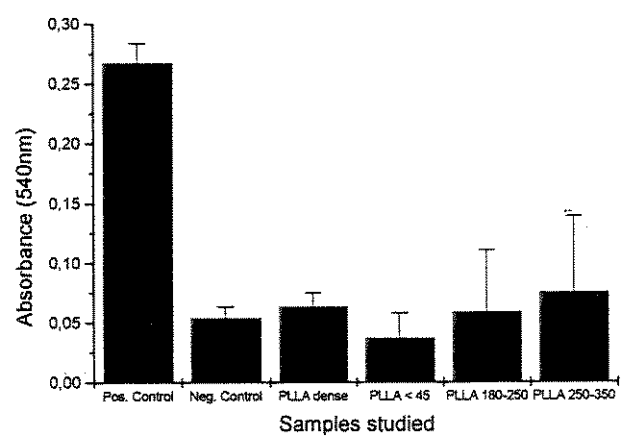

FIG. 1. The bar graph shows the adhesion of Vero cells on different PLLA membranes with 2 h of culture: positive control (polypropilene), negative control (silicone), dense membranes, less than 45 μm pore membranes, 180 to 250 μm pore membranes, and 250 to 350 μm pore membranes. All PLLA membranes studied showed similar adhesion patterns to the negative control used.

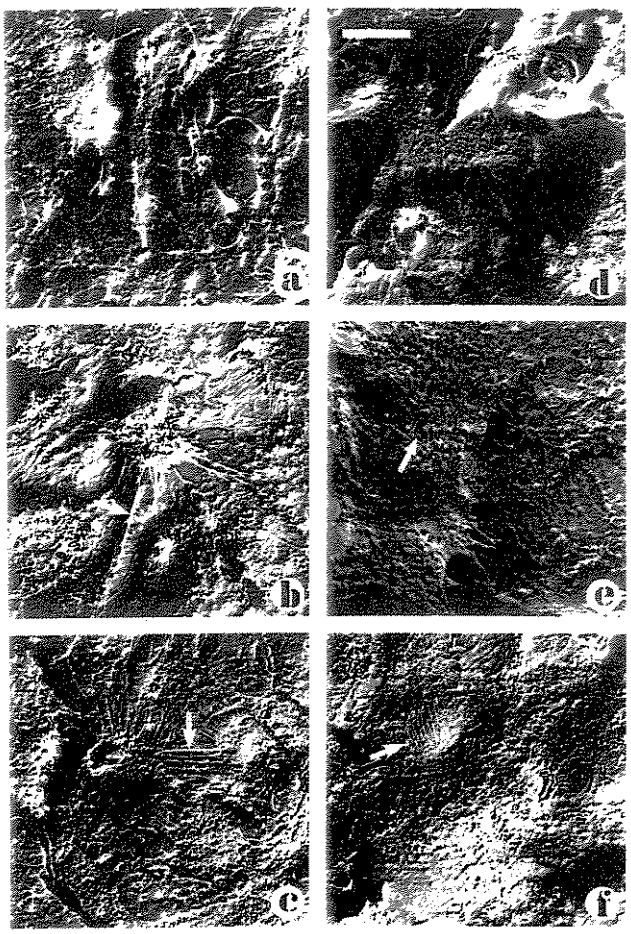

FIG. 2. The SEM images show Vero cells on different PLLA membranes cultured for 24 h: cells on glass coverslip **(a)** and cells on dense membranes **(b, c)** (note the cell processes **[arrow]** linking the cells to each other), cells cultured on less than 45 µm pore membranes **(d)**, and cells cultured on a 150 to 250 µm pore membrane **(e)** (note the fibrillar network-like material over the cells **[arrow]**), and cells cultured on a 250 to 350 µm pore membrane **(f)** (note the cell processes linking the cells **[arrow]**). The bar scale is 10 µm.

many microvilli and/or vesicles on their surface. In these samples, we also found cell processes linking the cells (Fig. 2f).

Immunocytochemical analysis

The morphological analysis made with toluidine blue showed flattened cells that grew on all PLLA membrane surfaces. The cells remained as a confluent monolayer. We did not find stratification on the surface of the different PLLA membranes. In the immunocytochemical test, we found a region rich in Type IV collagen and fibronectin (Fig. 3) produced by cells in all samples studied. The presence or the absence of a porous structure did not influence the production of these extracellular matrix molecules.

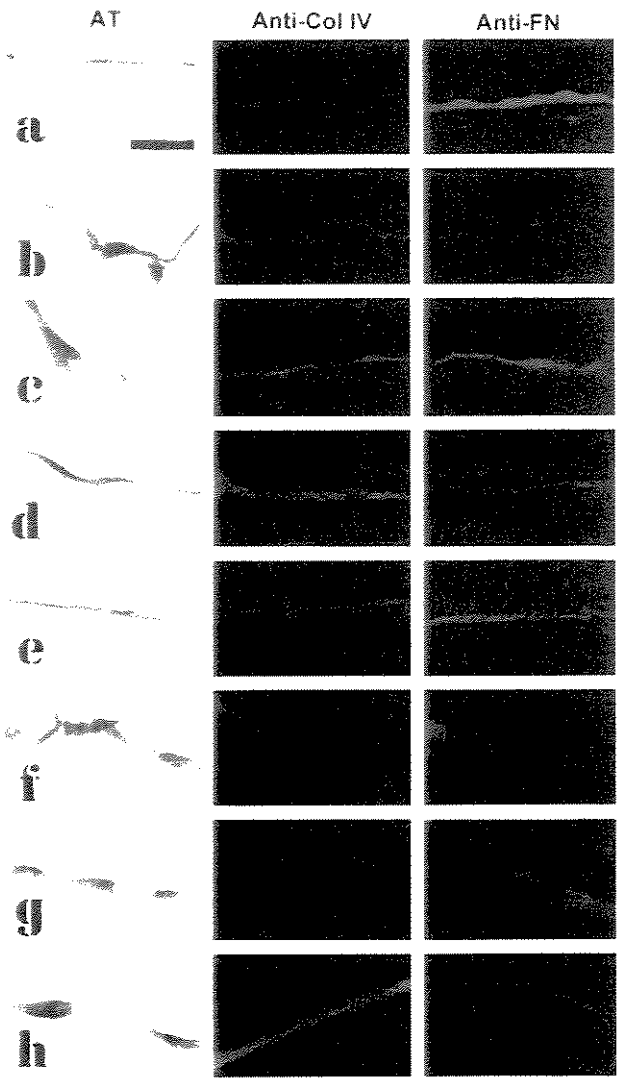

FIG. 3. Shown are the morphological (toluidine blue) and immunocytochemical (anticollagen Type 4 and antifibronectin) analyses of Vero cells cultured on PLLA membranes with different pore diameters: cells on dense PLLA membranes with 120 h of culture **(a)**, cells cultured on less than 45 µm pore PLLA membranes during 120 h **(b)**, cells cultured on a 150 to 250 µm pore PLLA membrane during 120 h **(c)**, cells cultured on a 250 to 350 µm pore PLLA membrane during 120 h **(d)**, cells on dense PLLA membranes with 240 h of culture **(e)**, cells cultured on less than 45 µm pore PLLA membranes during 240 h **(f)**, cells cultured on a 150 to 250 µm pore PLLA membrane during 240 h **(g)**, and cells cultured on a 250 to 350 µm pore PLLA membrane during 240 h **(h)**. The bar scale for all pictures is 25 µm.

DISCUSSION

The development of biomaterials that are capable of directing cell behavior is a rapidly improving research area. Applications of such materials are widespread and include devices for tissue replacement and regeneration as well as for cell culture substrates (2). The first step for cell interaction with a biomaterial is cellular adhesion. Mammalian cell adhesion

to polymer substrates is one of the key issues in tissue engineering, which rests on the ability to direct specific cell types to proliferate, migrate, and express physiological behaviors in order to yield a cellular architecture and organization performing the functions of the desired tissue (2,10). Thus, cell adhesion on PLLA devices was evaluated.

The PLLA membranes were less capable of stimulating cell adhesion than the positive controls used. Actually, the results of the adhesion assay were very similar to the negative controls (silicone), indicating that cells have difficulty attaching to this substrate. One explanation for this cell behavior on substrate is the hydrophobic characteristics of PLLA membranes. Surface hydrophobicity of polymer substrates has been shown to strongly influence cell adhesion, and it was reported that hydrophilic substrates promote the adhesion of cells whereas hydrophobic substrates do not (10–12).

Cell interaction with a biomaterial surface also depends on the composition and physical properties of the substratum (13). The adhesion of a cell to a substratum is modulated mainly by adhesion molecules and specific receptor structures on the surface to which they adhere (14). On a nonbiological surface, the adhesion is the consequence of protein adsorption on the substratum. Protein adsorption is a net of many complex interactions between and within all components, including the solid surface, the protein, and any other solutes present. These interactions include dipole and electric charge interaction forces, hydrogen bonds and electrostatic forces, and by topographical characteristics of polymer (15,16). Thus, the adhesion behavior of cells on PLLA membranes may be influenced, first, by the lack of receptors in the cell plasma membrane for the substratum, second, by the fact that the PLLA would have no electric charge in sufficient quantity to stimulate the adhesion of the cells, and, third, by the hydrophobicity of PLLA membranes.

The low adhesion capacity observed with Vero cells on PLLA membranes was expected by results previously reported with other hydrophobic biomaterials, such as poly(2-hydroxyethyl methacrylate) or their copolymer with methyl methacrylate and acrylic acid (17,18). Once the cells were capable of proliferating on PLLA substrates, they were able to attach to the polymer. Thus, we could conclude that cells adhere to PLLA slowly. On the other hand, low-adherent polymers are not necessarily a useless device. Mann et al. (19) recently showed that low-adherent materials are capable of stimulating the extracellular matrix production while in highly adherent biomaterials this capacity was decreased. Once the extracellular matrix production is a desirable condition for integration of polymer with the tissue structure in vivo, the characteristics of adhesion and extracellular matrix production must be evaluated to improve the efficiency of material in a tissue implantation.

The adhesion is a form of cellular communication and represents the way a cell senses its environment through contact. The interaction of cells with PLLA membranes is slow; once it occurs, the cells are capable of growing and proliferating on the PLLA membranes. We found a similar behavior pattern for adhesion and growth, as observed by SEM, of cells cultured on dense or porous PLLA membranes. It is possible that a larger pore diameter, supplying the cells with ample space to adhere, spread, and grow, gives them a culture condition similar to a dense membrane.

We found that, on all PLLA membranes studied, the cells are capable of growing and dividing up to the formation of confluent monocellular layers, but not multicellular layers. This behavior is similar to the normal growth pattern on nonbiological surfaces, such as a glass coverslip (20–22). On the other hand, it is different from growth patterns on a biological substratum, such as a three-dimensional Type I collagen gel (23) or dry collagen I sponges (24). The cells respond differently to substratum characteristics such as its composition, consistency, and flexibility (25). These characteristics determine whether the cells grow on bidimensional or three-dimensional environments. Thus, with different cell–substrate interactions, the cells respond with different growth patterns.

It has been shown that chondrocytes cultured on fibrous poly(glycolic acid) (PGA) or in porous PLLA with the pore size around 500 μm grew on both biomaterials, producing cartilage matrix composed of sulfated glycosaminoglycans and Type II collagen (4). PGA scaffolds also were implanted in cartilage defects of adult rabbits with or without chondrocytes cultured into them. It was found that cartilaginous repair tissue was observed after implantation of PGA either with or without cultured chondrocytes. Six months of repair was qualitatively better for cell–PGA allografts than for PGA alone (26). Similar results have been observed with bone cells. Osteoblasts cultured on biodegradable porous poly(lactic acid-co-glycolic acid) (PLGA) in three-dimensional scaffolds showed an increase in cell number, and the cells also showed the capacity of mineralized tissue formation (6). This same biomaterial, when implanted into rat mesentery, revealed the formation of mineralized bone-like tissue in the

constructs (27). In vitro bone formation also was investigated in porous PLGA scaffolds. In this study, the biomaterial had pore sizes ranging from 150 to 300 μm, 300 to 500 μm, and 500 to 710 μm. It was found that cells were capable of proliferating on biomaterial surfaces and made a deposition of mineralized matrix (5). In these experiments, variations of pore size did not promote behavioral alterations of the cells studied.

We found that morphological and growth patterns of cells were similar as described for cell culture on PLLA membranes obtained by the immersion precipitation process (28). In this study, we found that cell–substratum interactions were made by the production of extracellular matrix components. In all samples studied, we could find collagen Type IV and fibronectin. Type IV collagen is a member of the collagen family of proteins. It does not form fibrils but can create a network structure found in the basement membrane (29). The basement membrane is found in vivo, for example, between epithelial and connective tissues, separating each layer. Its production or destruction occurs normally through many physiological processes during differentiation and embryo development (30). The fibronectin is a glycoprotein of the extracellular matrix of connective tissues, but it can be expressed in many cultured cell types such as fibroblasts, mensenchymal cells, chondrocytes, muscle cells, and some types of epithelial cells (31). This protein is related to many biological processes such as cell adhesion, migration, and differentiation (31). The presence of pores, or the variation of their size, on PLLA membranes apparently does not influence the capacity of producing extracellular matrix molecules as reported here and in other experiments (4–6,26,27), but the pore structure is a desirable characteristic to improve the interaction of the polymer with the targeted tissue in an in vivo situation.

The basement membrane collagen Type IV is the extracellular material at the dermal–epidermal junction by which the epidermis adheres to native dermis or to regenerated connective tissue. Epidermal growth and differentiation are accelerated when viable fibroblasts are present in a dermal layer (32), and mesenchymal tissue may induce basement membrane formation (33). The fibronectin is an extracellular matrix protein related to epidermal differentiation. In multilayered epithelial tissue, it is present on the basal layer, and its expression is decreased with terminal differentiation of keratinocytes on the upper layer (34). Biodegradable materials have been used in experimental skin substitutes. PLLA polymers are related to a good dermal tissue restoration (35). A basement membrane formation, rich in collagen Type IV and laminin, by epidermal cells on copolymers of PLLA and PGA also was reported (36). Here, we found similar behavior of fibroblastic cells cultured on pure PLLA polymer. Thus, we suggest the possible utilization of PLLA for the development of grafts to improve skin restoration research.

CONCLUSIONS

Because porous devices are better indicated for in vivo restoration of damaged tissues, our results indicate that porous PLLA membranes showed good results. All PLLA membranes tested in vitro showed similar results on stimulating the production of extracellular matrix molecules, such as Type IV collagen and fibronectin. Previous results reported that, experimentally, the PLLA improves cartilage and bone regeneration. Our results suggest the possible utilization of PLLA in skin restoration because this polymer induced the production of some epithelial extracellular matrix molecules by fibroblastic cell, indicating that these cells alter their differentiation pattern on polymers. Research using animals should be directed toward this possible experimental approach.

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III.2.

Cytochemical and Proliferation Analysis of Fibroblastic Cells Cultured on Dense and Porous Poly(L-Lactic Acid) Membranes

Cytochemical and Proliferation Analysis of Fibroblastic Cells Cultured on Dense and Porous Poly(L-Lactic Acid) Membranes

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Abstract: The polyesters derived from α-hydroxi acids are experimentally employed for cell culture substrate, tissue restoration, and orthopedic prosthesis. The devices based on the poly(L-lactic acid) [PLLA] are the most frequently used mainly due to their atoxic characteristics and good biocompatibility. The objective of this work was to evaluate if some morphological characteristics of PLLA membranes (dense or with different pores diameters) could induce alteration on the growth pattern of cultured Vero cells, a fibroblastic cell line established from African green monkey (*Cercopithecus eothips*). It was observed that cells were capable of proliferate on dense and porous PLLA membranes in a similar way, except on lower pore diameters PLLA devices where cells showed a low mitotic rate. The cytochemical data showed high metabolic activity of cells on the different polymers. Taken together, our results showed that growth and proliferation pattern of fibroblastic cells on different PLLA device studied were satisfactory.

Key words: poly(L-lactic acid), membranes, scaffold, biomaterials, cell culture, cell proliferation.

Introduction: The utilization of bioabsorbable polymers for cell culture substrate represents an important research field for tissue engineering. A large variety of materials have been used in biological systems, where the polyesters derived from α-hydroxi acids are frequently employed [1]. The bioabsorbable material most commonly used is the device based on poly(L-lactic acid) [PLLA] polymers. The PLLA is a polyester used as support for cell culture or experimental treatment of many damaged tissues mainly due to their good biocompatibility [2]. Their degradation occurs by hydrolysis resulting in releasing of theirs atoxic degradation monomers that are eliminated from the organism by metabolic sources, such as citric acid cycle or by direct renal excretion [3,4].

Cell culture permits a fast, reproducible and trustful evaluation of the biological performance of the biopolymers. We have demonstrated the adhesion and differentiation behavior of fibroblastic cells on dense and porous PLLA devices [6]. Our objective in this work was the evaluation if those morphological characteristics of PLLA membranes could induce alterations on growth pattern of cells cultured on them. These parameters are important to evaluate the cell-biomaterial interaction for the prediction of the polymer integration with a target tissue once the polymer had been implanted *in vivo*.

Material and Methods: The dense membranes were prepared dissolving PLLA (Mw = 300,000, Medisorb Technologies, Cincinnati, OH, USA) in 10% chloroform (w/v) [MERCK KGaA, Darmstadt, Germany]. The drying was made by casting in a closed chamber with compressed dry air flow ($0.01 nm^3/h$) for 24 h. The porous PLLA membranes were made by dissolving PLLA in 10% chloroform with sodium citrate (Fluka Cheme,

Netherlands), which salt grain diameter was previously sieved (< 45μm, 180-250μm and 250-350μm) in order to achieve a film having a pore volume around 80%. The drying procedure was the same as described for the non-porous film. After drying, the films were washed in demineralized water for 24h to remove the salt and washed for 6h in ethanol. The polymers were then dried and maintained in vacuum until characterization. For morphological analysis, the membranes were fractured by immersion in liquid nitrogen, covered with gold with a sputter coater (Baltec SCD 050) and observed in a JEOL JXA-840A scanning electron microscope. The PLLA were also characterized by differential scanning calorimetric. The samples were heated from 25 to 200°C (10°C/min^{-1}) in STA 409 equipment (Netzsch Geratebau Gmbh). It was evaluated the vitreous transition temperature (*Tg*) and the melting temperature (*Tm*). The cristalinity level was determinate by: $[(\Delta H_{melting}/\Delta H_{100\%}]$ x100, where $\Delta H_{melting}$ is the melting enthalpy and the $\Delta H_{100\%}$ is the 93.7 J.g^{-1} [4,5].

For cell culture the dense or porous PLLA membranes were sterilized. The samples were washed three times in Ham F-10 medium (Sigma Chemical Co., St. Louis, MO, USA) and incubated in the same medium for 24h at 37°C before cell inoculation. Vero cells, a fibroblastic lineage, were used. These cells were cultured in Ham-F10 medium with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are recommended for studies of cytotoxicity and for cell-substratum interactions in biomaterial research [7].

For proliferation assay the method described by Murakami *et al.* was used [8]. 200μl of cell suspension (1.0 x 10^4 cell/ml) was inoculated on the different PLLA devices

in a 96 wells culture plate (Corning Corporation, Cambridge, MA, USA) at 37°C. The cells were cultured for 48h, 120h, 240h, 360h, washed with 0.1M phosphate buffered saline (PBS) in pH 7.4, fixed in formalin 10%, washed in PBS, and stained with crystal violet 0.05%. The samples were then washed with PBS and incubated with sodium citrate 0.1M (in 50% ethanol at pH 4.2). The wells were read in a Multiskan Bichromatic 1.06 microplate reader with 540nm wavelength. It was used as a proliferation positive control the culture plate itself and as a negative control some Teflon membranes. We read the absorbance of all experimental conditions in a cell-free condition for a dye staining control.

For cytochemical study, 1.0 x 10^5 cell/ml were cultured on the different PLLA membranes for 120 and 240h. The samples then were fixed in Karnovsky fixative, dehydrated with an ethanol series, cleared with xylene and included in paraplast. 5μm of sections were obtained and stained with toluidine blue at pH 4.0 (TB) or with xylidine ponceau at pH 2.5 (XP).

Results and Discussion: An area of research that has received increased attention is tissue engineering, in which functional tissue is restored from native or synthetic sources by using engineering principles. Biomaterials play an important role in many of these principles, serving as scaffolds to guide tissue regeneration or creating a new functional structure when damaged tissue does not regenerate. In order to be used in humans a biomaterial must be atoxic, to permit the cell adhesion, proliferation and, if is possible, the cell differentiation.

With morphological analysis of PLLA we could observe that all substrates had a smooth and regular lower surface because of the preparation procedure. We did not find

significance variations on membrane thickness. The dense PLLA membrane presents an irregular and rough upper surface, but the fracture analysis confirms that the material is compacted. The porous membranes showed a variation on pore diameters as expected. The fractures also showed a homogeneous pore distribution and their interconnections (Figure 1). We also determinate the polymer thermal properties (Table 1). We did not find significance variations on *Tg*, *Tm* or cristalinity level of different membranes as expected [5,9]. The materials have good thermal stability to be used as cell cultured scaffold or in an *in vivo* situation because their *Tg* occurs upper to physiological temperature. We also determinate the PLLA cristalinity, which is an important parameter for polymer degradation. The hydrolysis of material could be modulate by 2 factors: first, the localization of implant, because degradation is proportional to target tissue vascularization [3], and, second, the polymer crystallinity, once the water molecules enter preferentially in the amorphous and not in the crystalline regions of polymer [4].

We observed here that the fibroblastic cells did not show alterations on proliferation rate on different PLLA membranes, except on less diameter pore membranes (Figure 2). The cells grew until convergence and entered into pores, creating three-dimensional arrangements. Some studies showed that porous materials are able to stimulate cell proliferation and the uniformity of the distribution and the interconnectivity of the pores are important to facilitate the tissue formation in an organized network. We did not find cellular stratification on substrates. This data suggest that PLLA did not induce the lost of density/contact cell growth inhibition. Maybe this could explain the low mitotic rate found

in cells on less diameter pore membranes, because, the pore diameter of 45μm is quite bigger than cell size, what presents a little area for cell proliferation.

Different materials based on PLLA have been used as cell culture substrate. Condrocytes cultured on bioabsorbable scaffolds showed formation of a new cartilaginous matrix. On the different substrates used, the cells on poly(glycolic acid) have shown a better adhesion capacity, but on the PLLA, the cell proliferation was higher [10,11]. PLLA devices have been utilized to support the growth of endothelial cells. It was found that the polymers were capable of improve cell adhesion and proliferation without stimulate the platelet activation [12]. Ocular cells cultured on PLLA substrates showed also an increase on their proliferation and differentiation capacity [13]. Recently, it was reported that extracellular matrix proteins, such as fibronectin, laminin and collagen, could improve the cell multiplication on absorbable scaffolds [14]. We have previously demonstrated that Vero cells can produce fibronectin and collagen IV on PLLA membranes [6]. Maybe its behavior is related with the good proliferation pattern observed on this work.

With cytochemical analysis made with TB we observed basophilic cell monolayer. When stained with XP, we found cells with a great acidophily (Figure 3). The TB is a basophilic dye that can stain PO_4^-, SO_3^- and COO^- anions, and at pH 4.0, the PO_4^- are found only in DNA or RNA, while SO_3^- and/or COO^- groups occur in glycosaminoglycans [15,16]. We also stained our samples with alcian blue at pH 2.5, which evidenced only glycosaminoglycans. We did not find any stained group (dada not shown). Thus, cell basophily indicates that the cytoplasmatic staining into cells is due to high RNA concentrations, suggesting that cells are able to perform active protein synthesis. The XP at

pH 2.5 is anionic dye that can stain NH_3^+ groups detected in all protein. [15,16]. Thus, XP showed that cells had an abundant quantity of proteins, confirming the data obtained by TB. Taken together, cytochemical data indicates a high metabolic activity of cells on the polymers and that activity is not changed by the different PLLA membranes. Similar results were found in Vero cells cultured on others biomaterials [17,18]. Due to an intense search for better biomaterials, the clinical applications of reabsorbable polymers have been increasing. Various studies have been made to search for biomaterials that could help in repairing of many tissues [1,19]. With this knowledge, biomaterials could be used with cell culture techniques, to create tissue-like structures that simulate the mechanics and physiological characteristics of tissues manipulating healing to stimulate the natural tissue regeneration. For these proposes, the behavior of different cell types on the biomaterials needs to be known.

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LEGEND FIGURES

Figure 1. Scanning electron microscopy of different PLLA membranes. (A) Dense PLLA membrane, (B) less than 45μm pores PLLA membrane, (C) 180-250μm pores PLLA membrane, and (D) 250-350μm pores PLLA membrane. Scale bar: 250μm.

Figure 2. Growth curve of Vero cells cultured on dense or porous PLLA membranes. We could see the low proliferation rate on less diameter pore membranes.

Figure 3. Cytochemical analysis of cells cultured on different PLLA membranes. (A) and (I) dense PLLA membrane cultured for 120h; (B) and (J) with less than 45μm pores PLLA membrane cultured for 120h; (C) and (K) 180-250μm pores PLLA membrane cultured for 120h; (D) and (L) 250-350μm pores PLLA membrane cultured for 120h; (E) and (M) dense PLLA membrane cultured for 240h; (F) and (N) with less than 45μm pores PLLA membrane cultured for 240h; (G) and (O) 180-250μm pores PLLA membrane cultured for 240h; (H) and (P) 250-350μm pores PLLA membrane cultured for 240h. From (A) to (H) samples stained with AT pH 4.0, and from (I) to (P) stained with XP pH2.5. Scale bar: 25μm.

Figure 1

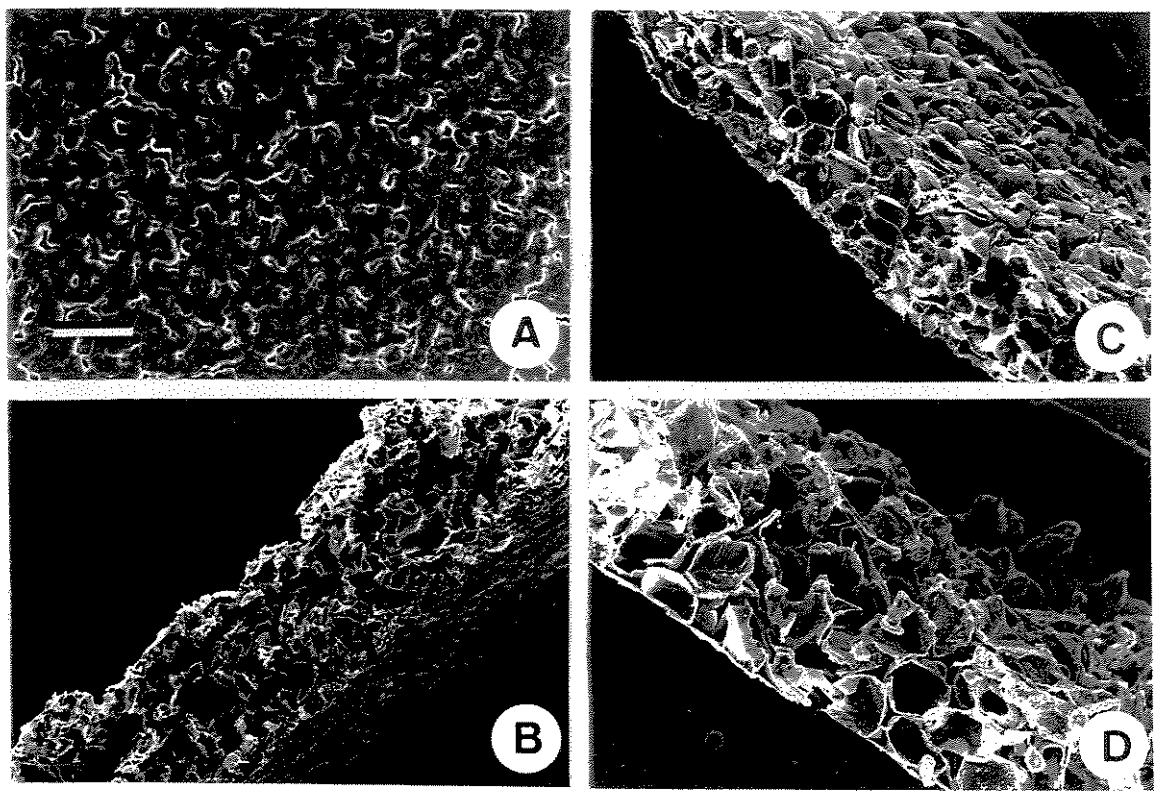

Figure 2

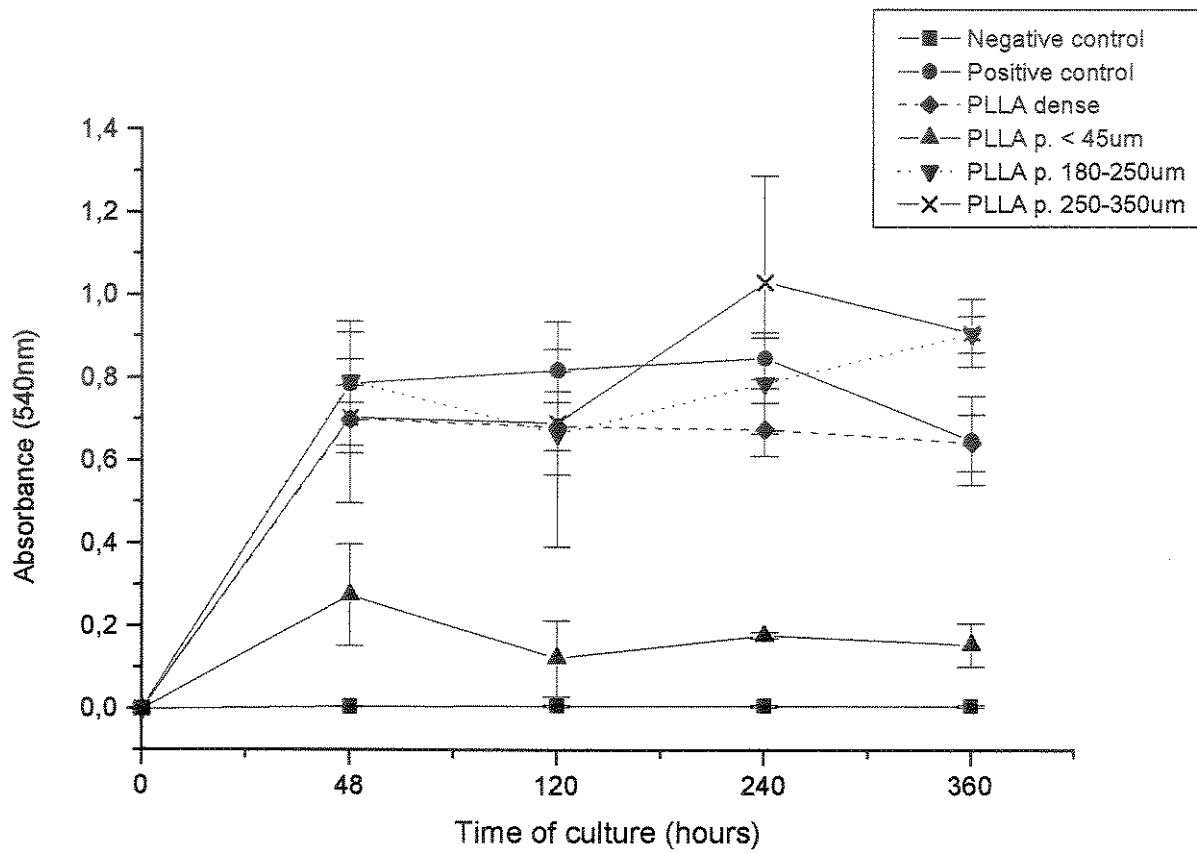
Negative control
Positive control
PLLA dense
PLLA p. < 45um
PLLA p. 180-250um
PLLA p. 250-350um
Absorbance (540nm)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
48
120
240
360
Time of culture (hours)

Figure 3

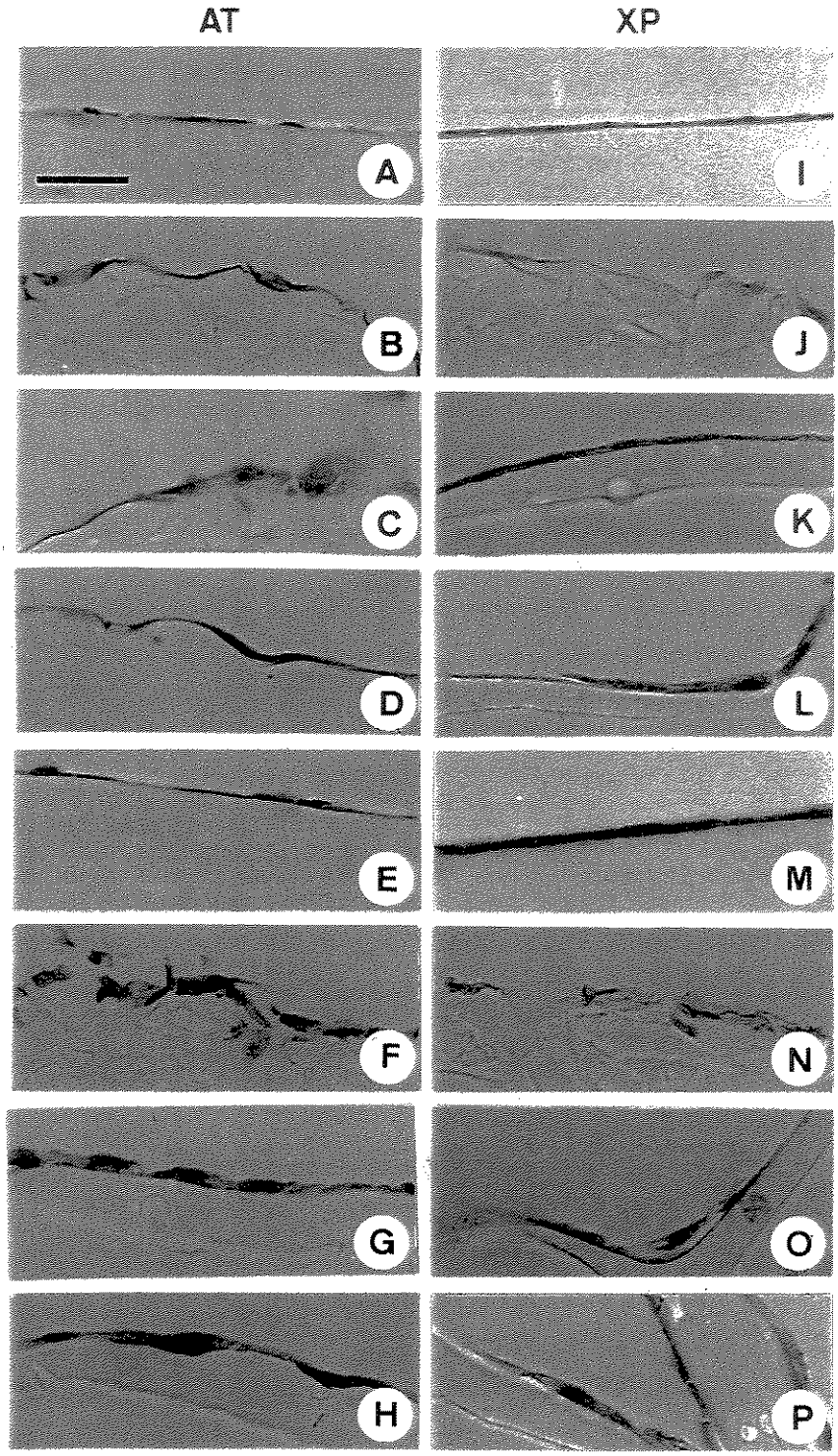

Table 1. Differential scanning calorimetric (DSC) analysis of dense or porous PLLA membranes.

PLLA membrane	***Tg*** **(°C)**	***Tm*** **(°C)**	**$\Delta H_{melting}$ (J/g)**	**Cristalinity (%)**
Dense	61	177	33	35
Pores < 45μm	60	178	38	40
Pores 180-250μm	59	178	37	40
Pores 250-350μm	59	178	35	38

Obs.: *Tg* represents the temperature of transition from solid to vitreous phase of PLLA devices; *Tm* is the melting point of substrates; $\Delta H_{melting}$ is the energy spent on PLLA fusion process.

III.3.

Adhesion and Morphology of Fibroblastic Cells Cultured on Different Polymeric Biomaterials

ADHESION AND MORPHOLOGY OF FIBROBLASTIC CELLS CULTURED ON DIFFERENT POLYMERIC BIOMATERIALS

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Running title: Adhesion of fibroblastic cells on biomaterials

Abstract

In vitro studies with of anchorage dependent cells allow the analysis of the interactions between the cell and the substrate by measuring the adhesion on material surface. Cellular adhesion is influenced by physical and chemical surface characteristics of the materials used as substrate for the cell culture. This work evaluates the morphological and chemical characteristics of different polymeric substrates in the adhesion and the morphology of fibroblastic cells. It was analysed the cellular growth on the surface of dense or different diameter pores poly(L-lactic acid) [PLLA] membranes, poly(2-hydroxy ethyl methacrylate) [polyHEMA], poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA] and poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)] hydrogels. The results of this study showed that cells adhered preferentially to more negativaly charged substrates and the polyHEMA hydrogels were found to be more adhesive than other samples studied. The pores present in the PLLA membranes did not interfere with the cellular adhesion capacity, but the cells have a distinguished morphlogical pattern for each membrane.

Keywords: Biomaterials, poly(L-lactic acid), polyHEMA, cell adhesion, cell growth, Vero cells.

INTRODUCTION

An area of research that has received increased attention recently is tissue engineering, in which functional tissue is restored from native or synthetic sources by using engineering principles. Biomaterials play an important role in many of these principles, by serving as scaffolds to guide tissue regeneration, releasing medicines and growth factors to stimulate tissue response, or create a new functional structure when damaged tissue do not regenerate. In this context, tissue engineering needed a clear understanding of cell responses to the biomaterial used for tissue restoration. With this acknowledge, biomaterials could be used with cell culture techniques, to create tissue-like structures that simulate the mechanics and physiological characteristics of tissues *in vivo*. Its devices could be also produced for manipulating healing and stimulate the natural tissue regeneration.

The devices that can be used in implants can be classified as biostable or permanent and bioabsorbable or temporary materials [1]. The biostable materials are the prosthesis, which are used to substitute the damaged body parts for unspecified period of time [2]. These type of materials need to retain their mechanical and chemical properties for years or decades *in vivo* [1]. On the other hand, in many cases the tissue need only the temporary presence of biomaterial support, for replacing tissue or to guide their growth during wound healing. The bioabsorbable polymers are components that are degraded *in vitro* and *in vivo*, disappearing along the time while the tissue is being repaired [2]. In both cases of the biomaterial utilization it is important to know the pattern of cell adhesion, growth and differentiation on these biomaterials.

One class of materials, which has been indicated for applications in tissue restoration, is the devices based on of poly(L-lactic acid) [PLLA] polymers. The PLLA is

biodegradable polyester frequently used as support for cell culture or experimental treatment of some damaged tissues mainly due their good biocompatibility [3, 4, 5, 6]. The PLLA degradation occurs by hydrolyses resulting in a gradually releasing of its degradation monomers[7]. PLLA devices have been prepared by different techniques such as phase inversion [8], addition of salt particles[9], plasticizing addition [10], incorporation of CO_2 [11], and by casting of solution [12].

Other biomaterial commonly used in tissue engineering research is the poly(2-hydroxy ethyl methacrylate) [polyHEMA]. This component form a hydrogel constituted by three-dimensional polymeric networks that are able to water swell without dissolving [13]. These materials present similarity to the extracellular matrix and, thus, represent a good model for cell culture studies with the objective of tissue restoration. The need to improve the mechanical characteristics of polyHEMA hydrogels for utilization as orthopedic implants has been studied. Chemical variations of this type of material have been used in the evaluation of the hydrophobic/hydrophilic properties such as the interaction between specific chemical groups that could promote changes on cell adhesion and growth pattern [14, 15, 16, 17, 18]. Furthermore, polyHEMA copolymers that simulate the articular cartilage matrix have been tested as alternative permanent prosthesis, and the presence of a $-COO^- H^+$ group in these copolymers structure introduces higher negative charges in polyHEMA changing the cell-polymer interactions [19, 20]

This work shows a study involving the evaluation of the structural and chemical characteristics of different polymeric substrates and its relation with cell interactions. It was evaluated the cellular adhesion, growth and morphological pattern of fibroblastic cells on surface of structural variations of PLLA or polyHEMA. The materials tested here were

dense and porous PLLA membranes, with a controlled variations on pore diameters, or polyHEMA, and their copolymers poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA] and poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)] hydrogels.

MATERIALS AND METHODS

Preparation of the biomaterials

Dense PLLA membranes: Poly(L-lactic acid) [PLLA] membranes were prepared by dissolving PLLA (with high pure an quality for medical utilization, Mw = 300,000, obtained of Medisorb Technologies International L.P., Cincinnati, OH, USA) at room temperature in 10% chloroform solution (w/v) [by MERCK KGaA, Darmstadt, Germany]. After polymer dissolving, the solution was transferred to Petri dishes. The drying procedure was made by casting in a closed chamber connected by compressed dry air flow ($0.01 nm^3/h$) for 24 h.

Porous PLLA membranes: The porous PLLA membranes were made by dissolving PLLA in 10% chloroform solution (w/v) at room temperature. Additionally, 40g of sodium citrate (Fluka Cheme, Netherlands) wich salt diameter previously sieved (grain diameter < 45μm; 180-250μm and 250-350μm) were added in portions of 10g to 70ml (PLLA-Chloroform) solution in order to achieve a film having a pore volume of approximately 80%. The drying procedure was the same as described for the non-porous film. After drying, the films were washed in demineralized water for 24h to remove the salt and subsequently washed for 6h

in ethanol. In both cases, the polymers were then vacuum dried and maintaied in dissicator for 5 days, to garantee the total withdrawal of the solvent.

PolyHEMA hydrogels: Three different hydrogels were used poly(2-hydroxy ethyl methacrylate) [polyHEMA], poly(2-hydroxy ethyl methacrylate)-cellulose acetate [polyHEMA-CA], and a poly(2-hydroxy ethyl methacrylate)-poly(methyl methacrylate-co-acrylic acid) [polyHEMA-poly(MMA-co-AA)] semi interpenetrating networks (sIPN). Hydrogel samples of 2 mm thickness sheet were obtained by thermal polymerization. Monomer, crosslinking agent (1.0% w/w) ant thermal initiator (0.5% w/w) were mixed under stirred and poured in a glass mold for polymerization. In the case of sIPN blend samples, the linear polymer, CA or poly(MMA-co-AA) was previously solved in the HEMA monomer, forming a polymer solution with 5.0% w/w concentration. After synthesis hydrogels were washed in distilled/deionized water to rinse residual monomer and initiator. Afterwards they were washed in a NaCl 0.15M solution until attaining a pH constant value equal to 7.0. The NaCl solution uptake of the different hydrogel samples was measured and expressed as the percentage of saline solution inside the gel at equilibrium. The fixed negative charge density (mEq COO^-/gel gram) was measured by titrimetric assay.

For cell culture utilization, all biomaterials used were sterilized and the sterility of samples was then tested. The samples were washed three times in Ham F-10 medium without fetal calf serum and then incubated in culture medium for 24h at 37°C before cell inoculation.

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were used. These cells were cultured in Ham-F10 medium (Sigma Chemical Co., St. Lois, MO, USA) supplemented with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are recommended for studies of cytotoxicity and cell-substratum interactions with biomaterial researchs [21, 22].

Cellular Adhesion Assay

A modification of Mosmann's method [23] was used. Briefly, the PLLA membranes or polyHEMA hydrogels, were incubated in a 96 wells plate (Corning/Costar Corporation, Cambridge, MA, USA) with culture medium for 24h at 37°C. After this incubation time, 100µl of a cell suspension (1.0×10^5 cell/ml) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was inoculated in the wells with different substrates. The cells were cultured for 2h at 37°C, washed twice with 0.1M phosphate buffered saline (PBS) in pH 7.4, at 37°C, and then received 100µl of medium without FCS and with 10µl of 3-(4,5-dimetiltiazol-2-il)-2,5 difenil bromete tetrazolium (MTT, Sigma). After 4h, 100µl of isopropanol acid (Isofar Ind. Produtos Químicos, Jacaré, RJ, Brazil) was added. The plate was read in a Multiskan Bichromatic Version 1.06 microplate reader at 540nm wavelength. As a positive adhesion control the culture plate itself (polypropilene) was used, while silicone adhesive membranes (Rhodiastic) served as a negative control. We also read the absorbance of all

experimental conditions (PLLA membranes, polyHEMA hydrogels, negative or positive controls) without cells for control of MTT reaction.

Light Microscopy analysis

The the cells were inoculated (1.0×10^5 cell/ml) on 24 well culture plates (Corning) with different biomaterials studied. After 48 hours of culture, the samples were fixed with paraformaldehyde 4% (in phosphate buffer 0.1M, at pH 7.2), and stained with Cresil Violet for morphological analysis. As control we used cells cultured on glass coverslip. We also made a cytotoxicity evaluation of substrates by the direct/indirect contact method [19, 22] by addition of glass coverslip into wells with the different biomaterials and for searching by signals of cell degeneration. All experiments were made in triplicate. The samples were observed in a Olympus IX-50 inverted microscope.

Scanning electron microscopy (SEM) analysis

For morphological analysis of different biomaterials used, the samples of PLLA and polyHEMA hydrogels were fractured by immersion into liquid nitrogen. The samples surface were gold sputtered and observed in a JEOL JXA-840 scanning electron microscope. For cell morphological analysis, 1.0×10^5 cell/ml were inoculated on PLLA membranes or on polyHEMA hydrogels, in Ham F-10 medium (Sigma) with 10% FCS (Nutricell). As a control, we used cells cultured on glass coverslips in the same culture conditions. After 24h, the samples were fixed in 2.5 % glutaraldehyde (Sigma) in phosphate buffer 0.1M, at pH 7.2, for 45 minutes at 4°C, and postfixed with 1% OsO_4 (Sigma) in the

same buffer for 2h, at 4°C. The specimens were then dehydrated through an ethanol series, critical point dried and gold sputtered. The samples were in a JEOL 300 scanning electron microscope.

RESULTS

Cellular Adhesion assay

The adhesion of fibroblastic cells was greater in the polyHEMA hydrogel than PLLA membranes. In all cases the cellular adhesion on the biomaterials was lower if compared to the case of the positive control, the polypropylene (Figure 1). The Figure 1a shows the cellular adhesion values on the hydrogels with different negative charge density. The adhesion values decreased while the negative charge density increased. The cell adhesion on porous PLLA membranes is showed in Figure 1b. We did not find significant variations on adhesion pattern of fibroblastic cells on dense and porous PLLA membranes.

Scanning electron microscopy analysis of different substrates

The Figure 2 shows the typical morphology of surfaces of polyHEMA hydrogel and dense or porous PLLA membranes obtained by SEM analysis. The hydrogel surface seems to be dense and smooth structure (Figure 2a) while the PLLA membrane presents a irregular and rough surface (Figure 2b). The morphology of the porous PLLA membranes can be seen from Figure 2c-2e. These membranes show a porous structure with different pore diameters. Porous PLLA membrane with diameter < 45μm (Figure 2c), diameter between 180 to 250μm (Figure 2d) and diameter between 250 to 350μm (Figure 2e).

Scanning electron microscopy of Vero cells growing on the different substrates

By SEM we observed cells forming a semi confluent layer on the glass coverslip. These cells showed commonly an elongated morphology with microvilli and/or cell prolongations on their surface (Fig. 3a). The cells cultured on polyHEMA hydrogel (Figure 3b) and dense PLLA membrane (Figure 3c) showed a less elongated morphology, also with microvilli and/or vesicles on their surface. In both cases it is observed flattened cells with cytoplasmatic prolongations linking the cells. On the less than 45μm pore PLLA membranes we could see flattened cells with the largest number of microvilli on their surface (Fig. 3d). On the 180-250μm pore PLLA membranes we found cells with an irregular morphology and many prolongations between the cells. Often these prolongations form a thin reticulated material on the cell surface (Fig. 3e-f). The cells cultured on the 250-350μm pore PLLA membranes were flattened, with many microvilli and/or vesicles on their surface. On these samples, we also found cellular processes linking the cells (Fig. 3g-h).

Light microscopy of Vero cells growing on the different substrates

In Figure 4 shows morphological aspects of Vero cells growing on the hydrogels with different negative charge density. We observed cells with irregular morphology on different substrates. Apparently we found cell more elongated on more negative charged hydrogels. The morphology of Vero cells on the PLLA membranes is showed on Figure 5. On those

substrates the observation is poor because this material is not translucent. In both cases, polyHEMA or PLLA biomaterials, we did not find signals of cell death.

DISCUSSION

The development of biomaterials capable of directing cell behavior is a improving research area. Applications of such materials are widespread and include devices for tissue replacement and regeneration as well as for cell culture substrates (Hubell 1995). The first step of cell interaction with a biomaterial is cellular adhesion. All the biomaterials used in this study showed a limited capacity of stimulating cell adhesion if compared to the positive controls used. The low adhesion observed on different substrates did not could be explained by any toxical effects of biomaterials over the cells, such demonstrated by light microscopic analysis.

The cellular adhesion is modulated mainly by adhesion molecules and specific receptor on cell membranes. On a synthetic surface, the cellular adhesion is the consequence of protein adsorption on the substrate, which is the result of many complex interactions including dipole and electric charge interaction forces, hydrogen bonds, electrostatic forces, hydrophilicity/hydrophobicity or surface free energy, and roughness and rigidity of the surface [10, 11, 18, 24]. We found that polyHEMA hydrogels were more adhesive than dense PLLA membranes. It is known that hydrogel show low values of surface tension, high wettability and a smooth surface, whereas the surface of PLLA membrane showed an irregular roughness surface with a globular morphology with concavities. These characteristics could explain preference of cells for hydrogel surfaces

than PLLA membranes. Other explanation for low cell adhesion on PLLA devices is its surface hydrophobic characteristics. It was reported that hydrophilic substrates promote the adhesion of cells, whereas hydrophobic substrates did not[4, 5, 7].

Some studies showed that porous materials are able to stimulate cell proliferation and the synthesis of extracellular matrix components. The uniformity of the distribution and the interconnectivity of the pores are important aspects to facilitate the tissue formation in a organized network, experimentally used in the repair of some types of damaged connective tissues. The variation in pore diameters could difficult the interactions between the cell and the substrate by reducing the area for cell attachment and thus, as a result there were a decreased of cellular adhesion. There were reports showing the cell behavior on large porous PLLA membranes, but the relation of variation of diameters of porous PLLA devices were not described. We found that variations on pore diameters of PLLA devices did not induce significant alterations on adhesion pattern of fibroblastic cells.

The applications of PLLA membranes for tissue regeneration have been studied in the last years. Some authors have shown the utilization of resorbable PLLA membranes in odontology, especially in periodontal disease. It was shown that membranes of this material are able to promote a guided tissue growth resulting in the osseous repair [6]. Other interesting utilization of resorbable devices based on PLLA is the adsorption of proteins on it, to stimulate cell growth and differentiation once the polymers were implanted *in vivo*. Using a poly(lactic acid-co-glycolic acid) [PLGA] matrix, Lee and collaborators [25] performed an experiment with the adsorption of a recombinant human bone morphogenetic protein (rhBMP-2), a protein able to promote the osteogenesis *in vitro* or *in vivo*. The rhBMP-2-PLGA matrix was capable of stimulation of new bone formation into osseous

fractures, improving the repair of the damaged tissue. The limitation of this approach was the size of the injury into bone [25].

The electrostatic interactions between cells and substrate are an important mechanism of adhesion once most of plasma membrane glycoproteins are negatively charged [26, 27]. In relation of polyHEMA hydrogels, it was verified that the negative charge density of material influences the cell adhesion. With increasing of negative charge density in material surface we obtained a decrease on cell adhesion and spreading. These results are in accordance with other reports that suggest that the positively charged materials are better indicate to adhesion, spreading and growth of cultured [17, 18, 28]. In the polyHEMA-poly(MMA-co-AA) blend, the COO^-H^+ groups from acrylic acid are ionized in the presence of the culture medium resulting in negative charges fixed to macromolecular network of hydrogel. This characteristic results in decreasing of the cell adhesion.

The results of this study confirmed the influence of the substrate surface properties, such as porosity and negative charge, on the fibroblastic cell adhesion. Although the biomaterials used did not stimulate cellular adhesion, the cells were capable to proliferate on theirs (dada do not shown). On the other hand, low adherent polymers are not necessarily useless devices. Low adherent biomaterials do not invalidate their biocompatibility and, in some cases, a low cell interaction is a desirable characteristic. In some experimental conditions low adhesive substrates are essential to maintain differentiated chondrocytes *in vitro* [29, 30]. Some researches believe that the presence of COO^-H^+ groups in some biabsorbable hydrogels cause a decrease with macrophages interaction, that could be a useful in some clinical applications [16, 31, 32] recently showed

that low adherent materials stimulate the extracellular matrix production while in highly adherent polymers this capacity was decreased. In this context, we have shown that fibroblastic cells cultured on dense or porous PLLA membranes are capable of production of extracellular matrix molecules [33]. Once the extracellular matrix production is a desirable condition for integration of polymer with the tissue structure *in vivo*, the characteristics of adhesion and extracellular matrix production must be evaluated to improve the efficiency of material for tissue implantation.

We found different morphological pattern in cells cultured on different substrates. The cells growing on PLLA membranes showed cell processes, some times liking each other, and a great number of microvilli on cell surface. These observations are similar as for the previously described for Vero cells cultured on PLLA membranes [33, 34]. The cells that grew on polyHEMA hydrogels showed flattened morphology, with microvilli and vesicles on cell surface. This morphological behavior was similar as previously described by Lombello et al. [28, 35]Alterations on cell morphology are related with the organization of cytoeskeletal network. It was previously reported that topography of polymers and rigid/flexible of its surface is clearly related with cell migration and functional activity of cytoskeletal [24, 36]. Thus, the physical characteristics of substrates could be related with morphological alterations observed here.

We also found that cells grew on different polymer forming a confluent monolayer. This behavior is similar to cell cultured on glass surfaces[37]. On the other hand, it is different from cell growth pattern than biological substrates, such as a three-dimensional type I collagen gel [38] or dry collagen I sponges [39] where the cells showed a multilayered structure formation. Thus, with different cell-substrate interactions, the cells

respond with different growth patterns. Once the morphology are related with cell function, there was the strong suggestion that cell are altering their differentiation pattern on different PLLA membranes or polyHEMA hydrogels used.

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Figure Legends

Figure 1 - Cell adhesion on different polymeric biomateial after 2h of incubation. a) cell adhesion on dense PLLA membranes on dense polyHEMA hydrogel, b) cell adhesion on porous PLLA membranes with different diameter pores, c) cell adhesion hydrogels with different negative charge density: polyHEMA (0,017mEq/g), polyHEMA-CA (0,02mEq/g) and polyHEMA-(MMA-co-AA) (0,11mEq/g). In all experimental samples we used a positive control (polypropilene) and a negative control (silicon adhesive).

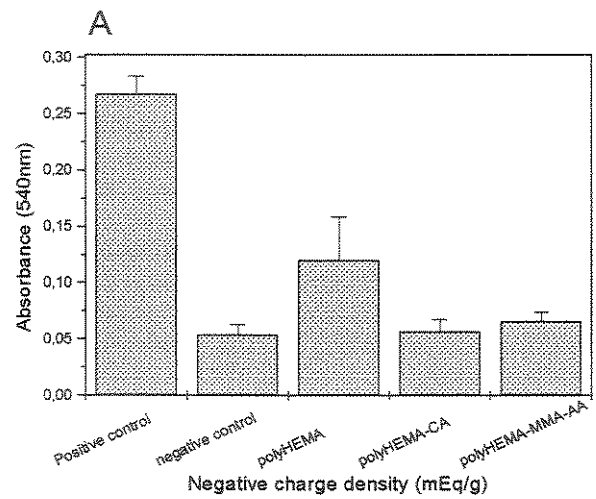
Figure 2 – Scanning electron microscopy of the different biomaterials used as substrate for cell culture. a) polyHEMA dense membrane surface, b) dense PLLA membrane, c) less than 45μm pores PLLA membrane, d) pores between 180-250 μm PLLA membrane, e) pores between 250-350 μm PLLA membrane. Barr scale: 100μm.

Figure 3 – Scanning electron microscopy of Vero cells growing on the different biomaterials used. a) on glass coverslip used as control of morphology, b) on polyHEMA dense membrane surface, c) on dense PLLA membrane, d) on less than 45μm pores PLLA membrane, e) and f) PLLA membrane with pores between 180-250μm, g) and h) PLLA membrane with pores between 250-350μm. Barr scale: 10μm.

Figure 4 – Ligth microscopy images of Vero cells growing on hydrogels membranes with different negative charge density values: (a) control, cells growing on glass coverships; (b) polyHEMA (0,017mEq/g); (c) polyHEMA-CA (0,02mEq/g) and (d) polyHEMA-(MMA-co-AA) (0,11mEq/g).

Figure 5 – Ligth microscopy images of Vero cells growing the different PLLA membranes. a) on glass coverslip used as control of morphology, b) on dense PLLA membrane surface, c) on less than 45μm pores PLLA membrane, d) on PLLA membrane with pores between 180-250μm, e) on PLLA membrane with pores between 250-350μm. In all samples b_1), c_1), d_1) and e_1) were shown the indirect cytotoxicity. Other samples were showed direct cytoxicity. We did not find any signal of cell degeneration on all samples studied. Barr scale: 10μm.

Figure 1


A
Absorbance (540nm)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Positive control
negative control
polyHEMA
polyHEMA-CA
polyHEMA-MMA-AA
Negative charge density (mEq/g)

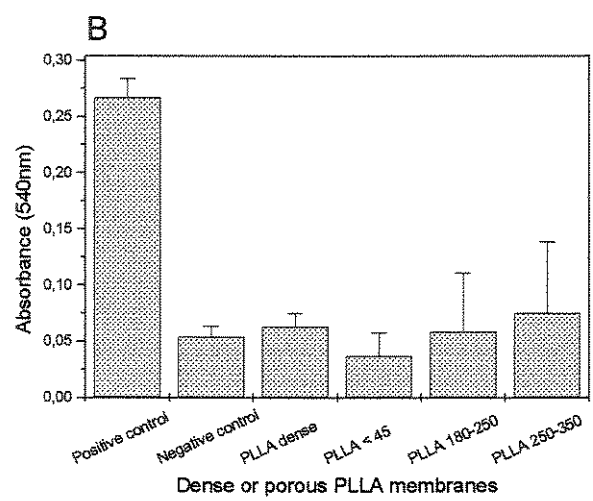

B
Absorbance (540nm)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Positive control
Negative control
PLLA dense
PLLA < 45
PLLA 180-250
PLLA 250-350
Dense or porous PLLA membranes

Figure 2

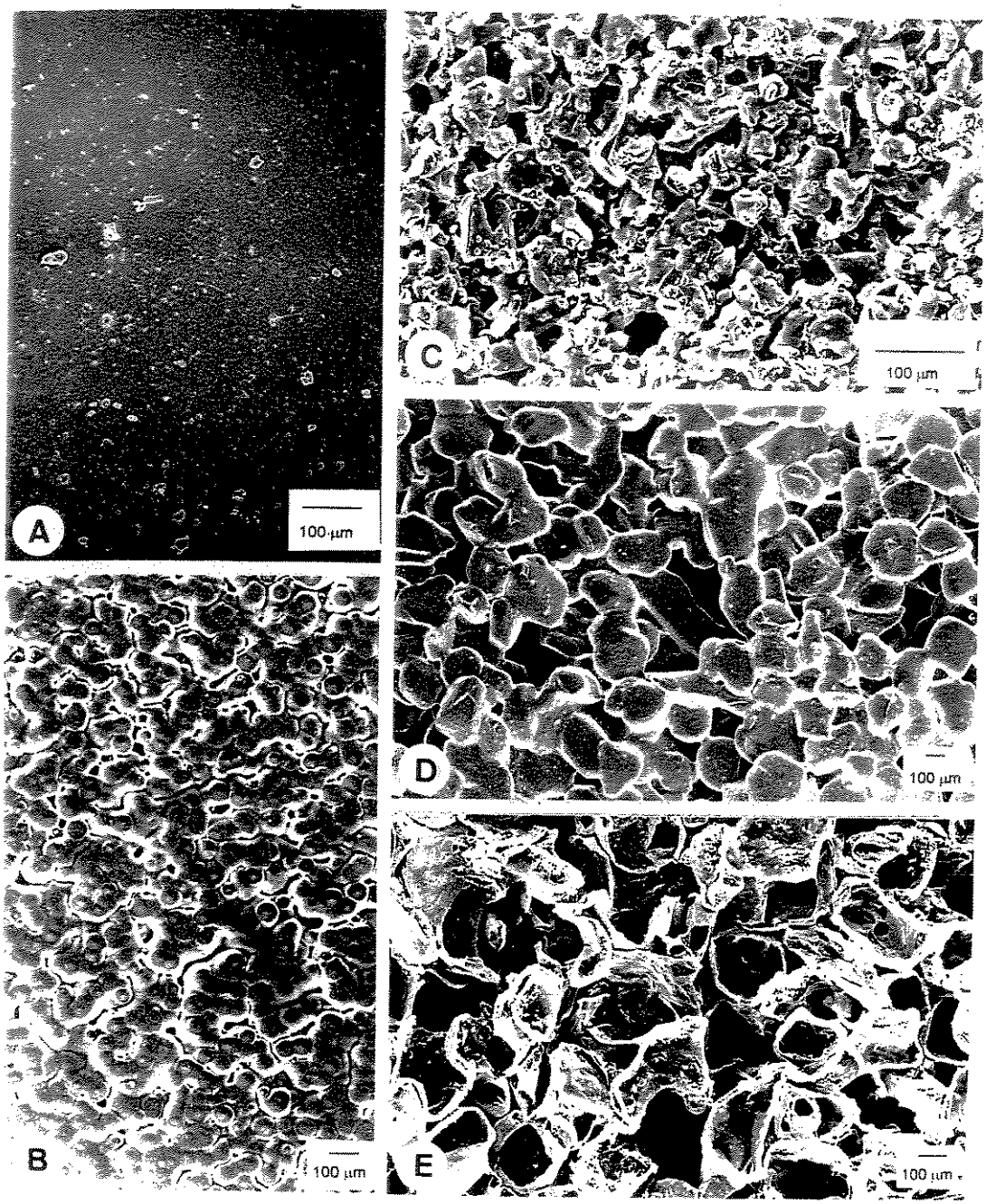


Figure 3

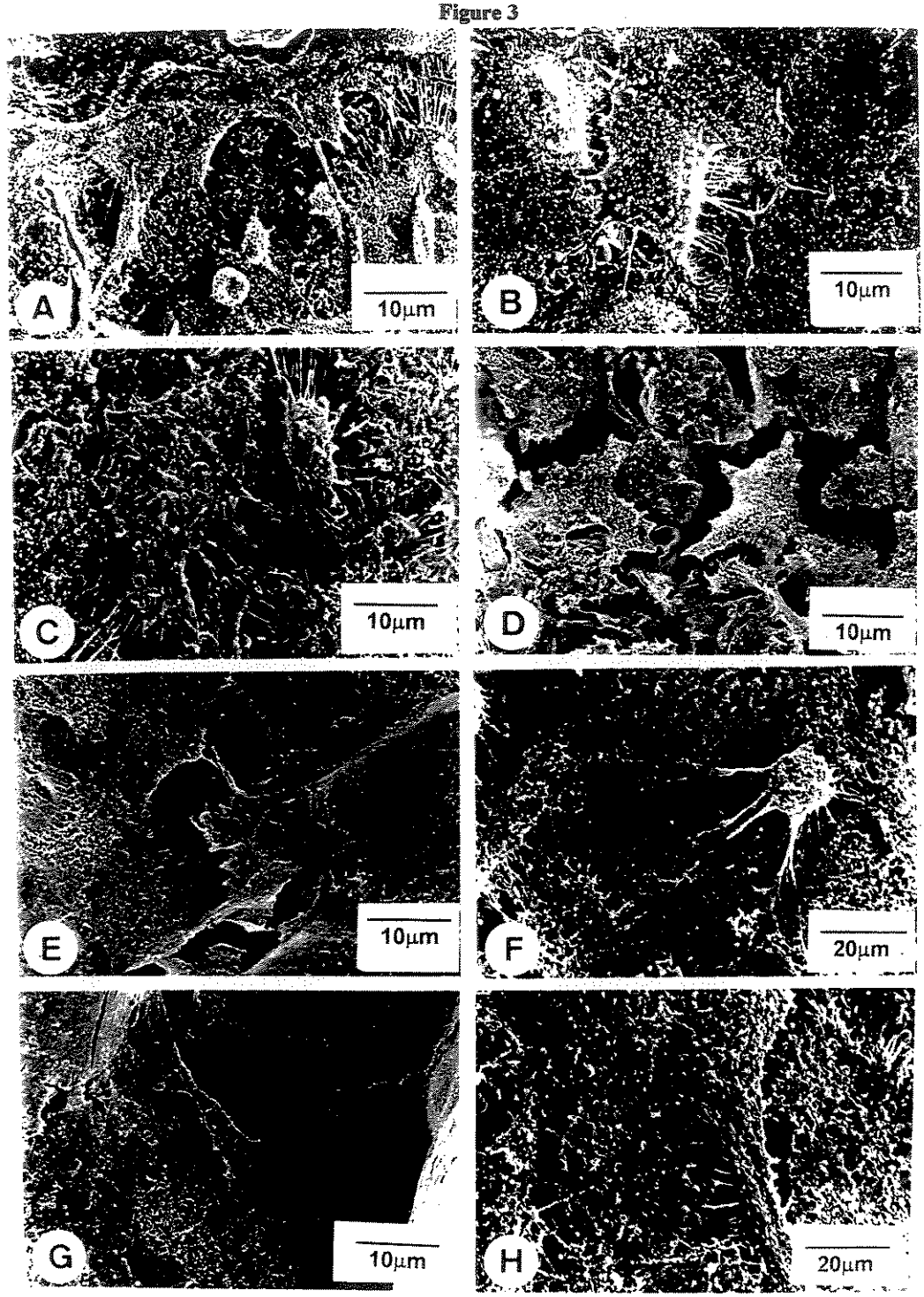


Figure 4

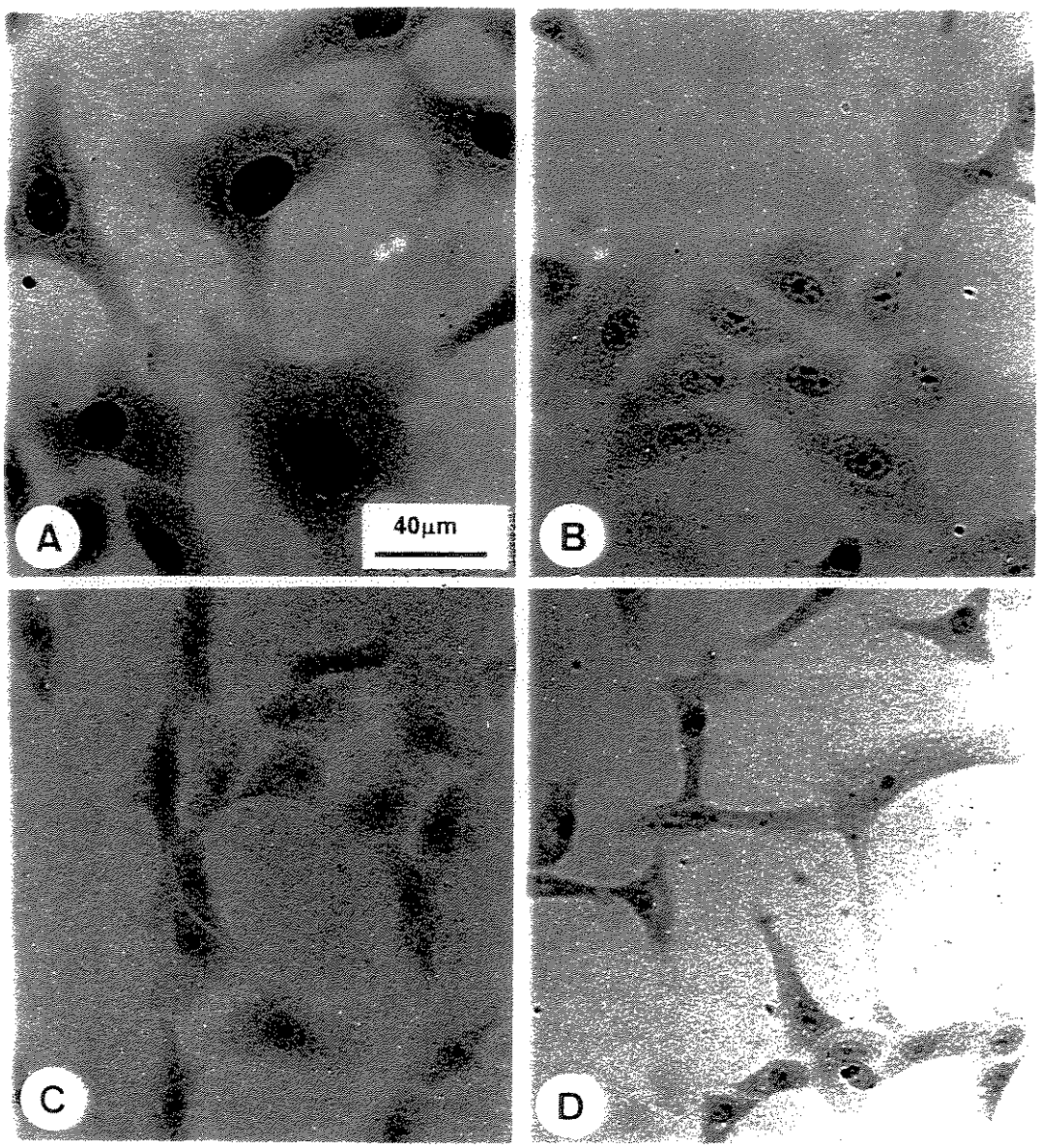
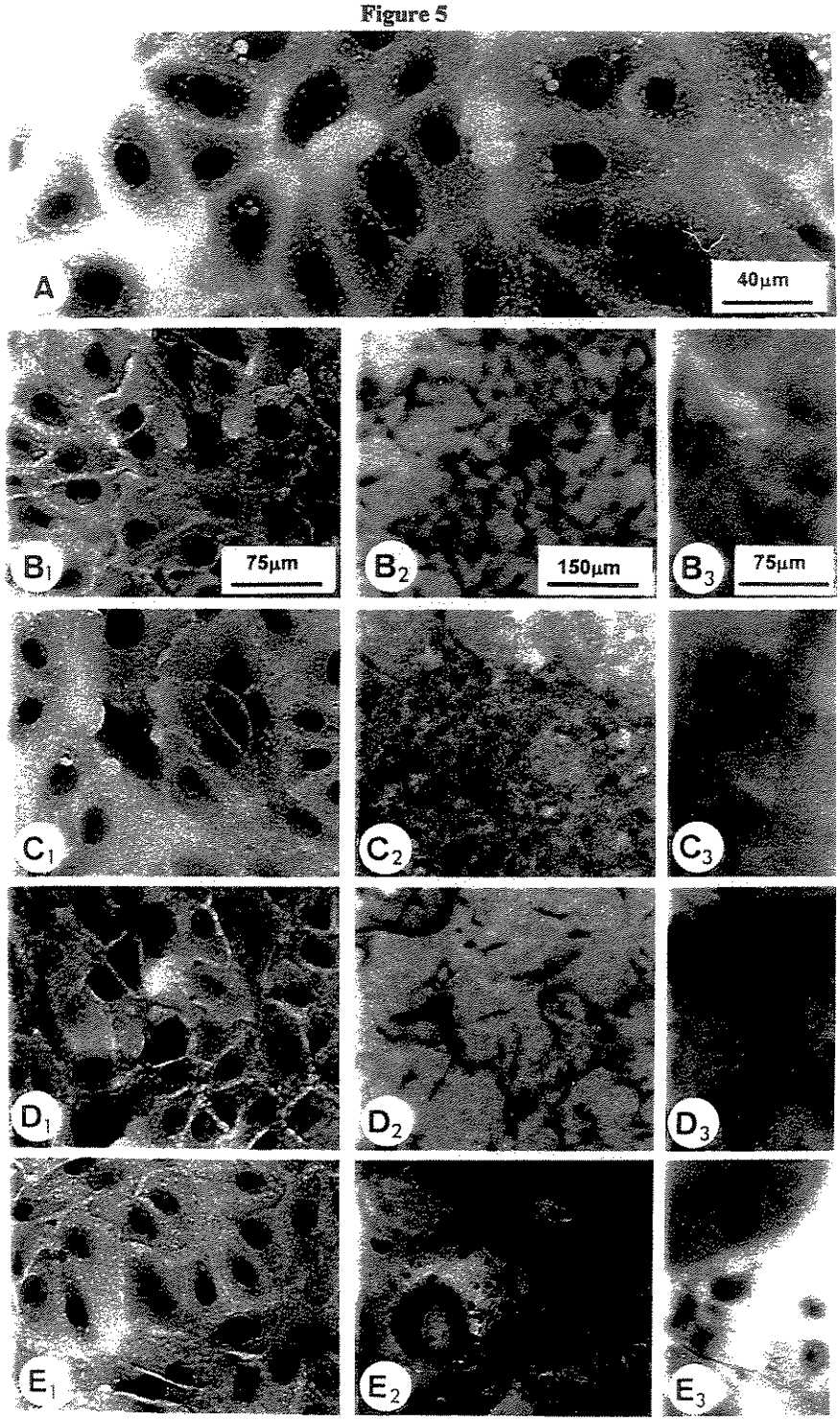


Figure 5



III.4.

Adhesion and Growth of Vero Cells Cultured on Poly(L-Lactic Acid)/Poly(Hydroxybutirate-co-Hydroxyvalerate) Blends

Adhesion and growth of Vero cells cultured on poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) blends

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Short title: Vero cells cultured on PLLA/PHBV.

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Key words: poly(L-lactic acid), poly(hydroxybutirate-co-hydroxyvalerate), blends, biomaterials, cell culture, cell growth.

Abstract

Vero cells were cultured on membranes of poly(L-lactic acid) [PLLA], poly(hydroxybutirate-co-hydroxyvalerate) [PHBV] and theirs blends in different proportions (100/0, 60/40, 50/50, 40/60 and 0/100). An evaluation of cell adhesion on the blends was carried out, as well as the cell growth pattern by scanning electron microscopy and cytochemical analysis of samples. The results of adhesion assay showed that the best results were obtained by blends (60/40, 50/50, 40/60) rather than pure polymers (100/0, 0/100). The SEM showed that after 24h the cells on PLLA/PHBV (100/0) and PLLA/PHBV (60/40) blends appeared rounded and preferably located in the porous areas. The PLLA/PHBV (50/50) blends had slightly flattened cells on the porous and smooth areas. PLLA/PHBV (40/60) blends showed flattened cells in the smooth areas. The PLLA/PHBV (0/100) blends which presented no pores also had spreading cells interconnected by thin filaments. The cytochemical analysis showed basophilic cells indicating a great amount of RNA and abundant quantity of proteins. Hence, we reported changes in cell morphology induced by alterations of the proportion of blend. This could indicate that the cells changed their differentiation pattern on various PLLA/PHBV blends. These properties could be used to promote the recomposition of one or more types of tissue.

1. Introduction

The development of new biomaterials aims at production polymers, which can be used as substitutes of damaged tissue. For a new material to be effectively used, assays are required which confirm its durability, no toxicity, and biocompatibility. Therefore, the understanding of these polymers interacting with the tissues where they have been implanted is extremely important [1].

This knowledgement could be used for the development of polymers designed to regenerate a specific tissues. In many cases, depending on the regenerative capacity of damaged tissues, the use of biodegradable materials is clinically recommended. In such situations, there is only a need for the temporary presence of biomaterials to supply support to the damaged area, or substitute it, so as to direct the growth and restoration of tissues [2]. Once regeneration is concluded, the implanted material is no longer required and the polymer could be degraded. In these situations, a large variety of bioabsorbable polymers have been used in biological systems, where the polyesters derived from α-hydroxi acids are the most frequently employed [3].

The poly(L-lactic acid) [PLLA] is a biodegradable polyester, which has been used experimentally as a support for cell culture or experimental treatment of some damaged tissues mainly due their very good biocompatibility. The poly(hydroxybutirate-co-hydroxyvalerate) [PHBV] is also a biodegradable polyester, produced by microorganisms, which has been investigated recently. The blend of these two polymers is a new approach that aims its biological evaluation for tissue engineering utilization.

The cell culture is a very important tool for the science of biomaterials. It provides a quick and rather reliable pre-selection so as to choose the best sample that will be tested in animals later on. Although the utilization of experimental animals is indispensable, this method, besides being too expensive, hinders viewing the direct effects of biomaterials on the stimulation of important parameters such as cell adhesion, growth and differentiation. Thus, our objective in this work is the evaluation of PLLA/PHBV blends as to their biological interactivity, specifically regarding the capacity that blends have in interfering with adhesion and growth of cells *in vitro*.

2. Material and methods

2.1. Preparation of poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) blends

The poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) [PLLA/PHBV] blends were produced in Department of Material Engineering, College of Mechanical Engineering, UNICAMP. Briefly, the blends were prepared by dissolving poly(L-lactic acid) (Medisorb Technologies International L.P., Cincinnati, OH, USA), Mw = 100,000 and poly(hydroxybutirate) with 12wt% hydroxyvalerate (Aldrich) separately in 5wt% methylene chloride (w/v). The polymer solution were mixed in different proportions (100/0, 60/40, 50/50, 40/60, 0/100) at room temperature. After evaporation of the solvent, the membranes were dried, washed with demineralized water during 48h. The polymers were then vacuum dried and maintained in a dissicator. In experiments of cell culture, all the PLLA/PHBV blends were sterilized overnight with 70% ethanol. The sterility of samples was tested. The samples were washed three times in Ham F-10 medium without

fetal calf serum and then incubated in the same culture medium for 24h at 37°C before cell inoculation.

2.2. Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were used. These cells were cultured in Ham-F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are a lineage that is recommended for studies of cytotoxicity and for cell-substratum interactions with biomaterials research [4, 5].

2.3. Cellular adhesion assay

For cellular adhesion analysis we used a modification of the method described by Murakami et al. [6]. Briefly, the different PLLA/PHBV bends (n=12) were inoculated in a 96 wells plate (Corning/Costar Corporation, Cambridge, MA, USA) in Ham F-10 (Sigma) for 24h at 37°C. After this incubation time, 200µl of cell suspension (1.0×10^5 cell/ml) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was inoculated on the different PLLA/PHBV blends. The cells were cultured for 2h in Ham F-10 with 10% FCS at 37°C, washed with 0.1M phosphate buffered saline (PBS) in pH 7.4, at 37°C, fixed in formalin 10% for 15 minutes, washed in PBS, and stained with crystal violet 0.05% (in methanol 20%) for 15 minutes. The samples were then washed twice with 0.1M PBS and incubated with sodium citrate 0.1M (in 50% ethanol at pH 4.2) for 30 minutes. The wells were read in

a Multiskan Bichromatic Version 1.06 microplate reader with 540nm wavelength. As a positive control, the culture plate itself (polypropilene) and as a negative control silicone membranes were used. We also read the absorbance of all experimental conditions (PLLA/PHBV blends, negative/positive controls) in a cell free condition for a dye staining control. Comparison of continuous variables for all groups was done with the ANOVA. When a significant difference was found ($p < 0.05$), the groups were compared using the Turkey's test.

2.4. Scanning electron microscopy (SEM)

The blends with different compositions were fractured by immersion into liquid nitrogen. The samples were covered with gold with a sputter coater (Baltec SCD 050) and observed in a JEOL JXA-840 scanning electron microscope. For cell morphology analysis, 1.0×10^5 cell/ml were inoculated on the different PLLA/PHBV bends in Ham F-10 medium (Sigma) with 10% FCS (Nutricell). As a control, we used cells cultured on a glass coverslip in the same culture conditions. After 24h of incubation, the samples were fixed in 3% glutaraldehyde (Sigma) in phosphate buffer 0.1M at pH 7.2 for 45 minutes at 4°C, and postfixed with 1% OsO_4 (Sigma) for 2hs at 4°C. The specimens were then dehydrated with an ethanol series, critical point dried (Balzers CDT 030) and coated with gold in a sputter coater (Balzers CDT 050). The coated specimens were viewed and photographed with a JEOL 300 scanning electron microscope. All experiments were made in triplicate.

2.5. Cytochemcial analysis

For cytochemical study, 1.0 x 10^5 cell/ml were cultured on the different PLLA/PHBV blends in Ham F-10 medium (Sigma) with 10% FCS (Nutricell). After 48, 120 or 240h of culture, the samples were fixed in Karnovsky (paraformaldehyde 4%/glutaraldehyde 2.5% in phosphate buffer 0.2M at pH 7.2), dehydrated with an ethanol series, cleared with xylene and included in paraplast. 5μm of sections were obtained. The sections were stained with toluidine blue at pH4.0 (Sigma), a basophilic dye which can bind with PO_4^-, COO^- and SO_4^- anionic groups, or with xylidine ponceau at pH 2.5, an anionic dye that can stain NH_3^+ cationic groups [7, 8, 9]. All experiments were made in triplicate.

3. Results

3.1. Cellular adhesion

The results obtained by cell adhesion assay, showed that all samples studied presented a lower capacity to stimulate the cell adhesion than the positive control used ($p<0.05$). Most of the PLLA/PHBV blends (100/0, 40/60 and 0/100) showed a cell adhesion capacity in a similar way than negative control. Only the 60/40 and 50/50 PLLA/PHBV blends proved to be more receptive to cell interaction ($p<0.05$). These results can be seen in Figure 1.

3.2. Scanning electron microscopy (SEM)

The Figure 2 shows the electromicrographs obtained by SEM of fractured blend surfaces in different proportions. The PLLA/PHBV (100/0) membranes were porous, with a rounded structure (Fig. 2a). The decreased concentration of PLLA in the blend composition, such as

(60/40) and (50/50) samples results in an increase in porosity (Fig. 2b and 2c, respectively). The PLLA/PHBV (40/60) proportion shows that the blend acquired a dense structure, with the presence of cavities on the surface (Fig. 2d). The PLLA/PHBV (0/100) blend is completely dense, as could be verified by its fracture (Fig. 2e).

In the SEM of cells cultured on the glass coverslip we could see the formation of a non confluent cell layer. These cells showed an irregular morphology, some times with a flattened and elongated form (Fig. 3a). On the PLLA/PHBV (100/0) blends we found cells growing preferentially in the porous areas. These cells were rounded, with microvilli on their surface. They also showed long and thin filaments (Fig. 3b). On the other hand, on the same membranes, the cells that grew on non porous areas, were flattened on to the substratum (Fig. 3c). On the PLLA/PHBV (60/40) blends we found cells with a growth pattern very similar to the former samples, although pores seen in blends were more irregular (Fig. 3d). On the PLLA/PHBV (50/50) bends, which unlike the previous samples, showed a very irregular surface, we could observe flattened cells with many filaments (Fig. 3e). Apparently the cells grew in equal proportion both on areas with or without pores. The PLLA/PHBV (40/60) blends displayed flattened cells which showed shrunk along theirs edges (Fig. 3f). We also found that on these samples the cells grew preferably on the non porous areas of the blend. On the PLLA/PHBV (0/100) samples, which do not present pores, we found spreading cells with many thin filaments interconnecting the cells (Figs. 3g and 3h).

3.3. Cytochemcial analysis

The results obtained with cytochemistry demonstrated that during the culture period, cells are able to reach confluence on the substrate and to enter into different PLLA/PHBV blends through the pores existing on the blend surface. In all samples studied, in the different culture times used, we detected cells highly stained by TB or XP, which are basophilic and acidophilic dyes, respectively. We did not find cytochemical alterations induced by the different PLLA/PHBV membranes on which the cells were cultured (Figs. 4a-4e) and (Figs. 5a-5e).

4. Discussion

The development of biomaterials that are capable of directing cell behavior is a growing research area. Its application includes devices for tissue replacement and regeneration as well as substrates for cell culture aiming tissue engineering. In the structure of the different polymers the presence of globular pores in the PLLA/PHBV (100/0) blend may be due to the low dilution of the solution during the membranes preparation process. This situation provides conditions for slow PLLA crystallization. With the presence of the solvent in the PLLA solution, globular bubbles are formed leaving pores in the membranes [10]. However, there may be two explanations for the behavior of PLLA/PHBV. First, it might not be a really solution but rather a suspension, thus, the polymer settles and is quickly united to the bottom of the plate, creating a dense membrane or, secondly, because poly(hydroxybutirate) crystal growth is extremely quick, starting from various points and soon forming dense membranes [10].

Since, the different PLLA/PHBV blends show structural variations, they could induce variations on cell growth. In this context, the results obtained by adhesion assays showed that all blends studied have a low capacity to stimulate cell adhesion. The PLLA/PHBV (60/40) and (50/50) blends were the exception, being more receptive to cell interaction. The low adhesion capacity of fibroblastic cells on PLLA membranes was previously reported [11]. In this context, our data indicates that addition of PHBV increases the cell affinity for the substrates. Thus, the PLLA/PHBV blends show better characteristics for tissue engineering than pure PLLA scaffolds.

The cell adhesion on biomaterials is extremely important in tissue engineering. Only upon adhesion to the substratum the cells can migrate and/or proliferate on it or even exert special physiological activities, such as the production of extracellular matrix or marker proteins of a given tissue [2, 12]. The fact that Vero cells showed low adhesion when compared to the positive control indicates that cell-polymer interaction is slower. In biological surfaces, adhesion is the consequence of protein adsorption to the substratum. These interactions involve electrical charge interactions, H bonds and electrostatic forces [13]. There are authors that try to associate the free polymer surface energy with cell spreading [14]. Others factors which can modulate cell adhesion are the types of chemical groups present on the polymer surface and its relationship between hydrophilicity/hydrophobicity [15]. Thus, cell adhesion to biomaterials is quite a complex process.

Once cell adhesion to different PLLA/PHBV blends was completed, the cell was able to grow on all the polymers in a satisfactory manner. However, the different blends seem to influence early cell growth patterns and morphology. Since the different blend

proportions are related to alterations of cell morphology and cell structure is intimately related with cell physiology, we could conclude that cells are changing their differentiation pattern on PLLA/PHBV blends. It has been recently shown that Vero cells grow on PLLA membranes with high porosity presenting a rounded morphology, with thin filaments. On the other hand, cells acquire a flattened morphology on a less porous PLLA membranes [11, 16]. These results are quite compatible with the results showed here. The kind of stimulus that comes from the PLLA/PHBV blends and, which induces changes in cell morphology is still unknown. The most remarkable factor is, perhaps, the blend chemical composition [12]. Some studies showed that spreading is stimulated by hydrophilic substrata. Other properties, such as the type of chemical groups present in polymers and its physical-chemical anisotropy have been proposed as factors present in synthetic polymers, which also induce cell behavior alterations in *in vitro* assays [15, 17]. Moreover, it has been also reported that biomaterial topography may induce morphological alterations of cells cultured on it suggesting modifications in cellular differentiation patterns [18].

We found that the cells were capable to proliferate until convergence on all PLLA/PHBV blends. They are also able to enter into pores, creating three-dimensional arrangements on the blend surface, even on those samples where cell affinity for the pores was smaller. Some work has shown that porous materials are able to promote cell growth. The uniform distribution and interconnection of porous structures is important for cell migration and the formation of organized network structures similar to a tissue [19, 20]. It is interesting to note that a similar growth pattern was reported when Vero cells were grown on dry collagen I sponges [21].

Cytochemistry has not shown alterations of cell behavior induced by different PLLA/PHBV blend proportions. The TB is a basophilic dye that can stain PO_4^-, SO_3^- and COO^- anions. At pH 4.0, the PO_4^- anions are found only in DNA or RNA, while SO_3^- and/or COO^- groups occur in glycosaminoglycans [7, 8, 9]. In all samples studied, we found basophilic cells. This indicates that the cytoplasmatic staining observed in the cells is due to high RNA concentrations, probably rRNA, suggesting that cells are able to perform active protein synthesis. The XP is anionic dye that can stain NH_3^+ groups. These radicals are cytochemically detected in protein groups. At pH 2.5, these ions are stained in all proteins [7, 8, 9]. Thus, XP shows that cells have an abundant quantity of proteins, confirming the data obtained by AT staining. Taken together, cytochemical data indicates a high metabolic activity of cells on the polymers and that activity is not changed by the different PLLA/PHBV blend proportions. Similar results were found in Vero cells cultured on others biomaterials such as poly(2-hydroxyethymethacrylate) [polyHEMA] hydrogels [22, 23] or glass coverslips [24]. Thus, we conclude that on the different PLLA/PHBV blends, the cells retain high metabolic activity.

Due to an intense search for better materials in the last years, the clinical applications of reabsorbable polymers have been noticeably increasing. Various studies have been made to search for biomaterials that could help in repairing articular cartilage [25] or bones [26]. In this way, the search for materials mimetising the behavior of these tissues will be responsible for the improvement of restoration. Bioabsorbable PLLA membranes have been experimentally used for this purpose with some promising results [27, 28, 29]. PLLA/PHBV blends are a new approach in literature. Usually blends present advantages in the physical and mechanical properties if compared to pure polymers. From

the results described in this work, they show very desirable characteristics for use in implants, with variations in blend proportion, the cells respond apparently changing their differentiation pattern. Theoretically, these could be used promote recovery of one or more types of tissue. New experiments will be developed to with this approach.

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LEGEND FIGURES

Figure 1. Adhesion of Vero cells cultured on different PLLA/PHBV blends proportions. All samples studied displayed the capacity to stimulate less adhesion than to the positive control used (*). The (100/0), (40/60) and (0/100) blends showed similar values to the negative control. Only the (60/40) and (50/50) PLLA/PLBV blends were significantly different to negative control used (**). For all samples the significance level used was < 0.05.

Figure 2. Scanning electron microscopy of different biopolymers used in this work. a) PLLA/PHBV (100/0) membrane; b) PLLA/PHBV (60/40) blend; c) PLLA/PHBV (50/50) blend; d) PLLA/PHBV (40/60) blend and e) PLLA/PHBV (0/100) blend. Scale bar: 100μm.

Figure 3. Scanning electron microscopy of Vero cells cultured on the different polymers studied for 24hs. A) Cells cultured on a glass coverslip, an inert surface used as control. The cells showed an irregular morphology. Elongated and flattened cells also could be seen. B) Cells on the PLLA/PHBV (100/0) membrane. We observed round cells growing in a pore, with microvilli and thin filaments. C) A non porous region of the same polymer. In this area the cells grew very flattened. D) Cells on the PLLA/PHBV (60/40) blend. Cells had a growth pattern similar to the previous

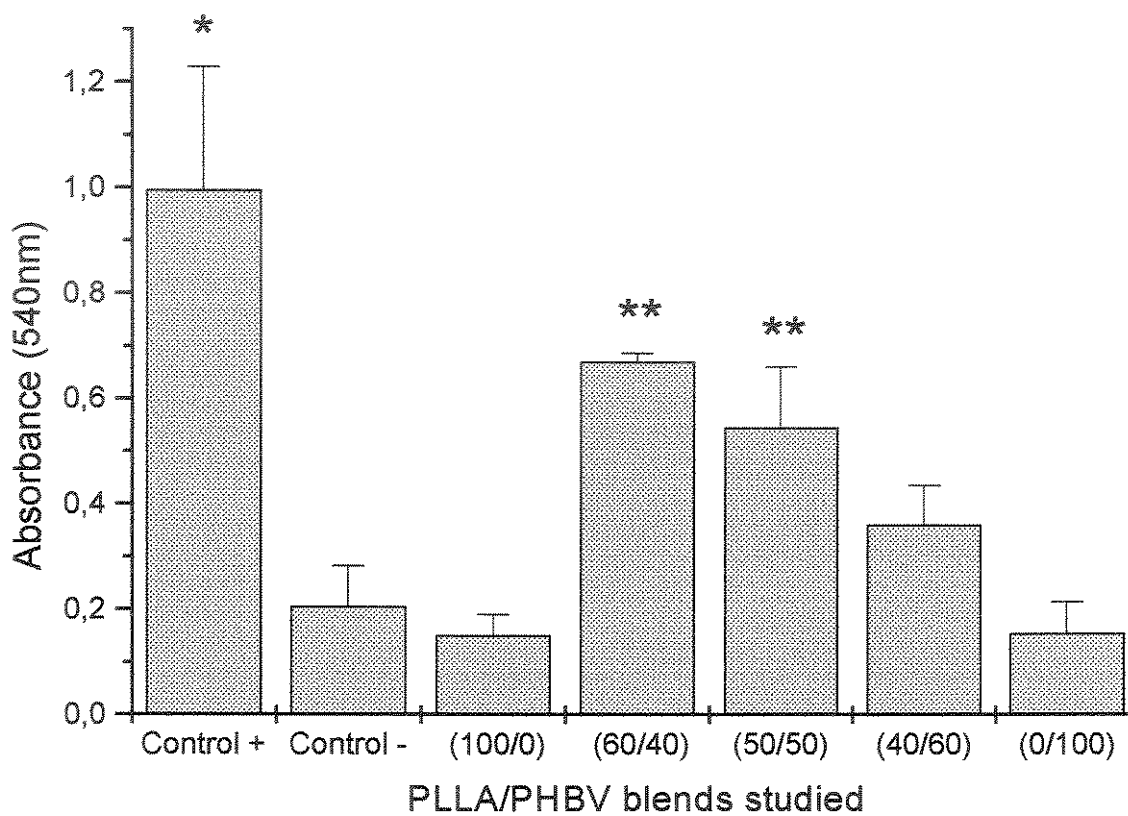
samples, although in the porous regions they were more irregular in structure. E) Cells on the PLLA/PHBV (50/50) blend. Flattened cells with filaments grow in equal proportions on porous or non porous areas was found. F) Cells on the PLLA/PHBV (40/60) blend. Flattened cells showed shrinkage at their edges and grew preferably on non porous areas. G) Cells on the PLLA/PHBV (0/100) membrane. In this case, we found spreading cells with many thin filaments linking the cells. H) The sample showing a cell on PLLA/PHBV (0/100) membrane, observed in greater magnification. Scale bar: 20μm for all pictures. The figures A) and G) in lower magnification than others samples.

Figure 4. Vero cells cultured on different PLLA/PHBV blends for 48h, 120h and 240h stained by toluidine blue at pH4.0. A) Cells cultured on PLLA/PHBV (100/0) blends. B) Cells cultured on PLLA/PHBV (60/40) blends. C) Cells cultured on PLLA/PHBV (50/50) blends. D) Cells cultured on PLLA/PHBV (40/60) blends. E) Cells culture on PLLA/PHBV (0/100) blends. In all samples and times of cultured studied we found basophilic cells growing on the surface of the different polymers. We did not find cytochemical alterations induced by the of the different proportion blend composition. Scale bar: 30μm.

Figure 5. Vero cells cultured on different PLLA/PHBV blend compositions for 48h, 120h and 240h stained with xylidine ponceau at pH 2.5. A) Cells cultured on a PLLA/PHBV (100/0) blend. B) Cells cultured on a PLLA/PHBV (60/40) blend. C)

Cells cultured on a PLLA/PHBV (50/50) blend. D) Cells cultured on a PLLA/PHBV (40/60) blend. E) Cells cultured on a PLLA/PHBV (0/100) blend. In all samples and times of culture studied we found acidophilic cells growing on the surface of different polymers. We did not find cytochemical alterations induced by the composition of the different blends. Scale bar: 30μm.

Figure 1


*
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1,2
1,0
0,8
0,6
0,4
0,2
0,0
Absorbance (540nm)
Control +
Control -
(100/0)
(60/40)
(50/50)
(40/60)
(0/100)
PLLA/PHBV blends studied

Figure 2

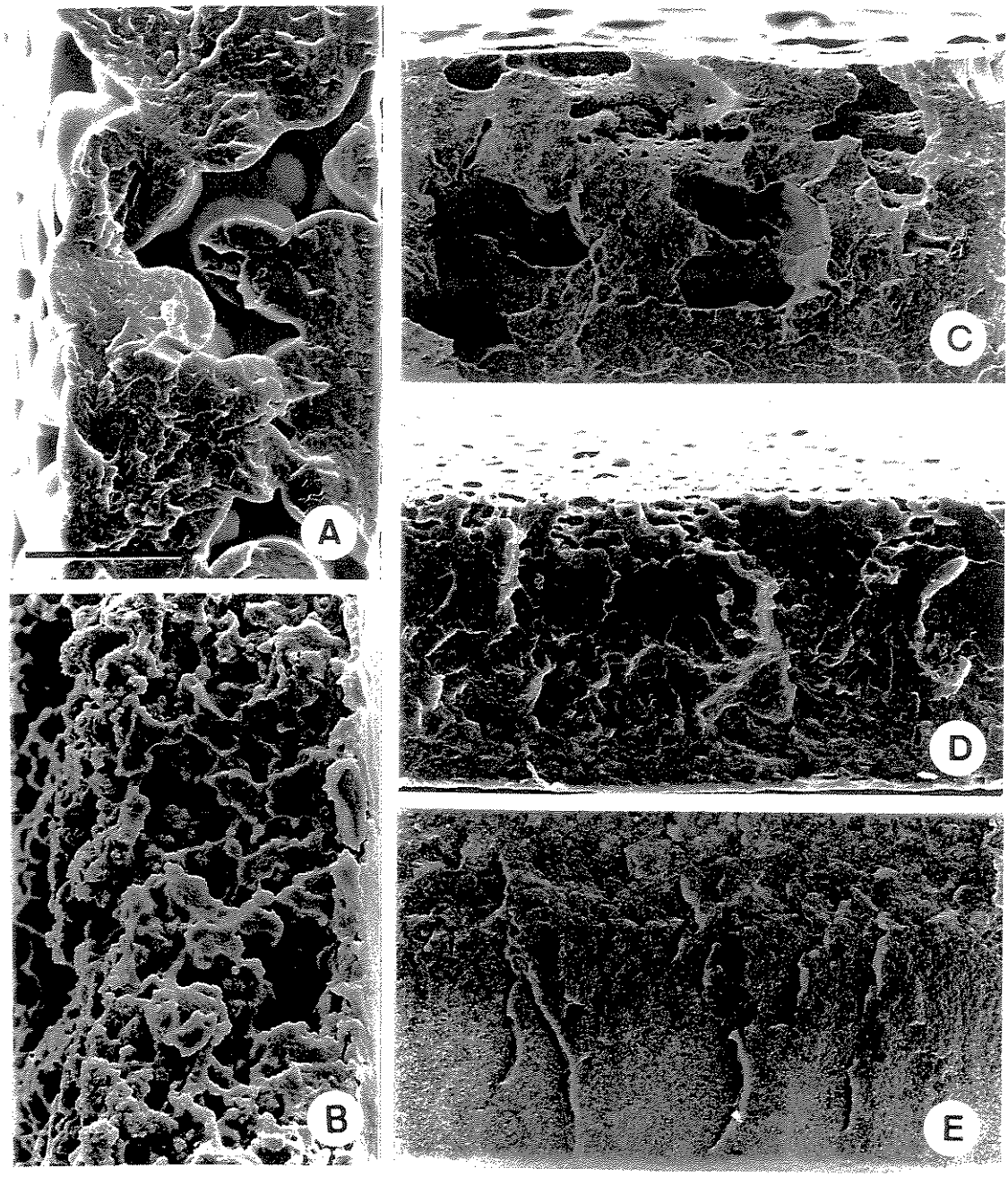


Figure 3

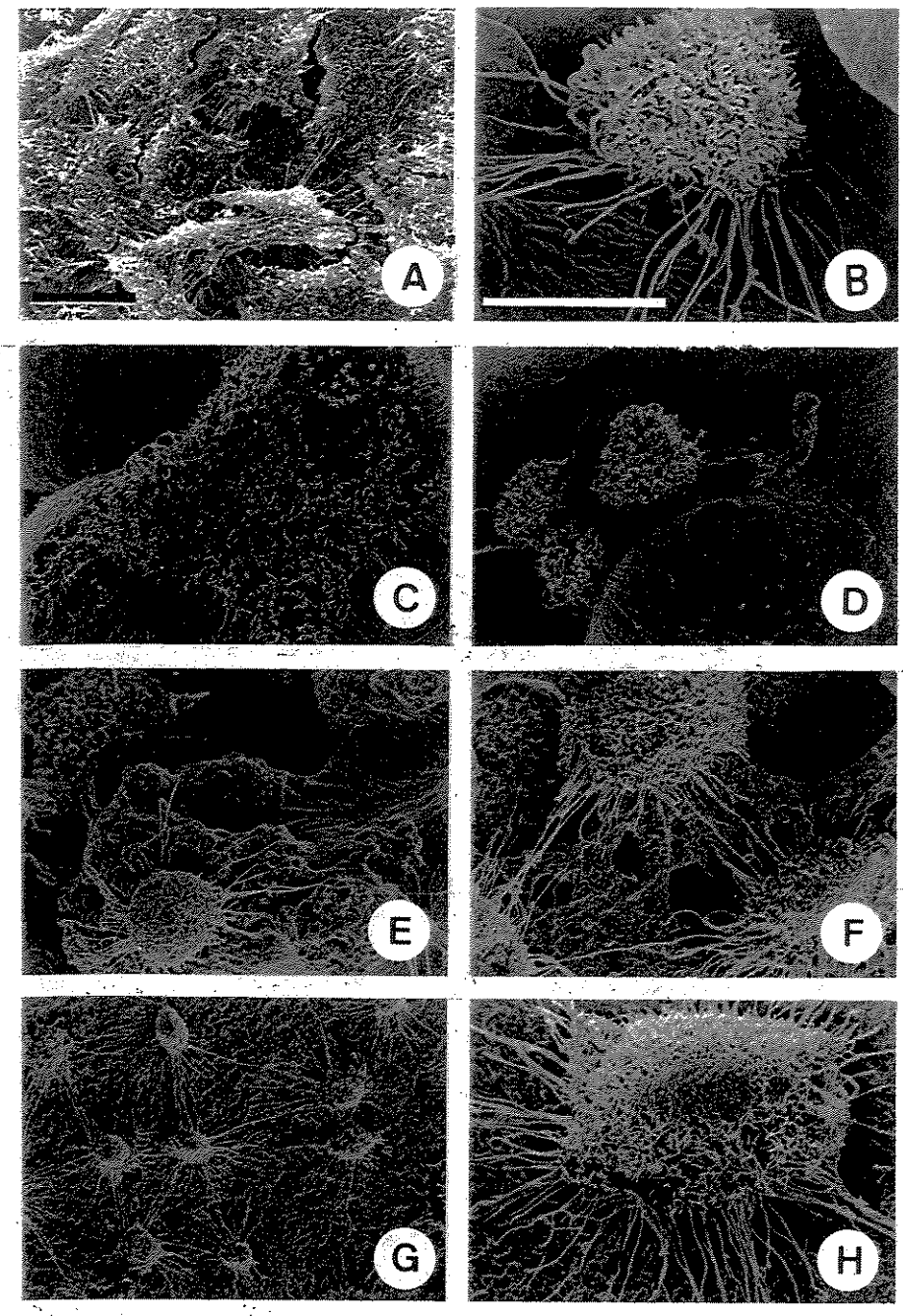


Figure 4

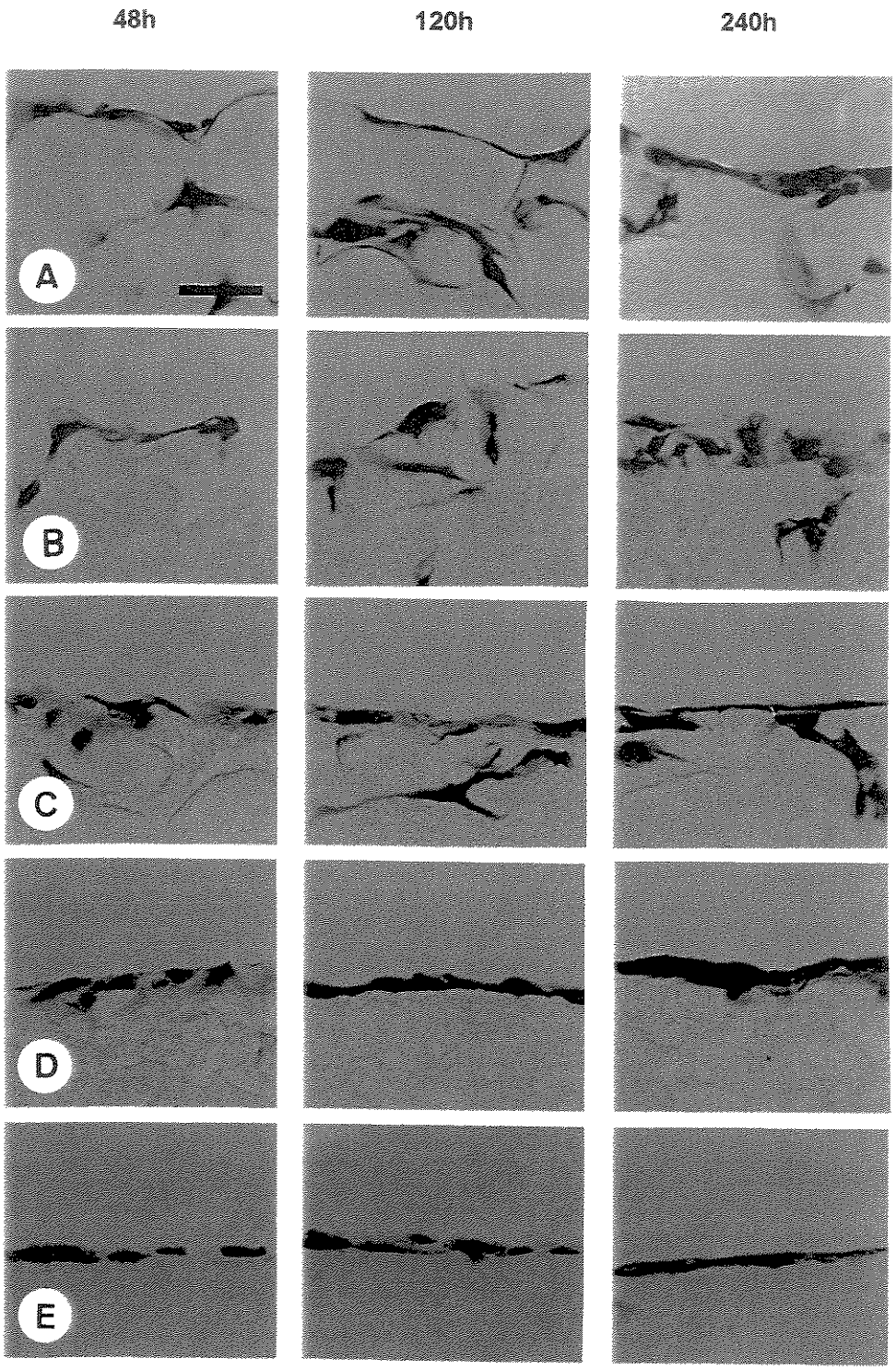
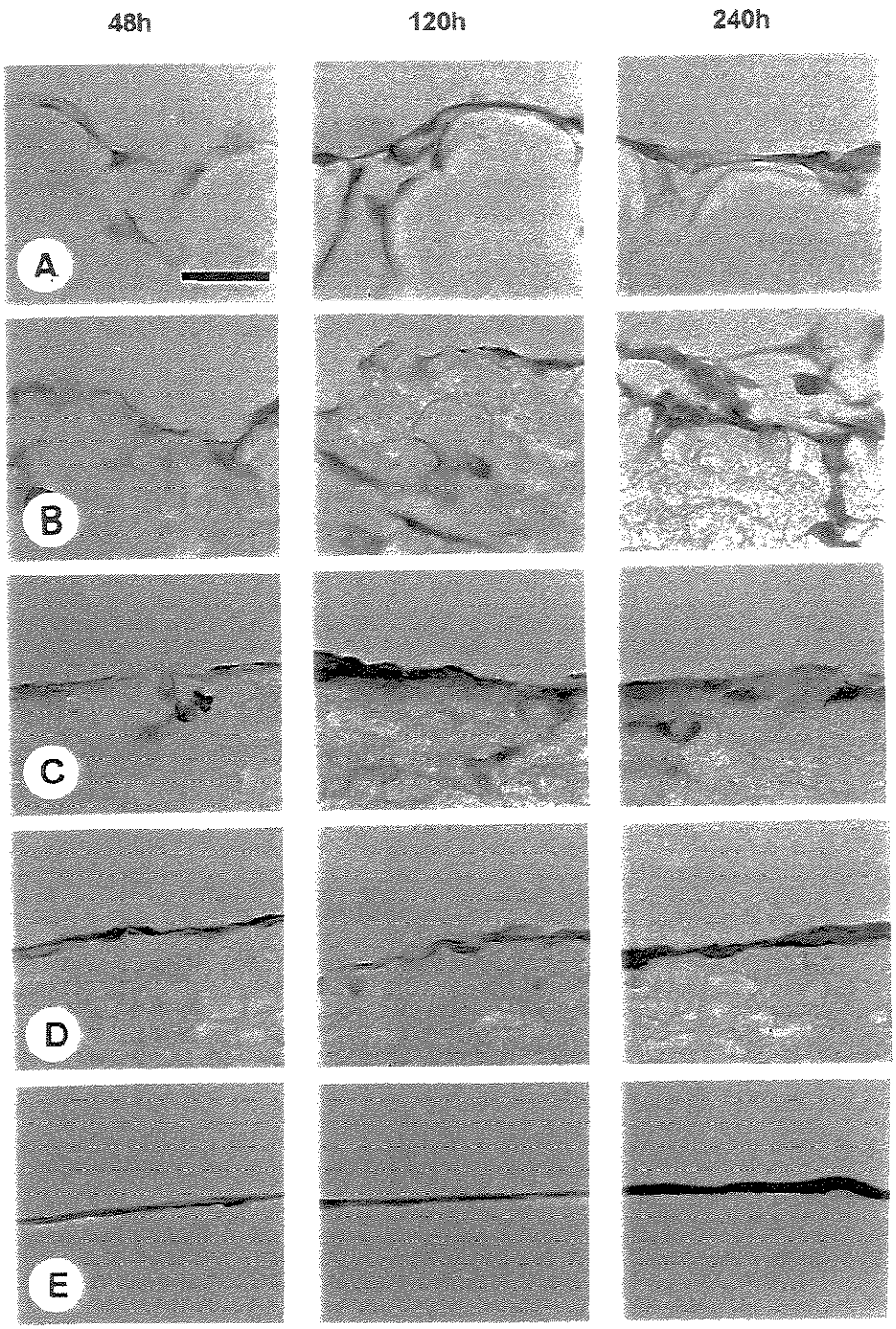


Figure 5



III.5.

Collagen IV and fibronectin production by Vero cells cultured on different poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) blends

Collagen IV and fibronectin production by Vero cells cultured on different poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) blends

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ABSTRACT

Vero cells were cultured on membranes of poly(L-lactic acid) [PLLA], poly(hydroxybutirate-co-hydroxyvalerate) [PHBV] and their blends in different proportions (100/0, 60/40, 50/50, 40/60 and 0/100). We made an evaluation by growth curve, morphological scanning electron microscopy and immunocytochemical to extracellular matrix components (collagen IV and fibronectin) in cells cultured on different samples studied. The SEM showed that the cells on PLLA/PHBV (100/0) and (60/40) blends initially growth preferable in the porous areas. The PLLA/PHBV (50/50) blends had cells in the porous and smooth areas in a similar way. PLLA/PHBV (40/60) blends showed cells on the smooth areas preferable. The PLLA/PHBV (0/100) blends, which presented no pores also had spreading cells interconnected by thin filaments. We also found that cells were capable of proliferating on all blends analyzed. The histological sections showed that cell grew as a confluent monolayer on different substrates. The imunocytochemical analysis showed that in all situation the cells were able to produce collagen IV and fibronectin. Thus, we conclude that PLLA/PHBV blends were non toxic. On the contrary, we found that blends were efficient on maintaining the cell proliferation as well as the production of extracellular matrix molecules on cells cultured on them.

Key words: poly(L-lactic acid), poly(hydroxybutirate-co-hydroxyvalerate), blends, biomaterials, cell culture, cell differentiation.

INTRODUCTION

In recent years, the necessity of performing tissue restoration is an important factor to the improvement of the patient's life quality. Most of the success experimentally obtained is attributed to the development of interdisciplinary approaches in tissue engineering. Tissue engineering is a research area that searches the functional restoration of damaged tissue obtained from native or synthetic sources by using engineering principles, witch create scaffolds to guide tissue regeneration, stimulate tissue recomposition, or develop new functional structure when damaged tissue does not regenerate. Synthetic and naturally occurring polymers are an important elements in new strategies for improvement of tissue restoration.[1,2] To select appropriate polymers to tissue engineering it is necessary to understand the influence of the polymer on cell viability, growth and function.[2]

Bioabsorbable polymers slowly degrade following implantation. This feature may be important for many tissue regeneration applications, since the polymer will disappear as functional tissue regeneration occurs.[3] The poly(L-lactic acid) [PLLA] is a biodegradable polyester which has been used experimentally as a support for cell culture or experimental treatment of some damaged tissues mainly due to their good biocompatibility. The poly(hydroxybutirate-co-hydroxyvalerate) [PHBV] is also a biodegradable polyester, produced by microorganisms, which has been recently investigated. The biological evaluation of these blend is the aims of our work for tissue engineering utilization.

The extracellular matrix (ECM) is a complex crosslinked network of proteic fibres, glycoproteins, glycosaminoglycans and proteoglycans. It serves to organize the space among cells, gives the mechanical and physiological properties to the tissues, and provides them environmental signals to direct cellular behavior. The knowledge of that cell-ECM

interactions is important in tissue engineering for the creation and use synthetic or natural polymers that could mimic the relationship between cell and ECM. We have previously demonstrated that PLLA polymers could induce variations on extracellular matrix production of fibroblastic Vero cell line. In the present work, we evaluated whether blends of PLLA/PHBV could induce similar behavior on the same cells cultured on them.

MATERIAL AND METHODS

Preparation of poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) blends

The poly(L-lactic acid)/poly(hydroxybutirate-co-hydroxyvalerate) [PLLA/PHBV] blends were produced in the Department of Material Engineering, College of Mechanical Engineering, UNICAMP. Briefly, the blends were prepared by dissolving poly(L-lactic acid) (Medisorb Technologies International L.P., Cincinnati, OH, USA), Mw = 100,000 and poly(hydroxybutirate) with 12wt% hydroxyvalerate (Aldrich) separately in 5wt% methylene chloride (w/v). The polymer solution was mixed in different proportions (100/0, 60/40, 50/50, 40/60, 0/100) at room temperature. After evaporation of the solvent, the membranes were dried, washed with demineralized water during 48h. The polymers were then maintained in vacuum dried until utilization. In experiments with cell culture, all the PLLA/PHBV blends were sterilized overnight with 70% ethanol. The sterility of samples was tested. The samples were washed three times in Ham F-10 medium without fetal calf serum and then incubated in the same culture medium for 24h at 37°C before cell inoculation.

Cell culture

Vero cells, a fibroblastic cell line established from the kidney of the African green monkey (*Cercopithecus aethiops*), obtained from the Adolfo Lutz Institute, São Paulo, Brazil, were used. These cells were cultured in Ham-F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C. Vero cells are a lineage that is recommended for studies of cytotoxicity and for cell-substratum interactions for biomaterials research.[4,5]

Scanning electron microscopy (SEM)

For cell morphology analysis, 1.0 x 10^5 cell/ml were inoculated on the different PLLA/PHBV blends in Ham F-10 medium (Sigma) with 10% FCS (Nutricell). As control, we used cells cultured on glass coverslips in the same culture conditions. After 24h of incubation, the samples were fixed in 3% glutaraldehyde (Sigma) in phosphate buffer 0.1M at pH 7.2 for 45 minutes at 4°C, and postfixed with 1% OsO_4 (Sigma) for 2hs at 4°C. The specimens were then dehydrated with ethanol series, critical point dried (Balzers CDT 030) and coated with gold in a sputter coater (Balzers CDT 050). The coated specimens were observed and photographed with a JEOL 300 scanning electron microscope.

Growth curve on different PLLA/PHBV bends

For cellular proliferation assay we used a modification of the method described by MURAKAMI et al.[6] Briefly, the different PLLA/PHBV blends were inoculated in a 96 wells plate (Corning Corporation, Cambridge, MA, USA) in Ham F-10 (Sigma) for 24h at

37°C. After this incubation time, 200µl of a cell suspension (1.0×10^4 cell/ml) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was inoculated on the different PLLA/PHBV blends. The cells were cultured for 48h, 120h, 240h and 360h with Ham F-10 with 10% FCS at 37°C, washed with 0.1M phosphate buffered saline (PBS) in pH 7.4, at 37°C, fixed in formalin 10% for 15 minutes, washed in PBS, and stained with crystal violet 0.05% (in methanol 20%) for 15 minutes. The samples were then washed twice with 0.1M PBS and incubated with sodium citrate 0.1M (in 50% ethanol at pH 4.2) for 30 minutes. The wells were read in a Multiskan Bichromatic Version 1.06 microplate reader with 540nm wavelength. As a positive control, the culture plate itself (polypropilene) and as a negative control Teflon membranes were used. We also read the absorbance of all experimental conditions (PLLA/PHBV blends, negative/positive controls) in a cell free condition for a dye staining control.

Immunocytochemical analysis

After detachment with trypsin-EDTA (Nutricell), 1.0×10^5 cell/ml were cultured on different PLLA/PHBV blends in Ham F-10 medium (Sigma) with 10% FCS (Nutricell). After 48h or 240h of culture, the samples were fixed in Karnovsky fixative (paraformaldehyde 4%/glutaraldehyde 2.5% in phosphate buffer 0.1M at pH 7.2), dehydrated with ethanol series, cleared with xylene and included in paraplast. 5µm sections were obtained. The sections were stained with crystal violet, for morphological evaluation of the samples. We used monoclonal primary anti-collagen IV (Sigma: clone Col-94, dilution 1:500) and anti-cellular fibronectin antibodies (Sigma: clone FN- 3E3, dilution

1:400) both obtained from mouse ascitic fluid. Secondary rabbit anti-mouse IgG FITC conjugate antibodies (Sigma: dilution 1:200) were also used. The material was washed in 0.1M PBS, in pH 7.4, at 37°C and incubated for 1h with bovine serum albumin 1% (BSA, by Sigma) in PBS to block nonspecific staining. The preparations were incubated in a moist chamber at 4°C with monoclonal anti-cellular fibronectin or anti-collagen IV. After washing in PBS at 37°C, the antigenic sites were observed using indirect marking with anti-mouse IgG FITC conjugated antibody treatment. The samples were observed with an inverted microscope, Olympus IX50 equipped for fluorescence analysis. Control experiments were performed with the omission of primary antibodies.

RESULTS

Scanning electron microscopy (SEM)

With the SEM analysis of the controls, we could observe areas with a confluent or a non confluent cell layer. The cell morphology observed was irregular with many prolongations. We found many microvillies on cell surface. When a confluent cell layer was observed, we could also see some cell processes linking the cells with others around (Fig. 1A). The cells that grew on PLLA/PHBV (100/0) showed a preferential growth in the pore areas rather than in non porous surfaces of the polymer (Fig. 1B). On these different regions, variations on cell morphology could be seen. On the porous areas the cells showed a round morphology with many microvillies. Some thin cell prolongations could also be observed over the substrate (Fig. 1C). On the other hand, the cells that grew on non porous regions showed a very flattened morphology. It was found less cell prolongations but microvillies could also be found all over the cell structure (Fig. 1D). On PLLA/PHBV (60/40) blends

we could see a similar morphological pattern to that observed on porous regions of PLLA/PHBV (100/0) (Fig. 1E). On PLLA/PHBV (50/50) blends, we found round cells with greater amount of microvillies on their surfaces. Cell prolongations were also found but in a increased number than on the previous samples studied (Fig. 1F). On PLLA/PHBV (40/60) blends, we observed very flattened cells with great quantities of microvillies. On that case, once the pore diameters were smaller, the cells were capable of growing over them. Many thin cell prolongations could also be seen (Fig. 1G). Finally, the cell morphology on PLLA/PHBV (0/100) was flat. Many cell processes linking the cells with others around could also be found (Fig. 1H).

Growth curve on different PLLA/PHBV blends

We observed that fibroblastic Vero cell line did not show alterations on proliferation pattern on different PLLA/PHBV blends. All samples studied showed better mitotic cell rate than the negative control used. Interesting to note that the pure polymers utilized (100/0 and 0/100) showed a similar proliferation rate. On the other hand, in (60/40) and (40/60) blends, we could observe a lower proliferative index. In all cases studied, the cells grew in a slower way than positive control. However, in the last time studied we observed a decrease on the cell number of the positive control, suggesting the senescence of the cell layer. This behavior was not found, in the different PLLA/PHBV blends (Fig. 2).

Immunocytochemical analysis

We could see that in all samples the cells grew until convergence and entered into pores, creating three-dimensional arrangements in the different blends. In all different times

studied, we could observe the deposition of collagen IV and fibronectin by the fibroblastic cells on all PLLA/PHBV blends. These data could be seen on Figs. 3 and 4.

DISCUSSION

The utilization of a bioabsorbable polymer for tissue engineering depends mainly on three parameters: first, the cell proliferation on substrate, second, extracellular matrix (ECM) production, and, third, scaffold degradation. In an ideal condition, the scaffold must degrade at the same time to the occurrence of damaged tissue regeneration. In a *in vitro* situation, the importance of ECM could be noticed once cell growth and differentiation in two or three-dimensional culture conditions require the presence of a structural environment by which the cell can interact. This environment is represented by the different components of ECM, a complex crosslinked network of glycoproteins, glycosaminoglycans and proteoglycans, that organize the space among cells, and provide them environmental signals to direct the cell behavior. The ECM is therefore not an inert product of secretory activity of cells, but a functional structure that can modulate cell morphology as well as cell proliferation, migration and differentiation.

For tissue engineering, there is a trend to create polymers that could simulate characteristics of ECM tissue on wherever they would be implanted.[2] In this context, the production of ECM components is an important event for cell-polymer interaction and the integration of that scaffold with the target tissue in an implantation site. The interactions between cells and ECM are not unidirectional. The cells are constantly accepting information from their environment and are frequently remodeling their ECM in response

to that signals. That complex type of cell-ECM interaction is called dynamic reciprocity.[7] Theoretically, the signals from artificial substrates could also stimulate alterations on cells growth in a similar way than ECM molecules, although the materials developed do not have all the properties of ECM when it is assembled by cells *in vivo*. On the other hand, an alternative form to biomaterial utilization is the creation of structures that induce the cells to produce new extracellular matrix components. MANN *et al.*[8] recently showed that low adherent materials are capable of stimulating the extracellular matrix production while in high adherent biomaterials this capacity was decreased. Once the extracellular matrix production is a desirable condition for the integration of polymer with the tissue structure *in vivo*, the characteristics of adhesion and extracellular matrix production must be evaluated to improve the efficiency of material in a tissue implantation.

The physicochemical properties of different materials could modulate the cell morphology and behavior. The signals from polymers that drive cell growth pattern are complex and could be originated from dipole and electric charge interaction forces, hydrogen bonds, electrostatic forces, hydrophilicity/hydrophobicity or surface free energy, and roughness and rigidity of the surface and surface tension.[9,10] Folkman and Moscona showed in a classic report that when the amount of poly(2-hydroxy ethyl methacrylate) (polyHEMA) added to a surface was increased, it became less adhesive and cell spreading capacity was changed on the surface. The results obtained suggest that cell shape, which was determinated by the adhesiveness of the surface, modulated cell proliferation.[11]

In this study, we could observe variations on cell morphology induced by physicochemical characteristics of substrates. On pure polymers, PLLA/PHBV (100/0) or (0/100), we found rounded or flattened cells, respectively. With the different proportions

blends, we could observe an intermediary cell morphological pattern, except on porous areas of PLLA/PHBV (100/0) or (60/40). In those regions, cell morphology observed was very flat, maybe because of the superficial tension on that regions. The morphology of Vero cells on pure PLLA scaffolds observed here where compatible with our results previously published.[12,13] The structure of fibroblastic cells on PLLA/PHBV blends or pure PHBV copolymers had not been described yet.

The occurrence of pores on biomaterial surface can also modulate the cellular proliferation rate. The pores represent an increased area for cell growth and division. In a *in vivo* situation the pore structure is a desirable characteristic that could create a three-dimensional region for tissue growth, increasing the tissue-polymer interaction. Some *in vivo* results had showed that porous membranes containing certain structural features were associated with enhanced new blood vessel growth.[14] Fiber meshes and foams of PLGA, PGA and PLLA have been used to create a three-dimensional environment for cell proliferation and to provide a structural scaffold for tissue regeneration.[15] In *in vitro* reports, the PLLA devices have been utilized to support the growth and proliferation of endothelial cells without stimulating the platelet activation.[16] Ocular cells cultured on PLLA substrates showed also an increase on their proliferation and differentiation capacity.[17] Recently, it was reported that extracellular matrix proteins, such as fibronectin, laminin and collagen, could improve the cell multiplication on absorbable scaffolds.[18] These data could explain the good interaction of Vero cells with bioabsorbable polymers used once some of these proteins were produced on substrates by cells. Interesting to note that, in our proliferation assay, we observed a similar multiplication rate on pure polymers, PLLA/PHBV (100/0) or

(0/100), in relation to PLLA/PHBV (50/50) blend. When the blends presented increased quantity of PLLA or PHBV, we found a trend for a reduction on cell mitotic rate.

In relation of ECM production, in all samples evaluated in this study we could find collagen IV and fibronectin production by fibroblastic Vero cells. The type IV collagen is a member of the collagen protein family. It does not form fibrils but can create a network structure found in the basement membrane.[19] The basement membrane is produced by differentiating epithelial cells and could be found *in vivo* between epithelial and connective tissues, separating each other.[20] Fibronectin is an protein of the extracellular matrix of connective tissues.[21] Fibronectin commonly has a amorphous organization, but in some situations, can assemble into a fibrous network in the ECM through interactions involving cell surface receptors and the fibronectin amino-terminal region.[22] The ability to bind to collagen ensures association between the fibronectin network and the scaffold of collagen fibrils. Fibronectin could be found in epithelial cells but in lower concentrations, and the decrease of its expression is associated with terminal epithelial differentiation.[23] We have previously demonstrated that Vero cells were capable of a great fibronectin synthesis but not of collagen IV.[24] Thus, our findings suggest that Vero cells acquired an intermediary differentiation pattern between epithelial and connective tissues.

The production of collagen IV by fibroblastic Vero cells on PLLA membranes was previously reported by us.[13] Other results showed that biodegradable materials have been used in experimental skin substitutes. PLLA is related to a good dermal tissue restoration.[25] A basement membrane formation, rich in collagen IV and laminin, by epidermal cells on copolymers of PLLA and PGA was also reported.[26] The presence of the pores structure or the variation of their size on PLLA/PHBV blends apparently does not influence the

capacity of producing extracellular matrix molecules. The production of other types of collagen on PLLA or PGA scaffolds was also reported. It was found that chondrocytes showed a higher collagen I synthesis on PLLA, although it was found a great collagen II deposition on PGA matrix.[27,28] This results suggest that PLLA could be used to fibrocartilaginous repair, and PGA should be indicated to hyaline cartilage regeneration. However, the PGA degrades much faster and PLLA was more stable.[29] On that way, the size of damage must be important parameter on the searching for the better polymer in a *in vivo* situation.

The contact among cells and extracellular matrix components play a critical role during the morphogenesis and wound healing. It is the continuous interactions among the cells and the surrounding matrix environment that lead the tissues to the acquisition and maintenance of differentiated phenotypes during the embryogenesis or tissue restoration. The ECM production by cells on biopolymers has a great relevance for tissue engineering once the ECM molecules give the substrate the capacity of maintenance of the cell viability and the induction of cell to differentiation. We did not find signals of cell degeneration that could indicate toxicity of the materials. On the contrary, we found that cells are capable of growing, proliferating and differentiating on substrates. Thus, we conclude that PLLA/PHBV blends present good qualities for cell culture substrate. For utilization on tissue engineering, those materials must be tested with experimental animals but, by results presented here, PLLA/PHBV blends showed desirable characteristics in an *in vivo* situation.

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FIGURE LENGENDS

Figure 1. Scanning electron microscopy of Vero cells cultured on different PLLA/PHBV blends. (A) Cells cultured on glass coverslip; (B) low magnification of cell over PLLA/PHBV (100/0); (C) round cell on porous region of PLLA/PHBV (100/0); (D) flattened cell on non porous region of PLLA/PHBV (100/0); (E) round cell on PLLA/PHBV (60/40); (F) cells linked by prolongation on PLLA/PHBV (50/50); (G) flattened cell growing over pores of PLLA/PHBV (40/60); (H) flattened cells linked by prolongation on PLLA/PHBV (0/100). Scale bar: 10μm.

Figure 2. Growth curve of Vero cells cultured on different PLLA/PHBV blends. In all samples studied, the cells grew in a lower way than positive control. On the other hand, we did not find proliferation rate on negative control used.

Figure 3. Morphological and immunocytochemical analysis of Vero cells cultured on different PLLA/PHBV blends for 48h. A) Cells cultured on PLLA/PHBV (100/0), B) Cells cultured on PLLA/PHBV (60/40), C) Cells cultured on PLLA/PHBV (50/50), D) Cells cultured on PLLA/PHBV (40/60), E) Cells cultured on PLLA/PHBV (0/100). The first column indicate cells stained by crystal violet, the second column anti-collagen IV and thirty column anti-fibronectin. Scale bar: 30μm.

Figure 4. Morphological and immunocytochemical analysis of Vero cells cultured on different PLLA/PHBV blends for 240h. A) Cells cultured on PLLA/PHBV (100/0), B) Cells cultured on PLLA/PHBV (60/40), C) Cells cultured on PLLA/PHBV (50/50), D) Cells cultured on PLLA/PHBV (40/60), E) Cells cultured on PLLA/PHBV (0/100). The first column indicate cells stained by crystal violet, the second column anti-collagen IV and thirty column anti-fibronectin. Scale bar: 30μm.

Figure 1

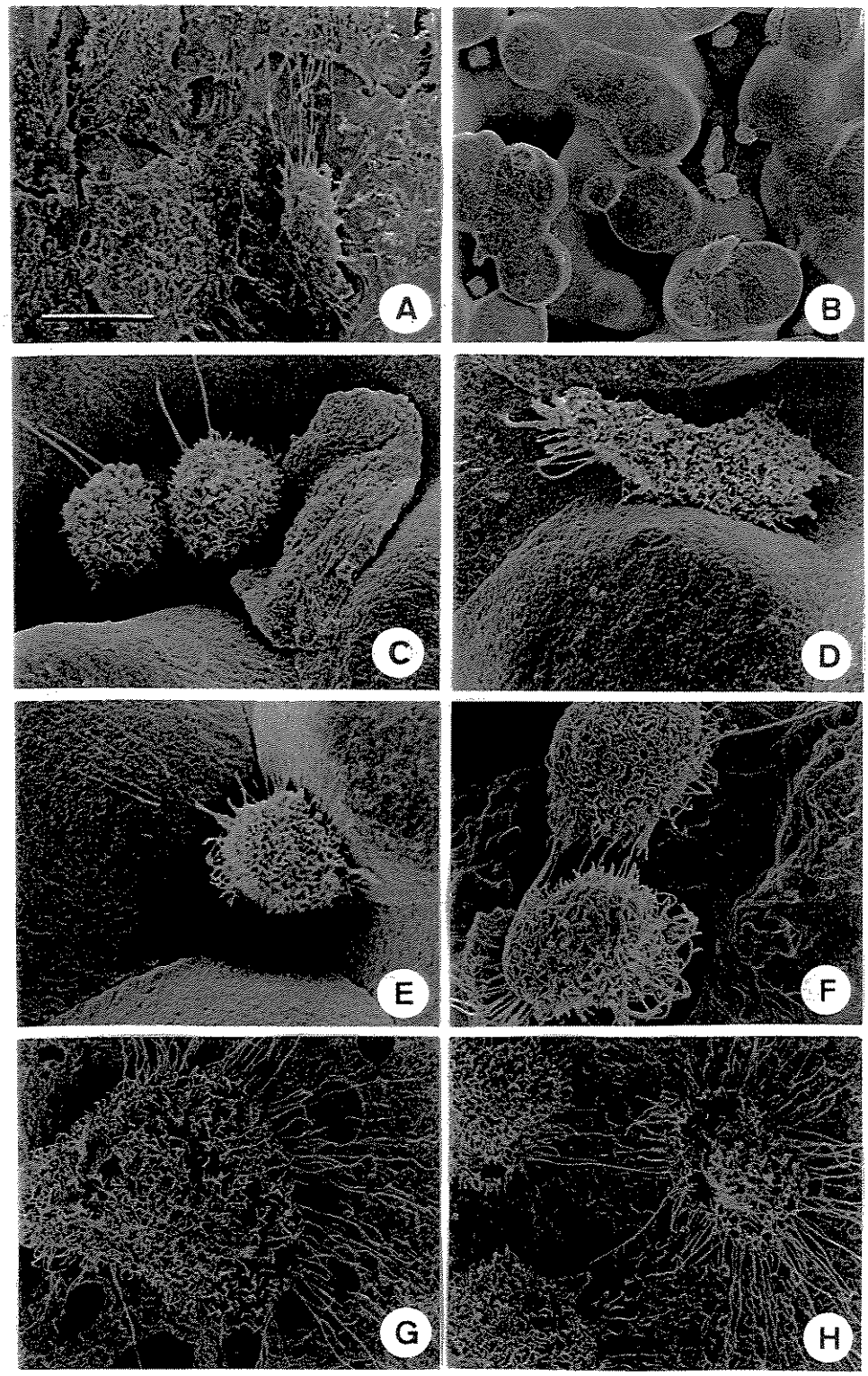


Figure 2

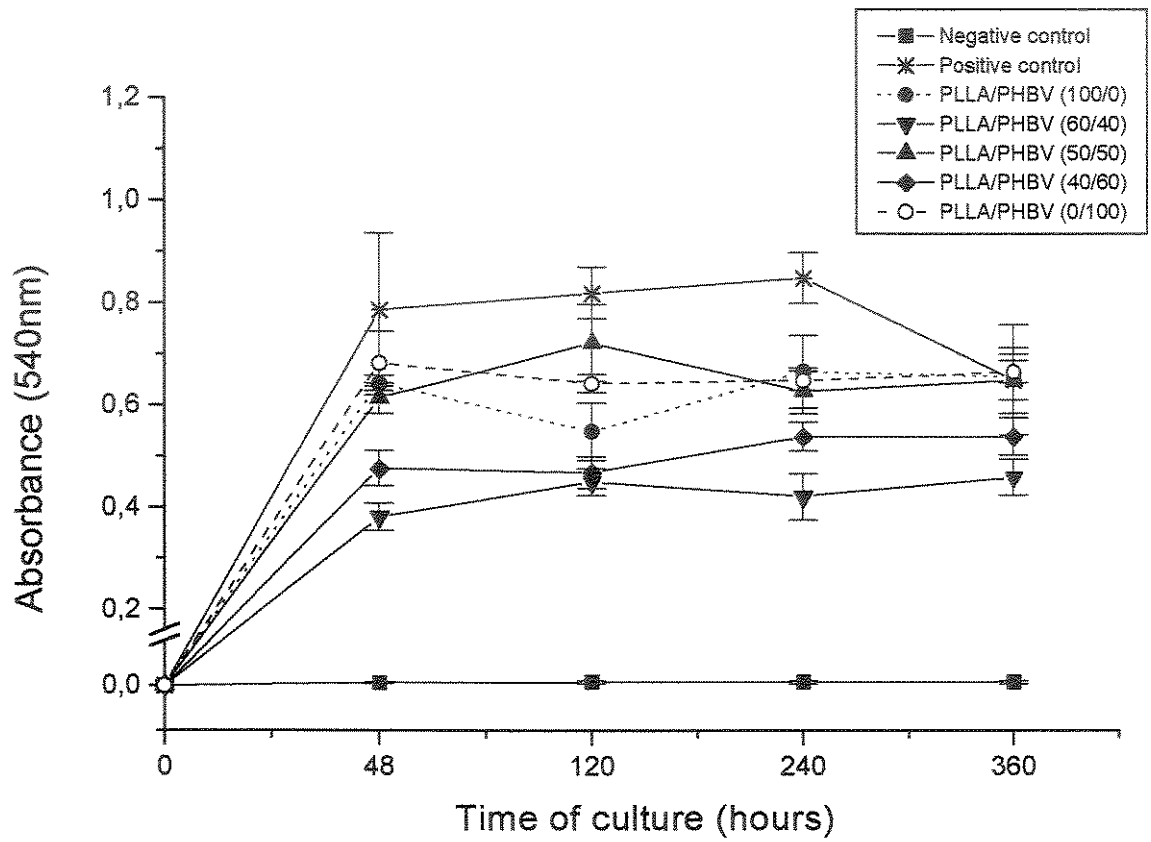
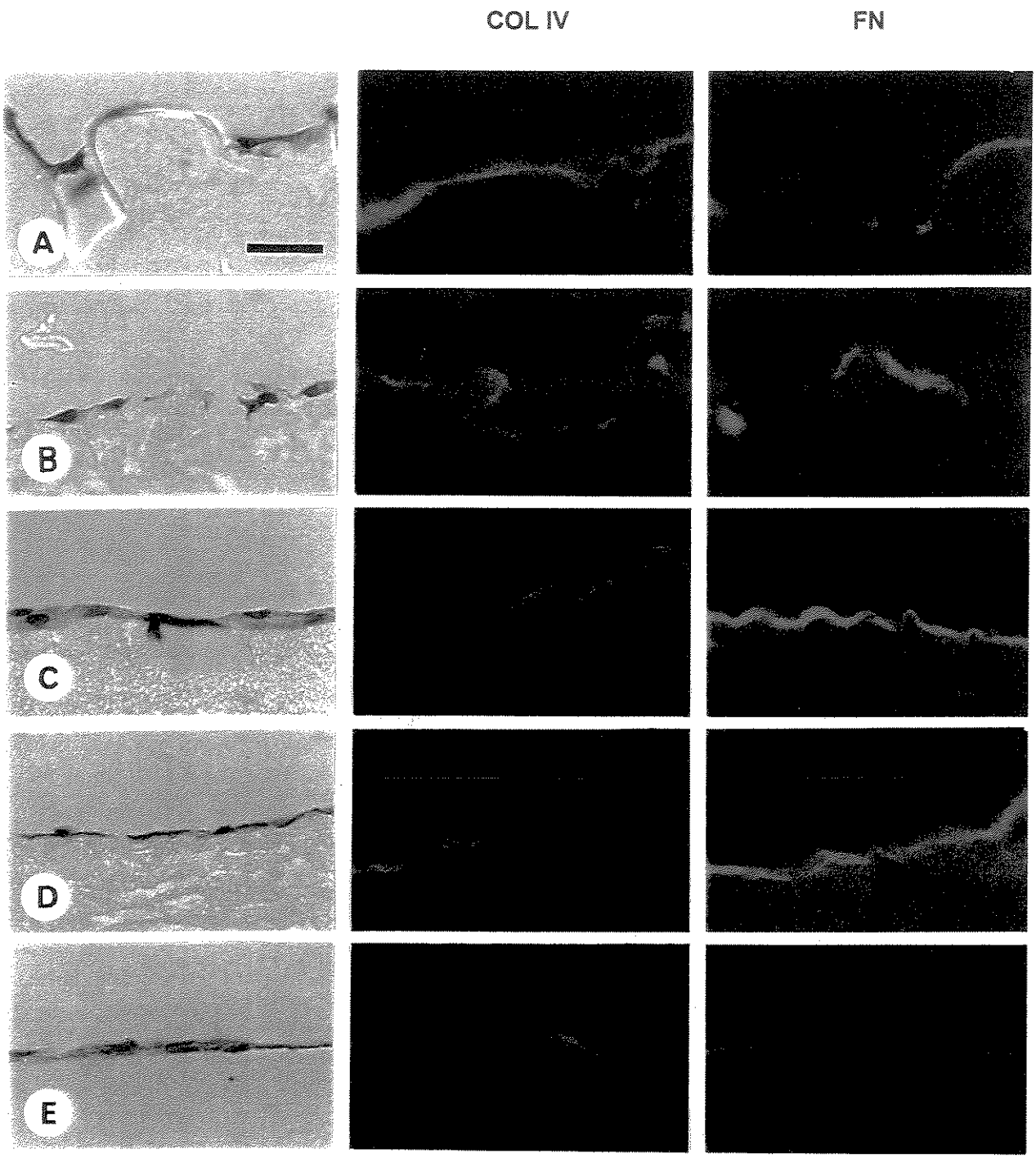
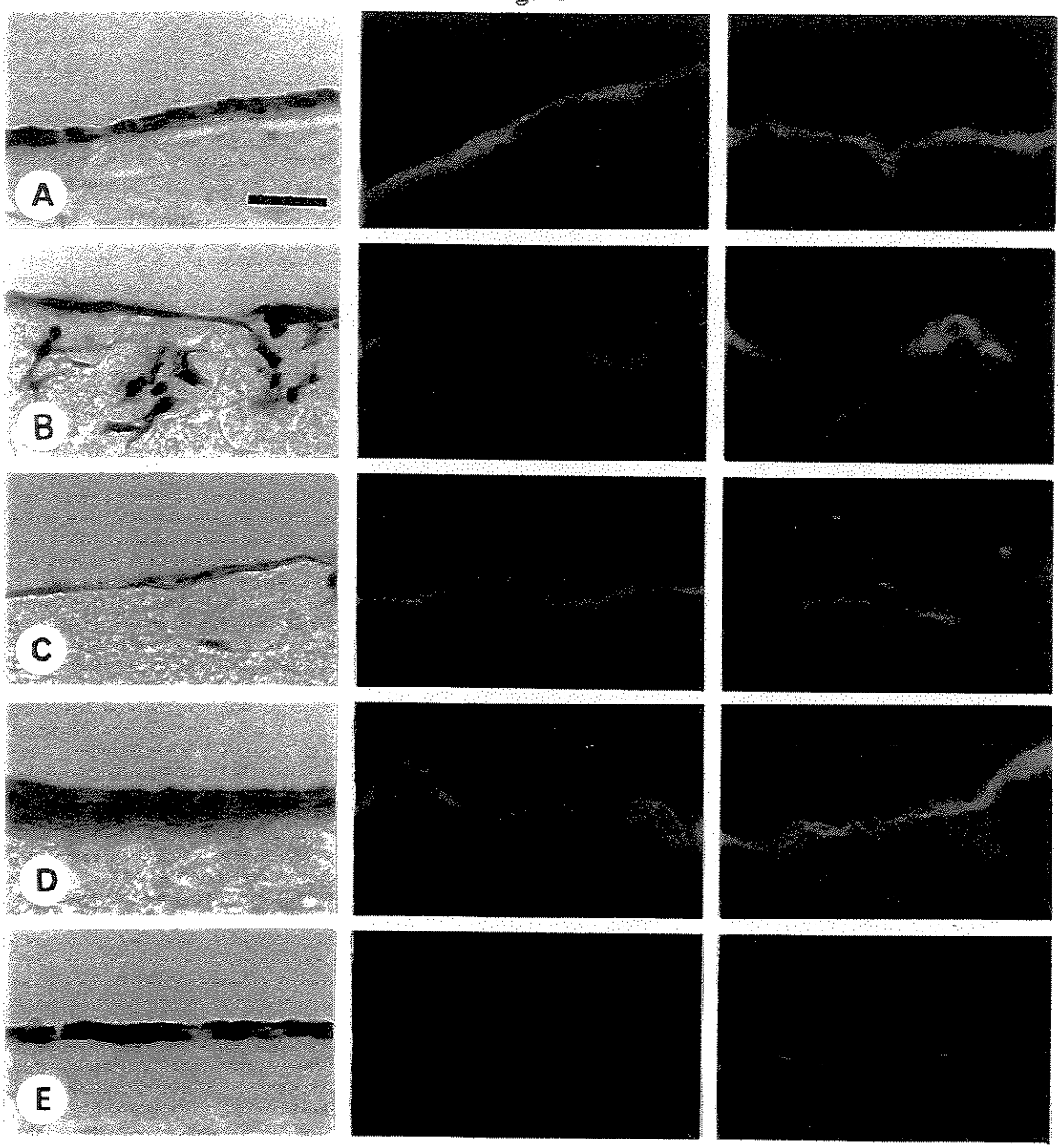

Negative control
Positive control
PLLA/PHBV (100/0)
PLLA/PHBV (60/40)
PLLA/PHBV (50/50)
PLLA/PHBV (40/60)
PLLA/PHBV (0/100)
Absorbance (540nm)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
48
120
240
360
Time of culture (hours)

Figure 3



COL IV FN

Figure 4



IV. CONCLUSÕES

1. Nenhum dos materiais estudados neste trabalho (membranas de PLLA densas ou com diferentes diâmetros de poros, e blendas de PLLA/PHBV em diferentes proporções) se mostrou tóxico para as células cultivadas sobre eles, uma vez que em todos os casos as células foram capazes de proliferar e produzir componentes de matriz extracelular sobre eles.

2. A morfologia celular, bem como sua taxa de proliferação, foram influenciadas pela presença de poros e por variações no diâmetro dos mesmos nas membranas densas ou porosas de PLLA. Em relação às blendas de PLLA/PHBV, as diferentes proporções de PHBV também foram capazes de alterar a morfologia das células cultivadas sobre os diferentes materiais, confirmando a sensibilidade das células Vero as características físico-químicas da superfície dos diferentes substratos.

3. As blendas de PLLA/PHBV (60/40, 50/50) mostraram-se mais eficazes em estimular a adesão celular do que os polímeros puros estudados (PLLA/PHBV 100/0 e 0/100). As células mostraram-se capazes de crescer e proliferar sobre as diferentes amostras de PLLA/PHBV, independente da variação dos polímeros presentes nas blendas.

4. Em todas as amostras estudadas, sejam membranas de PLLA densas ou com poros de diferentes diâmetros, e blendas de PLLA/PHBV em diferentes proporções (100/0, 60/40, 50/50, 40/60, 0/100) as células Vero foram capazes de produzir colágeno tipo IV e fibronectina, alterando em parte o seu padrão normal de diferenciação.

5. Nossos dados nos indicam que as membranas de poli(L-ácido láctico) que apresentam melhores resultados e mereceriam ser avaliadas *in vivo* são as amostras com poros variando entre 180-250μm e 250-350μm. Em relação as blendas de PLLA/PHBV, as amostras com proporções (60/40) e (50/50) foram as que apresentaram melhores resultados *in vitro*.

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VI. APÊNDICE

VI.1.

Avaliação da Citotoxicidade de hidrogéis de polihema: um estudo *in vitro*

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Avaliação da citotoxicidade de hidrogéis de polihema: um estudo *in vitro*.

Citotoxicity evaluation for polyhema hydrogels: an in vitro study.

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Resumo
Hidrogéis de poli(2-hidróxi etil metacrilato) reticulado e blendas do tipo redes semi interpenetrantes (sIPN) de poliHEMA com acetato de celulose e poliHEMA com poli(metacrilato de metila-co-ácido acrílico), desenvolvidas objetivando aplicação no reparo de defeitos da cartilagem articular foram submetidas a testes de citotoxicidade in vitro usando células VERO. Polipropileno e adevivo de silicone foram utilizados como controles negativo e positivo respectivamente. Foi avaliado a morfologia das células crescendo sobre amostras de hidrogéis e sobre lamínulas de vidro, bem como a presença e viabilidade das células presentes nos sobrenadantes (método de exclusão com azul Tripan). Todas as amostras de hidrogéis testadas apresentaram comportamento semelhante ao controle negativo, isto é, não apresentaram efeito citotóxico frente a linhagem celular testada.
Palavras-chave: citotoxicidade, hidrogel, cultura de células

Abstract
Hydrogels of polyHEMA and semi interpenetrating networks of polyHEMA with celulose acetate or poly(methyl methacrylate-co-acrylic acid) developed aiming articular cartilage repair were submited to "in vitro" citotoxicity tests using VERO cells. Polypropilene and silicone adesive were used as negative and positive control respectively. It was evaluated the morfology of cells growing on material samples and coverslips. The viability of cells presents in sobrenadants was evaluated using the Tripan Blue exclusion method. The behavior of cells on hydrogels was similar to that one presented by the the negative control, without citotoxicity effect.
Keywords: citotoxicity, hydrogel, cell culture

Introdução

Hoje em dia, com o crescente uso de biomateriais na prática médica e odontológica, torna-se cada vez mais importante o desenvolvimento e/ou implementação de testes para a avaliação da biocompatibilidade de materiais.

Os testes de citotoxicidade (Black, 1992) representam a fase inicial do teste de biocompatibilidade de um material com potencial para aplicações médicas, sendo utilizados em uma pré-seleção para detectar se o material em questão provoca morte das células ou outros efeitos negativos nas funções celulares. Diferentes métodos tem sido desenvolvidos e padronizados (Kirkpatrick, 1992), porém dependendo da especificidade do material, algumas adaptações devem ser feitas para que se possa tirar bons resultados da aplicação do teste.

Utilizam técnicas *in vitro* para identificar efeitos adversos que biomateriais ou dispositivos médicos em potencial possam acarretar à células, de maneira a torná-los impróprios para uso como tal. Para ser aprovado num teste de citotoxicidade *in vitro*, um material não deve causar a morte das células nem afetar suas funções celulares. Assim sendo, com o uso de técnicas de cultura de células (Freshney, 1989), os testes podem detectar se ocorre a lise das células, a inibição do crescimento celular e outros efeitos que possam ser causados nas células pelo material e/ou extrato oriundo do material.

Existem dois tipos de testes *in vitro* : métodos de contato direto e métodos de contato indireto. No primeiro, as células são colocadas em contato com o material em teste, sendo normalmente semeadas na forma de uma suspensão celular sobre o material. Já os métodos de contato indireto podem ser divididos em dois tipos: aqueles em que o material a ser testado é separado das células por uma barreira de difusão (ágar ou agarose) e o segundo tipo no qual substâncias são extraídas do material a ser testado, através de um solvente e colocada em contato com as células.

Normalmente utilizam-se nestes testes células de linhagens estabelecidas, devido a facilidade de obtenção e manutenção em laboratório (Northup, 1986). As linhagens celulares mais recomendadas para testes de citotoxicidade são a NTC clone 929, a Balb/3T3 clone A31; a MRC-5 a WI-38, a VERO, a BHK-21 e a V-79. Além destas, outras linhagens podem ser utilizadas se for possível demonstrar que os resultados obtidos são semelhantes (ISO 10993-5, 1992).

A avaliação da citotoxicidade pode ser feita através da análise da morfologia celular, da integridade da membrana celular (pela utilização de métodos com corantes vitais ou não), da proliferação celular, de atividade biossintética, etc (Freshney, 1989).

Existem diferentes protocolos padrões já estabelecidos para os testes de citotoxicidade, dentre os quais podem ser citados :

- ASTM F-813-83 - Método de contato direto para avaliação de materiais e dispositivos médicos frente à cultura de células;
- ASTM F-895-84 - Método de difusão em ágar de cultura de células para seleção de materiais por citotoxicidade;
- ISO 10993-5 - Avaliação biológica de dispositivos médicos - Parte 5 : Testes para citotoxicidade : métodos *in vitro*.

Além de aspectos referentes ao procedimento, os padrões normalmente especificam a linhagem celular, o meio de cultura e as técnicas para avaliação da citotoxicidade. Além disso, todos os protocolos padrões prevêem a utilização e especificam materiais a serem utilizados como controles positivo e negativo. Controle positivo é uma substância que apresenta efeito citotóxico de maneira reprodutível e controle negativo é o material ou substância que não produza efeito citotóxico.

Este trabalho relata uma das etapas de um estudo que vem sendo realizado visando o desenvolvimento de hidrogéis de poli(2-hidróxi etil metacrilato) - poliHEMA para aplicação no reparo de defeitos da cartilagem articular (Malmonge, 1997). Com o objetivo de obter um biomaterial capaz de mimetizar o comportamento mecanico da cartilagem articular natural, numa primeira etapa do estudo, diferentes hidrogéis foram sintetizados e caracterizados quanto ao comportamento mecânico (malmonge & Zavaglia, 1997). Nesta etapa do estudo, amostras de tres hidrogéis obtidos a base de poliHEMA foram submetidos a ensaio de citotoxicidade, conforme descrito a seguir.

Materiais e Métodos

Material

Foram submetidas ao ensaio três amostras de hidrogéis: poliHEMA reticulado, blenda de poliHEMA com acetato de celulose (AC) e blenda de poliHEMA com poli(metacrilato de metila-co-ácido acrílico) (poliMMA-co-AA). Os hidrogéis foram sintetizados por polimerização térmica conforme já descrito em trabalho anterior (Malmonge, 1997; Malmonge & Zavaglia, 1997).

- amostra A : poliHEMA reticulado (3.0% AR)

- **amostra** B : blenda poliHEMA - AC
- **amostra** C : blenda poliHEMA - poli(MMA-co-AA)

Como controle positivo foi utilizado filme obtido a partir de adesivo de silicone (Rhodiastic) e como controle negativo foi utilizado fragmento de placas de polipropileno. Tanto as amostras quanto os controles foram preparados na forma de discos com 10 mm de diâmetro e 2 mm de espessura, e a seguir lavados várias vezes com solução salina (NaCl 0.9%) e esterilizadas em autoclave a 120° C por 30 min. Cada amostra do material foi colocada sobre uma lamínula de vidro no interior de tubos de Leigthon (figura 1) e mantidos com meio Ham F 10, com 10% de soro fetal bovino (SFB) por 12 horas a 37° C, antes de receberem o inóculo celular.

Células

Células da linhagem VERO provenientes do Instituto Adolfo Lutz - SP, foram mantidas rotineiramente em meio Ham F 10 com 10% de SFB com repiques sendo efetuados sempre que a cultura atingiu a confluência. Alíquotas de 1 ml de uma suspensão celular em meio de cultivo contendo 10^5 células/ml foi inoculada em cada tubo de Leighton (figura 1), após a retirada do meio de lavagem. Os frascos foram mantidos a 37° C por 48 horas.

O experimento foi efetuado em triplicata, isto é 3 frascos de cultura por amostra, inclusive para os controles positivo e negativo.

Avaliação da citotoxicidade

Viabilidade celular *(método de exclusão pelo Azul Tripan)* - após 48 horas de incubação, amostras do meio de cultura em cada frasco foram coletadas, diluídas 1:1 em solução de Azul Tripan 0,1 % em salina 0.9 % e as células viáveis e não viáveis presentes no meio de cultura foram contadas em câmara de Neubauer.

Morfologia das células - Os discos e as lamínulas foram retirados dos tubos de Leighton, fixados em metanol:ácido acético (3:1), lavados rapidamente em metanol e secos ao ar. A seguir foram corados com cresil violeta 0,25% por 15 min, lavados em água, secos ao ar, diafanizados em xilol e montados sobre lâminas de vidro com Entelan (Merck) para observação ao microscópio.

Resultados e Discussão

O material utilizado como controle positivo, adesivo de silicone apresentou efeito citotóxico extremamente forte, após 48 h de cultura, pois praticamente não restaram células viáveis sobre as amostras e nem mesmo sobre as respectivas lamínulas. Na figura 3 podem ser observados aspectos degenerados das poucas células que permaneceram sobre o material ou lamínula. Para todos os frascos de cultura contendo o material controle positivo, foram detectadas células não viáveis em suspensão no meio de cultivo (figura 2)

Já no caso do controle negativo, fragmentos de placa de polipropileno, as células cresceram normalmente, tanto sobre as amostras quanto nas respectivas lamínulas (figura 4). A análise do meio de cultivo destes frascos mostrou ausência de células não viáveis em suspensão.

Conforme pode ser observado nas figuras 5,6 e 7, a morfologia das células crescendo sobre as lamínulas que foram incubadas junto com as amostras dos hidrogéis e as células que cresceram sobre as amostras dos diferentes hidrogéis não mostram alterações morfológicas acentuadas quando comparadas com o

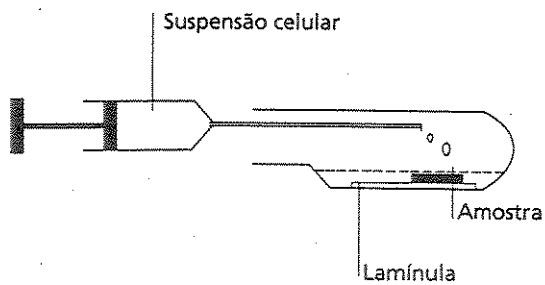

Figura 1: Esquema ilustrando o inóculo das células sobre a lamínula de vidro e amostra contidos em frasco de Leighton.

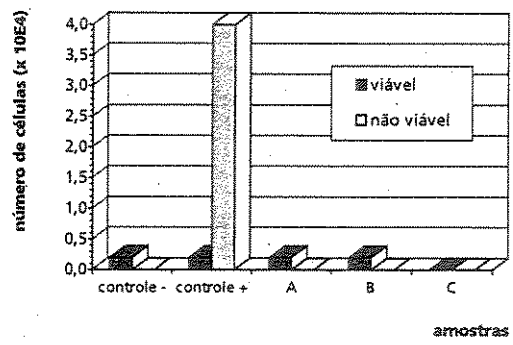

Figura 2: Quantidade de células em suspensão no meio de cultura (resultados representam a média de 3 amostras para cada frasco).

controle negativo (figura 4). Isto demonstra que os hidrogéis não exercem efeito citotóxico para as células que crescem sobre eles e não liberam substâncias tóxicas para o meio. A presença de células com morfologia um pouco mais alongada, que podem ser observadas (figuras 5b, 6b e 7b), crescendo sobre os hidrogéis é apenas resposta biológica das células VERO, pois as mesmas apresentam a característica de mudar de forma e/ou função *in vitro* de acordo com o substrato na qual estão aderidas (Wada & Vidal, 1991). Quanto a presença de células viáveis em suspensão no meio de cultura de algumas das amostras de hidrogel (figura 2), é fato considerado normal, pois as células que crescem em monocamada tornam-se redondas quando entram em processo de divisão celular, ficando menos aderidas ao substrato e podendo ser facilmente descoladas pelo manuseio do frasco de cultura (Adams, 1990).

A não citotoxicidade das amostras testadas pode ser concluída tendo em vista que apesar de menor número de células presentes sobre o substrato (hidrogel), em relação ao número de células presentes nas lamínulas, foi possível detectar células em processo de divisão e consequente proliferação celular . Além disso, as células que cresceram sobre as lamínulas em todos os casos apresentaram comportamento típico de células VERO.

Pode-se verificar que a adesão celular foi menor para as amostras de hidrogéis do que para as lamínulas de vidro. Além disso, a blenda de poliHEMA-poli(MMA-co-AA) apresentou a menor adesão celular entre os três hidrogéis testados. Segundo Folkman & Moscona (1978), apesar de não apresentar efeito citotóxico, o poliHEMA inibe a adesão celular. Segundo estes autores, o recobrimento de substratos com camadas de poliHEMA com diferentes espessuras podem modular tanto a extensão do espalhamento quanto o metabolismo celular.

Sabe-se que adesão e proliferação celular de diferentes tipos de células sobre substratos poliméricos

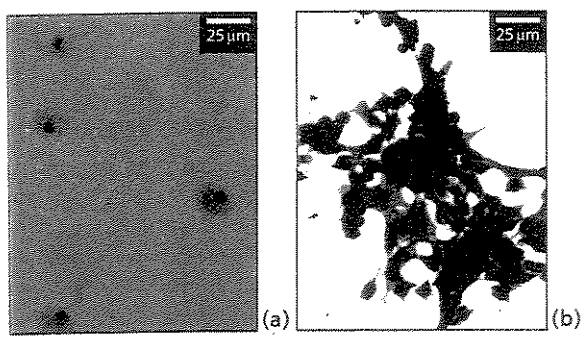

Figura 3: controle positivo , (**a**) material, (**b**) lamínula

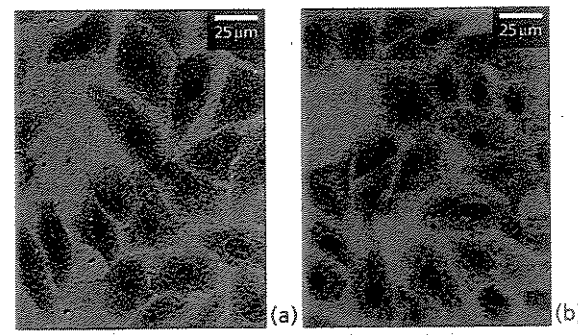

Figura 4: controle negativo, (**a**) material, (**b**) lamínula

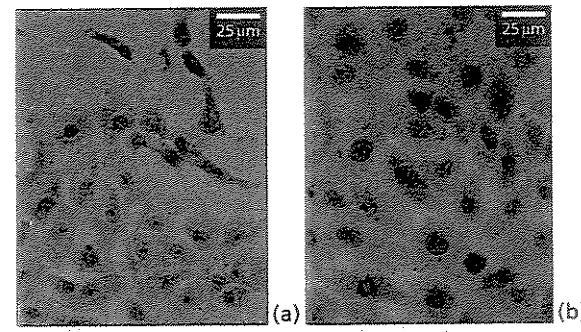

Figura 5: poliHEMA: (**a**) material, (**b**) lamínula

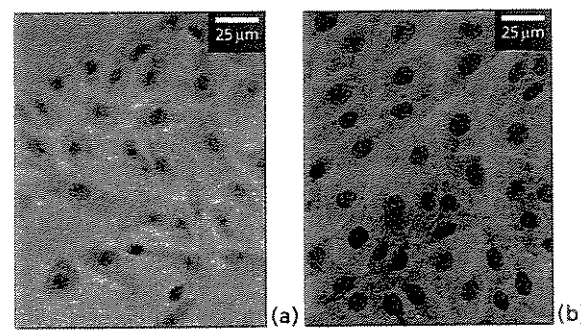

Figura 6: poliHEMA-AC: (**a**) material, (**b**) lamínula

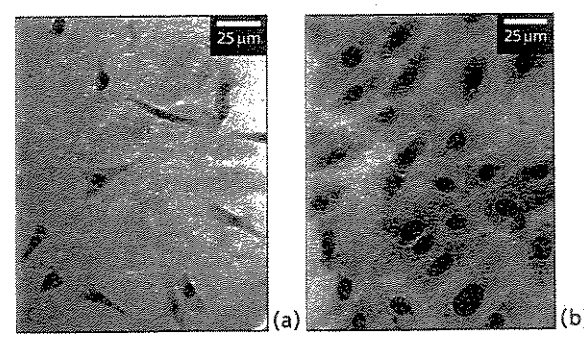

Figura 7: poliHEMA-poli(MMA-co-AA): (**a**) material, (**b**) lamínula

dependem das características de superfície de tais materiais tais como : molhabilidade, presença de grupamentos químicos específicos, carga, rugosidade e rigidez. Um grande número de pesquisadores vem estudando a interação entre diferentes tipos de células

in vitro e polímeros com diferentes características superficiais (Lydon e cols, 1985; Horbett e cols., 1988; Smetana, 1993 e Lee e cols., 1994).

Segundo Lee e colaboradores (1994), para superfícies com molhabilidade, rugosidade e rigidez semelhantes, aqueles que são positivamente carregados são mais propícios para a adesão, espalhamento e crescimento celular *in vitro* do que aqueles que são negativamente carregados ou mesmo neutros. Ainda segundo o autor, algumas proteínas presentes no meio de cultura como fibronectina e vitronectina, as quais exercem grande influência na adesão celular são mais facilmente adsorvidas nas superfícies positivamente carregadas, melhorando assim a adesão celular. Ainda segundo estes autores, a presença de grupamentos ácido carboxílico exerce efeito negativo na adesão, espalhamento e crescimento celular.

Smetana (1993), e Lio e colaboradores (1994), mostram que materiais positivamente carregados induzem a adesão celular, ao contrário dos materiais negativamente carregados. A hipótese levantada por Smetana é de que interações eletrostáticas entre a membrana celular e o substrato representam um dos mecanismos de adesão celular ao substrato, uma vez que as glicoproteínas presentes na membrana celular são negativamente carregadas. Recentemente, vários pesquisadores tem verificado a importância das glicoproteínas, em particular a fibronectina, na adesão e espalhamento celular (Culp, 1978 e Grinnel & Feld, 1981).

Assim, para a blenda de poliHEMA-poli(MMA-co-AA), a presença de grupamentos (COOH), provenientes do ácido acrílico os quais no meio de cultura encontram-se ionizados, constituem cargas negativas fixas a matriz, o que provavelmente leva a diminuição da adesão celular por mimetizar os estímulos normalmente fornecidos pelas glicoproteínas presentes na matriz extracelular dos tecidos.

O fato de um substrato apresentar menor adesão celular *in vitro* não significa que sua biocompatibilidade seja menor. Ao contrário, alguns autores acreditam que a presença de grupos (COOH) em hidrogéis não reabsorvíveis diminuem a interação do material com macrófagos, sendo portanto mais interessantes para algumas aplicações clínicas (Smetana, 1993 e Smetana, 1990).

Segundo Smetana (1993), as propriedades hidrofílicas/hidrofóbicas de polímeros e a ocorrência de grupos funcionais carregados podem influenciar a interação das células com o substrato e consequentemente a função celular. Os autores relatam ainda que a presença de grupos ácido carboxílico (COOH) no hidrogel, semelhante ao que ocorre na matriz extracelular natural, permite a este polímero participar no controle da função celular, mimetizando os estímulos normalmente realizados pela matriz extracelular natural.

Conclusões

A metodologia utilizada mostrou-se adequada para a determinação da citotoxicidade de hidrogéis.

Hidrogéis de poliHEMA reticulado, bem como de blendas de poliHEMA-AC e poliHEMA-poli(MMA-co-AA), não apresentaram efeitos citotóxicos frente a cultura de células VERO *in vitro*.

Agradecimentos

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VI.2.

Differential Schwann cell migration in adult and old mice: an in vitro study

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Short communication

Differential Schwann cell migration in adult and old mice: an in vitro study

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Abstract

The influence of aging on Schwann cell (SC) proliferation, migration and viability was studied in vitro. SCs were cultured in Ham F-10 medium enriched with 20% fetal calf serum (FCS), 40% FCS or collagen I gel plus 20% FCS. The migration of adult mice derived SCs was stimulated with FCS and collagen. With aging, SC migration, multiplication and viability decreased, indicating that ideal culturing conditions should be adjusted.

Theme: Development and regeneration

Topic: Glia and non-neuronal cells; Aging process

Keywords: Schwann cell; Cell culture; Collagen; Cell migration; Aging; Mouse

Peripheral nerve regeneration occurs as a result of a series of events, which involve axonal regeneration and reorganization of the extracelullar microenviroinment [2,4,5,11]. After nerve lesion, Wallerian degeneration takes place distally to the lesion. This process is characterized by macrophage invasion and Schwann cell (SC) multiplication, mainly into the distal stump [1,2,8]. Such newly produced SCs organize themselves within the basal lamina left by degenerated axons, originating the so-called 'bands of Büngner'.

Taking into account that with aging, nerve regeneration is less successful [6,10] and considering the importance of the SC for the development of this process, the aim of this study was to investigate in vitro its multiplication and migration capacity with aging. Also, we have investigated the importance of collagen and serum factors for SC migration and multiplication.

For this study, sciatic nerves from adult (8 months old, $n=5$) and old (2 years old, $n=5$) C57BL/6J male mice were used. After dissection, the nerves were reduced into fragments about 1 cm long and washed in Ham F-10 medium (Sigma) supplemented with 20% fetal calf serum (FCS) and 100 μg/ml of gentamicin (Schering-Plough). The fragments were then cut in smaller, 2 mm long pieces and cultured in culture plates with six wells (Corning/Costar) at 37°C for 20 days. Three different experimental conditions were used: (1) Ham F-10 medium supplemented with 100 μg/ml of gentamicin and 20% FCS, (2) Ham F-10 medium supplemented with 100 μg/ml of gentamicin and 40% FCS, or (3) Ham F-10 medium supplemented with 100 μg/ml of gentamicin and 20% FCS in well culture plates coated with 1 ml of collagen I gel. The collagen was extracted according to the method described by Schor [9] and was prepared with 0.9 ml of collagen solution, 0.05 ml of 4% $NaHCO_3$ and 0.05 ml of 10 times concentrated Ham F-10 medium (Sigma). Within the incubation period, the total number of migrant cells was evaluated on days 1, 2, 4, 6, 8 and 10 in all experimental conditions. On the 20th day, the culture medium was collected and the non-adherent cells were counted with an Olympus IX-50 inverted microscope with a phase contrast system.

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After 15 or 20 days of culture, the samples were fixed in 10% formalin for 1 h and washed in phosphate buffered saline (PBS) 0.1 M in pH 7.2 at 37°C. In order to block nonspecific staining, the specimens were incubated for 1 h with 1% bovine serum albumin (BSA, Sigma) in PBS, washed and the monoclonal anti-S-100 antibody (dilution 1:300) was applied. The samples were rinsed and the anti-rabbit CY-3 secondary antibody was added.

In all experimental conditions, cells from adult or old animals were able to migrate from the explants. The cell migration pattern from adult or old animals was very similar except that in general, the cells from adult animals showed a more intense migration rate than those from old animals. Also, an increase of migrating cells was proportional to the increase of FCS concentration. The cells that grew on collagen I gel showed the highest migration rate (Fig. 1).

After 15 days of culture, we observed SCs migrating from explants to the culture plate or to collagen I gels in all experimental conditions. The Schwann cells labeled with anti-S-100 antibody showed a bipolar morphology with thin- and long-cell prolongations, except in the nuclear region (Fig. 2). SC counting revealed that explants from adult animals cultured with 20% FCS plus collagen I gel or 40% FCS displayed an increase of SCs when compared to samples cultured only with 20% FCS. On the other hand, the explants from old animals showed a decreased SC number in relation to adult animals. An important finding was that the number of SCs derived from old animals did not increase substantially when the medium was enriched with FCS or collagen I (Fig. 1A).

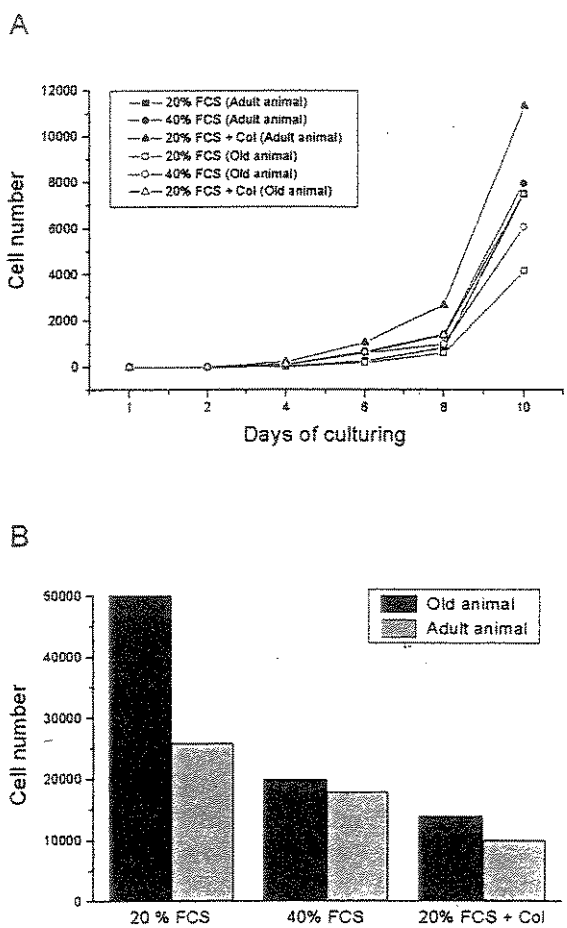

Fig. 1. (A) Migration of cells from the sciatic nerves explants from adult or old animals in the different experimental conditions. (B) Counting of non-adherent cells found in the culture medium after 20 days of culture.

Schwann cell autograft produced in vitro has been reported as a novel method for repairing long gaps [3,5] following extensive peripheral nerve lesions. However, with aging, there is evidence that SCs decrease in their capacity to multiply and produce neurotrophic factors, basal lamina components and myelin [12]. Considering the relative importance of SCs for nerve regeneration and taking into account that a percentage of peripheral nerve lesions occur in middle age or elderly individuals, we have investigated the behavior of SCs obtained from old mice, submitted to different experimental conditions. Under these experimental conditions, it was possible to determine if extracellular stimuli would be able to increase the survival, migration and proliferation rates of the SCs.

With regard to the migration of SCs, both adult and aged cells started migrating around the fourth day, but with different rates. Basically, migration in the old mouse-derived cells (OMDC) and adult mouse-derived cells (AMDC), when cultured with FCS 20 and 40%, was similar up to the sixth day. On the other hand, culturing with collagen stimulated SC migration only in the AMDC. These findings show that the SCs retain its migratory ability with aging but the capacity to respond to extracellular stimuli may be reduced. This fact can be related with the capacity to synthesize and express receptors for matrix components, such as collagen, fibronectin and basal lamina elements, which are essential for cell migration [7,11].

In this context, we observed that the viability of the SCs in culture was greatly increased when the medium was supplemented with FCS 40% or with collagen plus FCS 20% (Fig. 1B). Such results reinforce the hypothesis that the absence of extracellular stimuli as well as the relation with other cell types which synthesize trophic substances, as well as cytokines, may be a strong factor in changing the behavior of SCs when in vitro. With regard to the SCs from old mice, the viability assay showed that these cells are even more sensitive and almost all of them detached from the plate after 20 days of culture. Interestingly, when the medium was supplemented with FCS 40%, cell detachment was considerably reduced. The results were even better when collagen was added. Taken together, our results reinforce the fact that cultured SC behavior is altered compared to that shown during in vivo Wallerian degeneration. On the other hand, enriching the medium

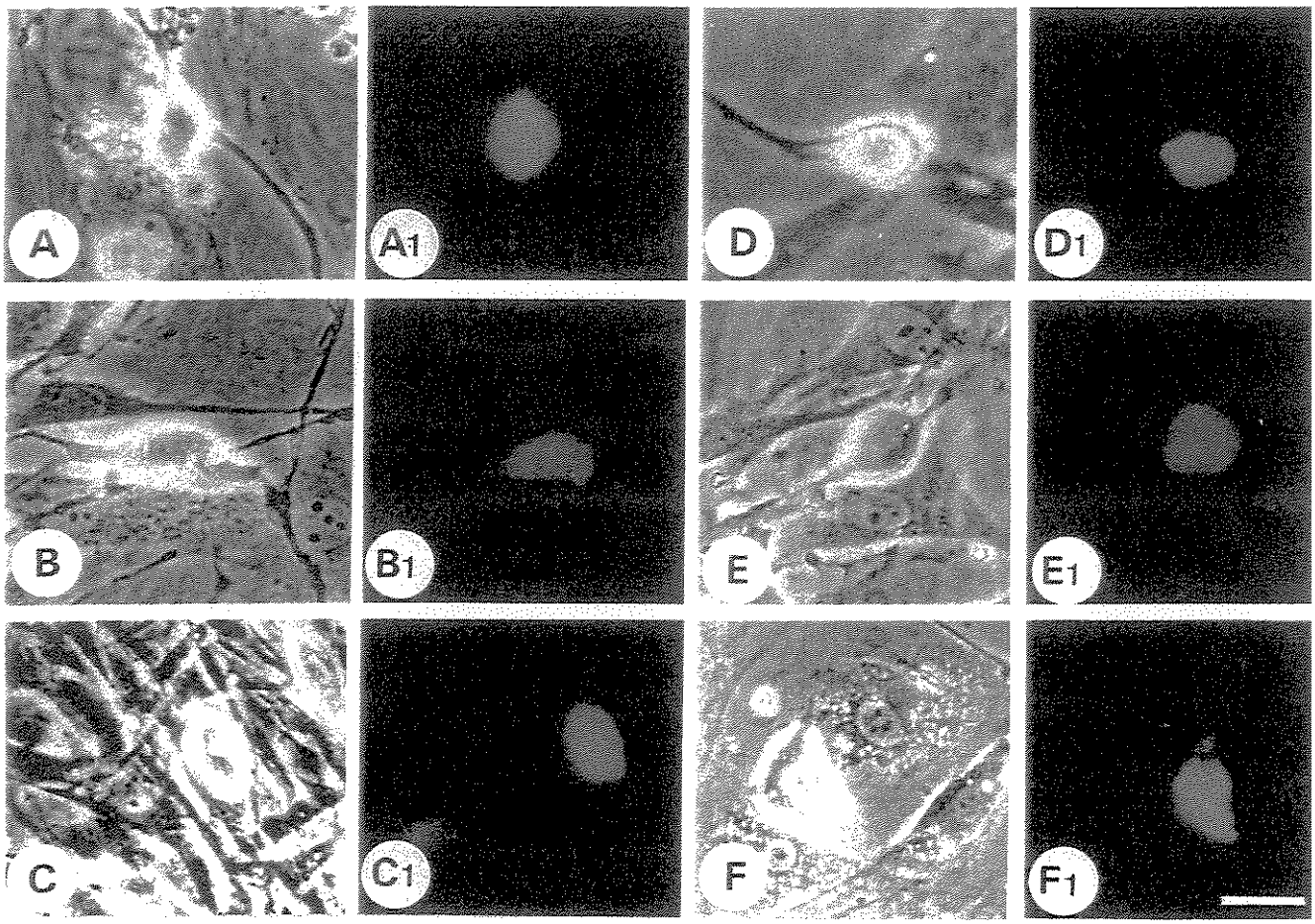

Fig. 2. Phase contrast and immunocytochemical labeling (S-100) of the Schwann cells cultured in the different experimental conditions. (**A**–**C**) represent adult derived cells and (**D**) to (**F**), old mouse-derived cells. (**A**), (**A1**), (**D**), (**D1**), cultured with 20% FCS. (**B**), (**B1**), (**E**), (**E1**), cultured with 40% FCS. (**C**), (**C1**), (**F**), (**F1**), cultured on collagen I gel with 20% FCS. Scale bar=25 μm.

with extracellular matrix components as well as trophic substances may increase cellular viability and make the cell behavior closer to the in vivo conditions. Also, with aging, all the behavioral alterations are sharper and cellular migration as well as multiplication and viability in culture are greatly reduced. These facts should be taken into account and studied further in order to define ideal culturing conditions for SCs at different donor ages, which will be crucial for the development of nerve repair techniques employing cultured SCs.

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