UNIVERSIDADE ESTADUAL DE CAMPINAS

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AVALIAÇÃO *IN VITRO* DE TUBOS DE PVC RECOBERTOS UTILIZADOS EM PROCEDIMENTOS DE CIRCULAÇÃO EXTRACORPÓREA

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

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Dedico este trabalho

aos meus pais e minha irmã

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RESUMO

Cirurgias cardiopulmonares necessitam de conectores para a circulação extracorpórea (CEC) do sangue do paciente. Os contatos entre o sangue e superfícies artificiais causam alterações adversas como, por exemplo, a deposição de proteínas, a adesão e destruição de glóbulos vermelhos, a adesão plaquetária com sua conseqüente agregação e a coagulação sanguínea.

Buscando produzir superfícies artificiais homogêneas e inertes para a CEC, tenta-se desenvolver superfícies hemocompatíveis. Deste modo, os tubos e muitos oxigenadores utilizados neste procedimento cirúrgico estão sendo revestidos por heparina, um anticoagulante amplamente utilizado nos procedimentos cirúrgicos cardiovasculares e, mesmo por lipídeos, que mimetizariam as membranas biológicas que normalmente entram em contato com o sangue e que não produzem nenhuma reação citada anteriormente, o que se acredita poder reduzir as complicações desse procedimento cirúrgico, uma vez que, podem diminuir ou até mesmo extinguir as reações adversas. Além disso, os revestimentos das superfícies sintéticas poderiam permitir a redução do uso de heparina sistêmica, uma vez que esta pode trazer complicações hemorrágicas ao paciente após a CEC.

Assim, este trabalho compreendeu o estudo do comportamento de células da linhagem Vero, e de culturas primárias de células sanguíneas humana quando cultivadas sobre tubos de cloreto de polivinila (PVC) revestidos com heparina e com lipídeos dimiristoil fosfatidilcolina (DMPC) e dimiristoil fosfatidiletanolamina (DMPE). O objetivo final foi verificar a interferência desses recobrimentos nas reações e processos envolvidos com a hemocompatibilidade e citotoxidade dos tubos de PVC, propiciando o desenvolvimento de

um produto nacional o que, por sua vez, diminuiria o custo da CEC, uma vez que os tubos revestidos disponíveis no mercado são importados, o que encarece tal procedimento cirúrgico.

Nossos resultados demonstraram que o processo de esterilização utilizado sobre esses materiais é importante, uma vez que podem causar falhas no recobrimento, permitindo agregação celular, além de causarem reações químicas as quais também contribuem para o processo citado anteriormente.

O recobrimento de heparina mostrou-se mais eficiente em relação aos fosfolipídios (DMPC e DMPE) no que diz respeito à proliferação celular, uma vez que, apesar de permitir alguma adesão celular, não proporciona um espalhamento das células sobre os substratos, o que é essencial para a proliferação celular.

No entanto, após tempos prolongados de cultura não se encontram células aderidas à superfície dos polímeros em nenhuma das situações experimentais. Isto pode estar sendo causado pela liberação de ftalatos para o meio de cultura. Estes são plastificantes utilizados na confecção dos tubos de PVC e são, conhecidamente, tóxicos. Apesar disto, os recobrimentos reduziram a taxa de degeneração celular quando comparados aos tubos não revestidos, o que nos permitiu concluir que os recobrimentos diminuem o efeito citotóxico encontrado no PVC.

ABSTRACT

Cardiopulmonary surgery requires connectors for the extracorporeal circulation (ECC) of the patient's blood. Blood contact with artificial surfaces may cause side effects such as protein adsorption, adhesion and destruction of red cells, platelet adhesion as well as their aggregation and consequently, blood coagulation.

Searching for homogeneous artificial surfaces for ECC, the development of hemocompatible surfaces have also been undertaken. In this way, tubes and some of the oxygenators used in this procedure have been coated by heparin (an anticoagulant largely used in cardiovascular surgeries) and even by lipids (which may mimetize the biological membranes that normally get in contact with blood and do not produce any of the reactions cited above). It is believed that these coatings can reduce the complications of this procedure, since they can decrease or even extinguish the common side effects. Moreover, the coating of synthetic surfaces could allow the reduction of the systemic heparinization which can cause severe hemorrhage in the patients after the ECC.

So, this project studied the behavior of Vero cells and blood cells when plated over (poly)vinyl chloride (PVC) tubes coated by heparin and phospholipids as Dimyristoyl phosphatidylcholine (DMPC) and Dimyristoyl phosphatidylethanolamine (DMPE) in order to observe the interference of these coatings in the reactions and processes involved with hemocompatibility and cytotoxicity of PVC tubes, which are important for the development of a national product that could reduce the ECC cost, since all the coated material available on the market is imported.

Our results showed that the sterilization process used on these materials is important, because it can cause grooves in the coating, allowing cellular aggregation, and even react with them which would also contribute to the above cited process.

The heparin coating was more efficient than the phospholipids ones (DMPC and DMPE) concerning cellular proliferation since, although, it allowed some cellular adhesion, it did not allow the spreading of those cells, which is essential to their proliferation.

However, after prolonged culture time, we did not find cells adhered to any of the coated surfaces. This could have been caused by the release of phthalates into the culture medium. Phthalates are platicizers used in PVC tubes and are known to be toxic. Despite this fact, the coated tubes reduced the degeneration index when compared to the uncoated ones, which allowed us to conclude that the coatings reduce the cytotoxic effect found in PVC.

INTRODUÇÃO

FISIO-PATOLOGIA DA CEC

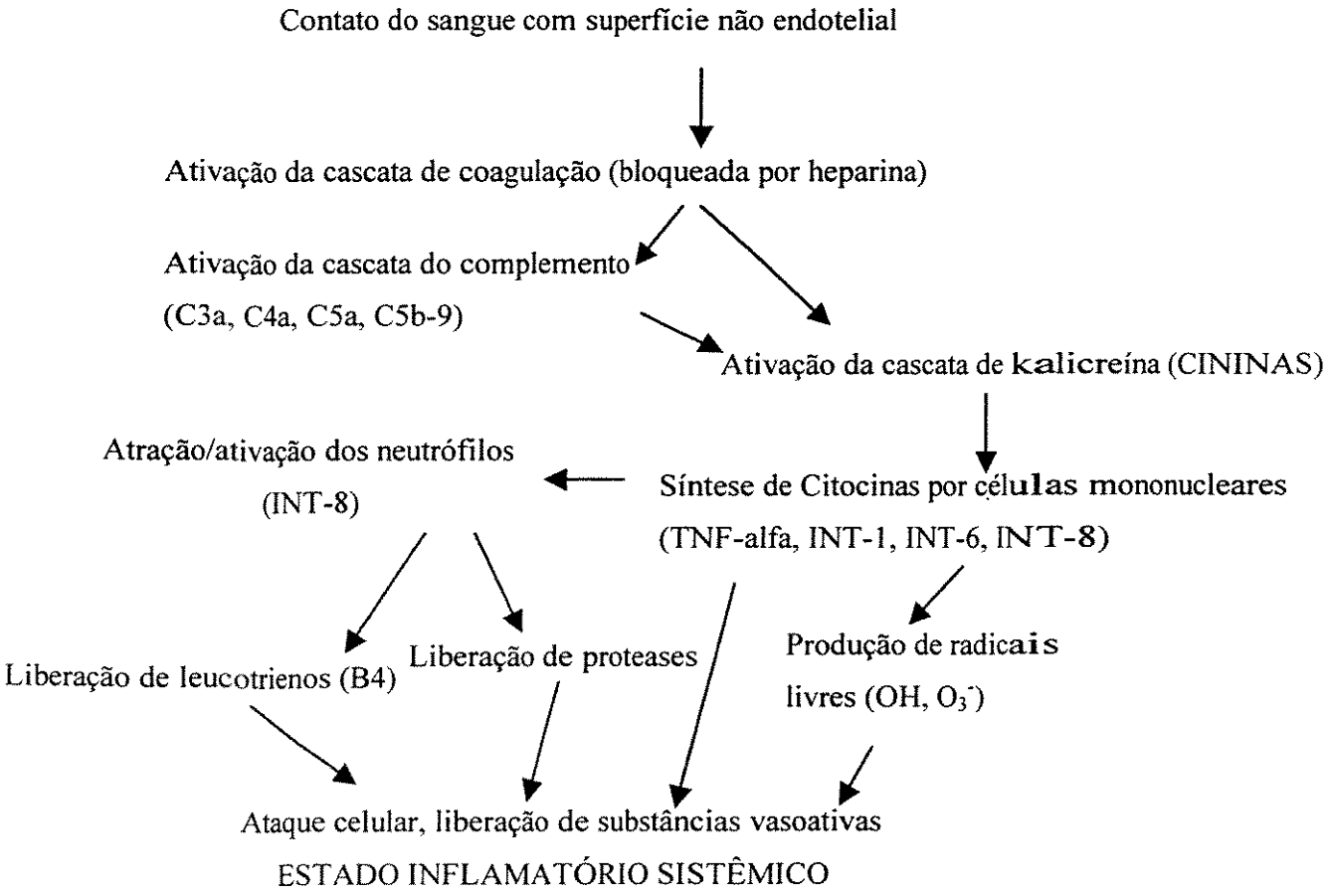
A CEC ocasiona significativas alterações na fisiologia normal do organismo que são dependentes da hemodiluição, da hipotermia e do contato do sangue com os tubos do circuito e dos oxigenadores a que estão submetidos os pacientes durante este procedimento cirúrgico (SOUZA et al., 1995).

A hemodiluição é empregada com o objetivo de diminuir os efeitos da perfusão com o sangue do paciente, notadamente, as reações decorrentes de incompatibilidade entre elementos presentes no sangue de vários doadores. Portanto, ela é responsável pela menor ocorrência de hemólise e de perda sanguínea pós-operatórias, bem como pela preservação do número de plaquetas (UTLEY et al., 1981).

Já a hipotermia tem sido amplamente utilizada com o objetivo de reduzir o consumo de oxigênio pelos tecidos e órgãos vitais. No entanto, ela produz diversas alterações como o aumento da viscosidade sanguínea, aglutinação de hemácias e disfunção plaquetária (UTLEY et al., 1981).

O contato do sangue com superfícies não endoteliais na CEC desencadeia a produção e liberação de diversas substâncias com efeito cardiovascular e a ativação de determinados sistemas de proteínas plasmáticas (MURIITHI et. al., 2000; WENDEL et. al., 1999; PEKNA et al., 1994; BUTLER et al., 1993), o que causa um quadro clínico chamado "Síndrome de Resposta Inflamatória Sistêmica" (SIRS). Tal quadro compreende uma série de reações como a ativação do sistema complemento, principalmente C_{3a} , C_{4a} , C_{5a} , $C_{5b\text{-}9}$, as quais aumentam a permeabilidade vascular e ativam os leucócitos e neutrófilos (sistema

imunológico). A liberação de cininas promove a síntese de citocinas por células mononucleares como TNF-alfa (Fator de Necrose Tumoral) e INT-1, INT-6, INT-8 (interleucinas 1,6 e 8, respectivamente). As citocinas promovem a atração e a ativação dos neutrófilos que liberam Interleucina 8, Leucotrienos (B_4), Proteases e Radicais Livres (OH, O_3^-) (vide esquema 1). Todos esses mediadores do processo inflamatório ativados promovem então um ataque celular, criando um processo inflamatório sistêmico, responsável por um aumento significativo da mortalidade e morbidade em cirurgias cardíacas (MILLER et al., 1997). A expressão clínica da S.I.R.S. pós CEC pode ser observada pelas alterações que ocorrem em vários órgãos e sistemas, as quais estão listadas na tabela 1.



Esquema 1 Sequência metabólica da Síndrome de Resposta Inflamatória Sistêmica pó

Além disso, com a adsorção de proteínas plasmáticas aos materiais utilizados na CEC, a cascata de coagulação pode ser desencadeada, com a conseqüente formação de trombos, o que pode ocasionar um bloqueio total ou parcial do tubo, caso o coágulo não seja liberado. Quando este coágulo é liberado passa a ser chamado de êmbolo, o qual pode deslocar-se pelos capilares de órgãos vitais, bloqueando-os e impedindo a circulação sanguínea e ocasionando danos aos tecidos, enfartes ou mesmo a morte do paciente (YOUNG et al., 1988).

Tabela 1 – Expressão clínica dos mediadores bioquímicos.

Complemento	Coagulopatias
INT – 6, INT- 8	Isquemia do Miocárdio
Radicais Livres	Fibrinólise
Cininas	Vasoplêgia
TNF-alfa	Necrose Celular

MECANISMO DE COAGULAÇÃO SANGUÍNEA E AÇÃO DA HEPARINA E DE LIPÍDEOS

O sangue é composto por células sanguíneas e o plasma no qual as células encontram-se suspensas. Entre os tipos celulares, estão os eritrócitos, ou glóbulos vermelhos; as plaquetas e os linfócitos (vide tabela 2). Já o plasma é uma solução aquosa composta por diversas substâncias como aminoácidos, vitaminas, hormônios, lipoproteínas, dentre outros. As principais proteínas plasmáticas são a albumina, as alfa, beta e gama globulinas (as quais são conhecidas como anticorpos) e o fibrinogênio, que atuará no processo de coagulação.

Tabela 2 – Produtos e funções das células sanguíneas

TIPOS CELULARES	PRODUTOS PRINCIPAIS	FUNÇÕES PRINCIPAIS
ERITRÓCITOS	HEMOGLOBINA	TRANSPORTE DE CO_2 E O_2
NEUTRÓFILOS	GRÂNULOS COM ENZIMAS	FAGOCITOSE DE BACTÉRIAS
EOSINÓFILOS	SUBSTÂNCIAS FARMACOLOGICAMENTE ATIVAS	DEFESA CONTRA PARASITAS E MODULAÇÃO DO PROCESSO INFLAMATÓRIO
BASÓFILOS	GRÂNULOS CONTENDO HISTAMINA E HEPARINA	LIBERAÇÃO DE HISTAMINA E OUTROS MEDIADORES DA INFLAMAÇÃO
MONÓCITOS	GRÂNULOS COM ENZIMAS	FAGOCITOSE E DIGESTÃO DE PROTOZOÁRIOS, VÍRUS E CÉLULAS SENESCENTES
LINFÓCITOS B	IMUNOGLOBULINAS	GERAÇÃO DE ANTICORPOS
LINFÓCITOS T	INTERLEUCINAS (SUBSTÂNCIAS QUE MATAM CÉLULAS E CONTROLAM ATIVIDADE DE OUTROS LINFÓCITOS)	MATA CÉLULAS INFECTADAS POR VÍRUS
CÉLULAS NATURAL KILLER	SUBSTÂNCIAS QUE PROMOVEM PERFURAÇÕES NAS MEMBRANAS DE CÉLULAS ALVOS, MATANDO-AS	MATA ALGUMAS CÉLULAS TUMORAIS E CÉLULAS INFECTADAS POR VÍRUS
PLAQUETAS	FATORES DE COAGULAÇÃO DO SANGUE	COAGULAÇÃO DO SANGUE

Um dos principais responsáveis pela coagulação sanguínea são as plaquetas. No início do processo de coagulação, o vaso lesado expõe o colágeno, que é um ativador das plaquetas. As plaquetas ativadas tornam-se aderentes, mudam da sua forma original de disco achatado

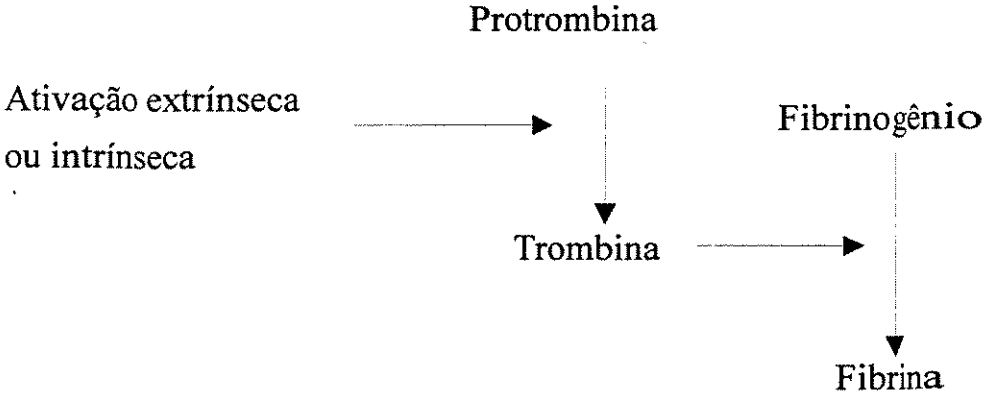
para a forma esferoidal, com inúmeros prolongamentos que partem de sua superfície, semelhantes a pseudópodes, e liberam tromboxane A_2 (TA_2) e difosfato de adenosina (ADP) (COLMAN, 1990).

O TA_2 provoca vasoconstrição com o objetivo de diminuir o sangramento e aumenta a liberação de ADP por parte das plaquetas. A função do ADP é ativar as plaquetas vizinhas, desencadeando um processo crescente de ativação plaquetária que leva à formação do coágulo. Os prolongamentos superficiais das plaquetas facilitam tanto a aderência plaquetária ao colágeno do vaso lesado quanto a agregação entre elas. Com isto, é formado o tampão plaquetário. A seguir, ocorre a formação de uma malha de fibrina que aprisiona as células do sangue e as plaquetas, originando o coágulo firme.

O processo de coagulação pode ser iniciado através da via denominada intrínseca, que se origina no próprio sangue circulante. O traumatismo vascular lesa o endotélio dos vasos e expõe o colágeno presente na região que ativa o fator XII (Fator de Contato ou Fator Hageman), que é clivado em duas cadeias enzimáticas (fator XII ativado). Uma vez ativado, este fator estimula uma seqüência de reações das proteínas do sistema de coagulação que culminam na transformação do fibrinogênio em um complexo de filamentos insolúveis de proteína, a fibrina. As malhas do aglomerado de fibrina retêm plaquetas e hemácias para formar o coágulo (vide esquema 2).

A via extrínseca é ativada pela tromboplastina tecidual liberada pelo tecido ou vaso traumatizado. O fator tecidual (proteína presente na membrana e superfície de muitas células, disponível no plasma somente quando há algum dano vascular) interage com o fator VII e, em conjunto, catalisam a conversão do fator X, que em presença de outros fatores e cálcio, ativa a ação da trombina sobre o fibrinogênio. O início da coagulação normalmente ocorre pela via extrínseca. Porém, na CEC, a superfície artificial do sistema

sofre a adsorção de proteínas plasmáticas, tornando o fator XII mais susceptível à digestão enzimática (clivagem) e ativação (ARNANDER et al., 1986), desencadeando a formação de microtrombos, apesar da heparina circulante.



Esquema 2 – Esquema do processo de coagulação do sangue

Além disso, a camada protéica adsorvida pelos materiais influencia a deposição de diferentes tipos celulares, incluindo as plaquetas. Algumas das proteínas adsorvidas são: fibrinogênio, fibronectina e trombospondina. O fibrinogênio apresenta grande capacidade de promover adesão de plaquetas em superfícies implantadas, uma vez que a membrana de cada plaqueta apresenta receptores para determinadas proteínas plasmáticas, incluindo o fibrinogênio (UEDA et al., 1995). Também, desta maneira, as superfícies sintéticas iniciam o processo de coagulação.

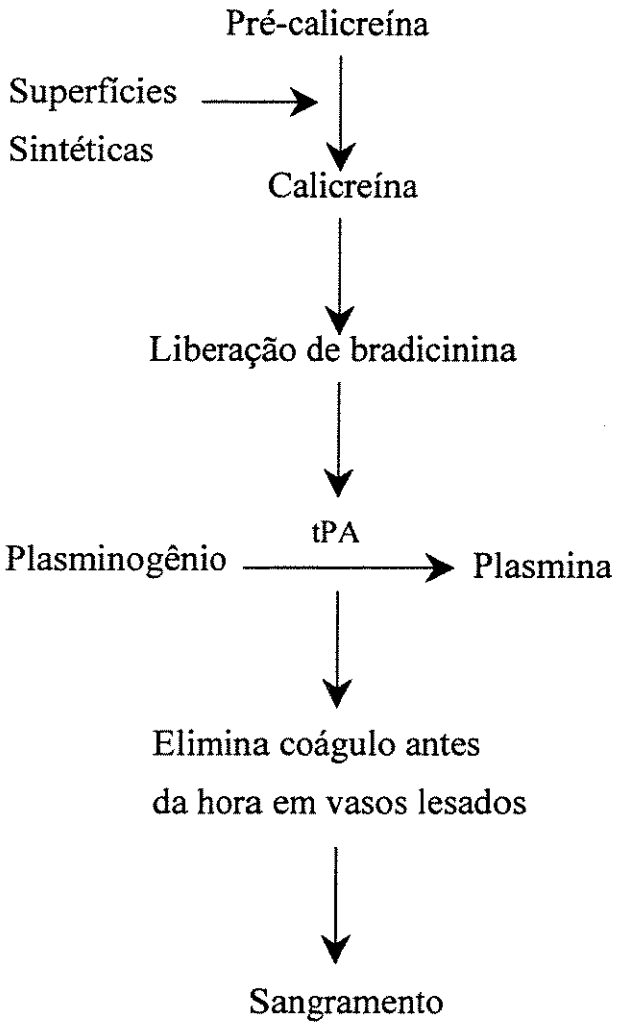
Os microtrombos são uma das causas da fibrinólise que pode levar ao sangramento. No coágulo formado existe grande quantidade de plasminogênio que, em 24 horas, será transformado em plasmina para eliminar o coágulo e restaurar o fluxo no vaso já cicatrizado. Este processo é chamado fibrinólise.

O contato do sangue com as superfícies sintéticas ativa o processo de coagulação com transformação da pré-calicreína em calicreína, que por sua vez estimula a liberação de bradicinina. Essas duas substâncias estimulam o ativador do plasminogênio tecidual (tPA) que converte plasminogênio em plasmina (TANAKA et al., 1989). A trombina formada durante a CEC também ativa o tPA (vide esquema 3).

Existem mecanismos que regulam a coagulação, restringindo o processo ao local do dano vascular. A antitrombina III (AT III) é o mais importante modulador da coagulação, pois se liga à trombina e a inativa, além de também inativar os fatores IX, X, XI e XII.

A principal ação da heparina consiste na estimulação da AT III, ligando-se à sua molécula, tornando-a cerca de 1000 a 2500 vezes mais potente na função de inibir a trombina, bem como de acelerar sua atividade na inibição dos fatores da via intrínseca da coagulação (BULL et al., 1975). No entanto, a heparina não previne a perda de plaquetas durante a CEC (VIDEM et al., 1991) e nem mesmo previne a aderência de plaquetas ao tecido subendotelial (SALZMAN et al., 1980). Além disso, não se pode esquecer dos efeitos colaterais sobre a protamina utilizada como substância antagônica à heparina que serão citados posteriormente (BRUINS et al., 2000).

Já o recobrimento dos tubos da CEC com lipídeos que estão, normalmente, presentes nas membranas de células como eritrócitos e plaquetas, age no sentido de tornar as superfícies sintéticas não trombogênicas, evitando a ativação, desta forma, da cascata de coagulação no paciente.



Esquema 3 - Esquema do processo de fibrinólise

CONDUTAS ALTERNATIVAS: REVESTIMENTOS EM COMPONENTES DO CIRCUITO EXTRACORPÓREO

A redução da trombogenicidade e o aumento da biocompatibilidade das superfícies artificiais podem ser obtidos revestindo-se os materiais de dispositivos médico-hospitalares com agentes antitrombogênicos. Estes revestimentos são utilizados em filtros arteriais, oxigenadores, conjuntos de tubos extracorpóreos, catéteres e reservatórios de sangue.

A maioria dos revestimentos utiliza como agente antitrombogênico a heparina (NIIMI et al., 1999), um glicosaminoglicano com massa molecular em torno de 15000 Daltons. Apesar da sua utilização sistêmica como anticoagulante durante o procedimento da CEC, ela tem ação limitada em minimizar as alterações causadas pela ativação dos sistemas e elementos do sangue. Além disso, a heparina pode causar hemorragia severa pós-operatória (BOROWIEC et al., 1992) e sua ação anticoagulante deve ser neutralizada com a utilização de protamina, que, por sua vez, produz diversos tipos de reações ou efeitos colaterais, como reações hemodinâmicas, alérgicas ou anafiláticas, e as reações que ocorrem por liberação do complemento (KIRKLIN, 1986).

Os resultados obtidos com recobrimentos de superfícies com anticoagulantes estão diretamente relacionados ao modo como a molécula de heparina é ligada à superfície do material. HOFFMAN, em 1987 e SVENNING *et al.*, em 1992 demostraram a importância dessa questão para que os benefícios desejados com o recobrimento sejam alcançados. Foram desenvolvidos vários tipos de revestimentos com heparina ativa imobilizada, onde a heparina ligada covalentemente ao substrato confere maior estabilidade ao revestimento.

Ultimamente, como revestimento alternativo, está sendo estudada a utilização de lipídeos que mimetizam a camada externa de membranas plasmáticas que normalmente entram em contato com o sangue sem desencadear reação adversa. Estudos revelaram que as estruturas da camada externa de eritrócitos e de plaquetas são similares e não ativam normalmente a cascata de coagulação (DURRANI et al., 1986). A maior fração lipídica da camada externa dos elementos celulares em questão é composta por esfingomielina e fosfatidilcolina, sendo que 89% destes lipídeos possuem como cabeça polar o grupo fosfotidilcolina. Apesar das diferenças em suas funções biológicas, essas células apresentam similaridades nas

orientações de seus fosfolipídios. Aqueles que contêm o grupo colina estão presentes em maior proporções na superfície externa das células, enquanto que os carregados negativamente (os quais apresentam características pró-trombogênicas, como a fosfatidilserina) encontram-se confinados em sua maioria na superfície interna da bicamada. Portanto, os materiais revestidos por fosfolipídios devem apresentar a porção polar lipídica da superfície externa das membranas (não apresenta atividade pró-coagulante) em suas superfícies.

O recobrimento por heparina, neste estudo, é feito pela simples adsorção do agente antitrombogênico nos tubos de PVC. Uma das extremidades do tubo é bloqueada e uma solução final de 800 UI/ml., feita a partir da diluição da heparina/ cloreto de benzalcônio (Sigma Chemical Co. St. Louis MO, USA – Sigma H-7280) de mucosa intestinal porcina (peso molecular entre 3.000 e 25.000 Daltons) em álcool isopropílico 100% é colocada em seu interior. Após 10 minutos em contato, a solução é retirada e o tubo é seco em estufa a 50°C. O tubo encontra-se, desta forma, com um recobrimento iônico de heparina de espessura de aproximadamente 2-3 μm, visualizado por microscopia eletrônica de varredura (MEV) de um corte transversal do material recoberto.

Já o recobrimento de lipídeos utiliza técnicas um pouco mais complexas e pode ser feito por acoplamento covalente de grupos fosforilcolina (CHAPMAN et al., 1985) ou por deposição seqüencial de monocamadas de moléculas anfifílicas sobre um substrato sólido utilizando a técnica de Langmuir-Blodgett (MARRECO, 1999; ALVES, 1999; ROBERTS, 1990). Esta última apresenta algumas vantagens perante outros métodos, como a deposição de filmes em substratos de diversos tamanhos e formas, formação de bicamadas, além do controle da densidade lipídica das monocamadas. No entanto, tal procedimento é de difícil

aplicação em escala industrial. Pensando em produzir materiais mais viáveis industrialmente, procuramos adaptar o recobrimento lipídico de forma que ele seja reprodutível (como na técnica de Langmuir-Blodgett) e eficiente. Desta forma, os materiais a serem utilizados nesse estudo foram recobertos por lipídeos, utilizando-se placas de PVC obtidas através do aquecimento a 180°C em forno, a partir de tubos de PVC utilizados em circulação extracorpórea, prensados entre duas superfícies lisas de vidro. As amostras foram então submetidas à adsorção dos fosfolipídios dimiristoil fosfatidilcolina (DMPC) (Sigma P-7331) e dimiristoil fosfatidiletanolamina (DMPE) (Sigma P-5693), ambos em solução de 0.5 mM em álcool etílico 70%. A adsorção foi realizada mergulhando-se as amostras em solução por 10 minutos. Deste modo, pode ser obtido um recobrimento de espessuras variadas, visualizado por microscopia de força atômica (MARRECO, 1999).

Apesar desses grandes avanços na melhoria dos circuitos extracorpóreos, ainda se faz necessária uma completa avaliação de sua interação e reatividade com os elementos sanguíneos, para a obtenção de materiais nacionais de boa qualidade os quais posteriormente possam ser comercializados.

OBJETIVOS

Neste trabalho, avaliou-se a influência de superfícies de PVC recobertas com heparina e com os fosfolipídios fosfatidilcolina (DMPC) e fosfatidiletanolamina (DMPE), utilizados na CEC, em células mantidas "in vitro". Assim, os objetivos do projeto foram:

- Avaliar a citotoxicidade dos materiais desenvolvidos;
- Comparar a biocompatibilidade entre tubos recobertos e não recobertos;
- Avaliar as alterações morfológicas das células cultivadas sobre os polímeros recobertos ou não;
- Comparar a eficiência dos recobrimentos de heparina com os de lipídeos.

Baseado nos objetivos acima mencionados, a avaliação dos resultados obtida apresenta-se na forma de artigos publicados ou submetidos à publicação, anexados a seguir.

ARTIGOS ANEXOS

Anexo I

MOREIRA, P.L.; WADA, M.L.F.; NOVELLO, W.P., Importance of uniform heparin coating on biopolymers. **Artif. Organs 24(3)**, 209-211, 2000.

Anexo II

MOREIRA, P.L.; GENARI, S.C.; MORAES, A.M.; WADA, M.L.F. Cellular adhesion on uncoated and different coated PVC tubes used in Extracorporeal Circulation (ECC), submetido ao **Journal of Biomedical Materials Research.**

Anexo III

MOREIRA, P.L.; GENARI, S.C; WADA, M.L.F.. Morphological analysis of a cell line on uncoated and different coated PVC tubes used in Extracorporeal Circulation (ECC), submetido ao **Journal of Thoracic and Cardiovascular Surgery**

ANEXO I

Artificial Organs
24(3):209–211, Blackwell Science, Inc.

Importance of Uniform Heparin Coating on Biopolymers

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Abstract: Cardiopulmonary surgeries need connectors for extracorporeal circulation. The patient's blood in contact with the tube surfaces modifies its plasmatic proteins, promotes platelet aggregation, and activates the complement system, unleashing thrombus formation. Thus, it becomes necessary for an anticoagulant to keep the circuit free from these events. Heparin is the anticoagulant used even after reports about its disadvantages. Platelet adherence seems to be very dependent on the quality from the surfaces that can promote cellular proliferation, aggregation, and thrombosis. In this study, we compare the quality of the heparin-coated and uncoated surfaces. We used a blood cell culture and scanning electron microscopy (SEM) to visualize the platelet aggregation. It was concluded that there are groove areas that permit platelet adherence, and if they are not coated totally by the heparin, aggregation still occurs although in lower scale than on the uncoated tubes. **Key Words:** Extracorporeal circulation—Heparin—Platelet adherence—Groove areas—Heparin-coated tubes—Uncoated tubes.

Despite the progress in extracorporeal circulation (ECC) technologies, they are not protected against deleterious effects. Contact between the blood and artificial surfaces in the ECC triggers activation of the complement, coagulation, and fibrinolytic systems which leads to qualitative and quantitative changes of blood elements (1). One approach to minimize such deleterious effects, which can lead to permanent dysfunction of many organs (2), is to improve the biocompatibility of ECC circuits. Surfaces recovered with heparin have been shown to improve thromboresistance, to inhibit the alternative pathway of complement activation, and to improve biocompatibility both in vitro and in vivo (3). It also permits the use of lower doses of systemic heparin, which can cause hemorrhagic complications (4).

The anticoagulant effect of heparin results from its ability to bind and activate a natural inhibitor of the serine proteases involved in the intrinsic coagulation system in addition to blocking the action of thrombin on platelets (5). However, heparin does not prevent platelet loss during the ECC (6) or the adherence of platelets to subendothelial connective tissue (5) or to artificial surfaces in vitro (3). It seems probable that even the cellular activation rather than the adherence is extremely dependent on the quality of the surfaces and that the adherence happens next to areas that promote cellular activation, once this activation can promote the release of essential factors for the process.

This study shows that a whole coating of heparin on the biopolymer surfaces can decrease the tendency of platelet aggregation, which is increased when this coating is not uniform (that is, clearly shown in the coated grooves).

MATERIALS AND METHODS

Polymer preparation

The samples were uncoated (Fig. 1a) or heparin-coated (Fig. 1b and c). These samples were sterilized in 70% alcohol for 48 h. This procedure caused the grooves in the heparin coating tubes (Fig. 1c). After sterilization, the tube pieces were washed for 15 min 3 times in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, U.S.A.) medium. Next, the samples were added to the blood cell culture.

Blood cell culture

Five ml of a healthy donor's blood was taken by heparinized syringe, adding 15 drops of the blood in each culture bottle with 5 ml of RPMI 1640 (Sigma)

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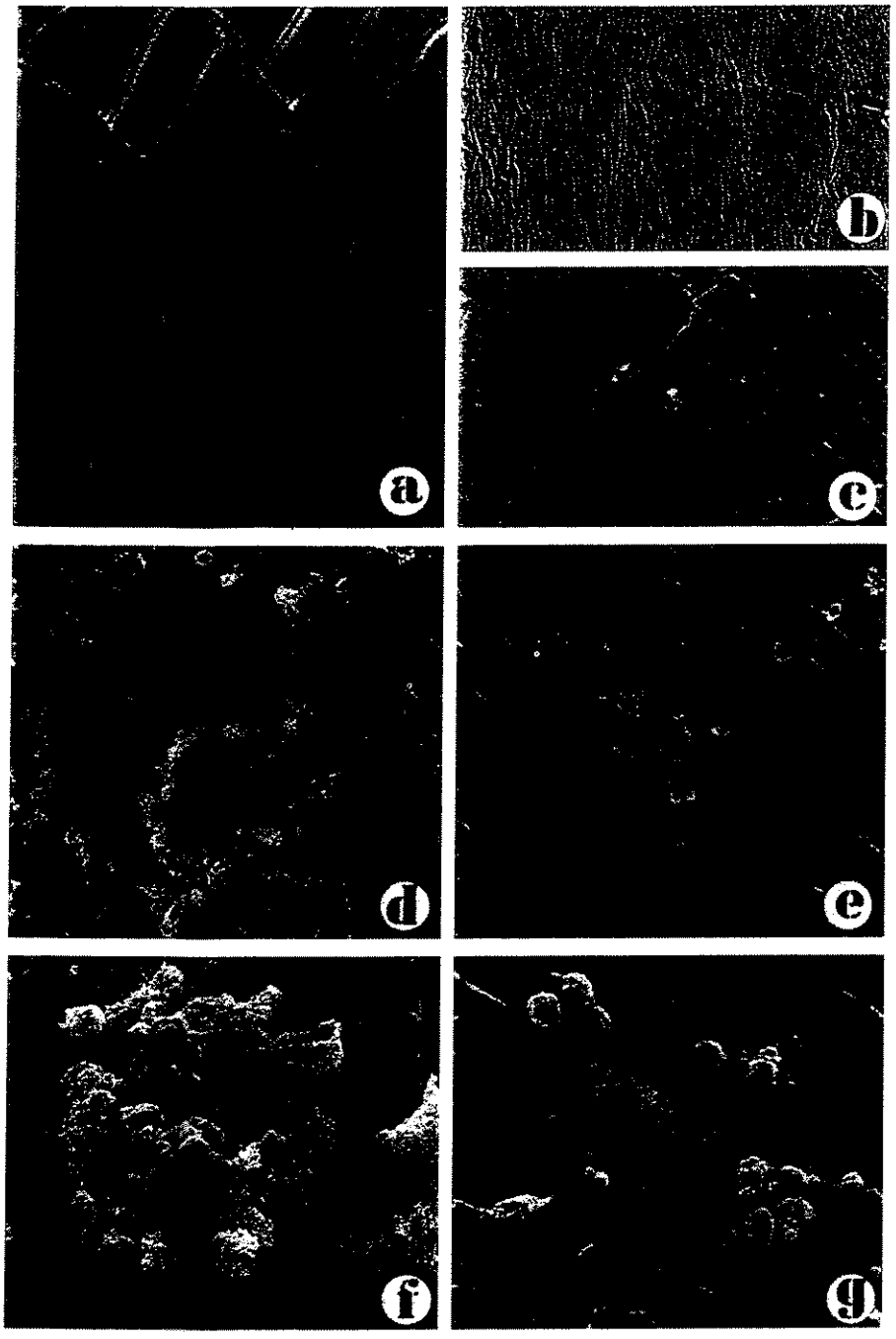

FIG. 1. Micrographs show blood cells grown over the heparin-coated and uncoated tubes. Control of the uncoated polymer **(a)**, control of the heparin-coated polymer before the treatment with alcohol **(b)**, control of the heparin-coated polymer after the treatment with alcohol **(c)**, cells over uncoated polymers **(d)** and **(f)**, and cells over the heparin-coated polymers **(e)** and **(g)**. For **(a–e)** the magnification is x270 and for **(f)** and **(g)** x760.

medium, 20% of bovine fetal serum (Nutricell Nutrientes Celulares Ltda., Campinas, São Paulo, Brazil), and 0.3 ml of fitohemaglutinine (Sigma) in addition to the samples in the heparin-coated and uncoated tubes. The control bottles differed due to the lack of blood cells. The bottles were lightly agitated and incubated at 37°C. After 70 h of incubation, the materials were taken out of the culture and were processed by scanning electron microscopy (SEM).

Scanning electron microscopy

The materials were fixed with Paraformoldehyde/Glutaraldehyde (Merk KgaA, Darmastadt, Germany) 2.5% in phosphate buffer 0.1 *M* pH 7.4 for 2 h, then washed in phosphate buffer, postfixed in osmium tretroxide (Sigma) 1% (7), and dehydratated in ethanol series. The material was critical point dried (Balzers CPD030) and gold sputtered (Balzers SCD050).The observations of the specimens were performed with a JEOL JXA-840A microscope.

RESULTS AND DISCUSSION

As can be seen in Fig. 1a, the noncovered polymer has some grooves on its surface, which are responsible for platelet adherence because in this area aggregation occurs, causing coagulation (Fig. 1d and f) and thrombosis which reassert the dependence of the quality of the surfaces. In this way, it is vital to use some coating to avoid this process. The heparin coating prevents contact between the regions of the polymer that promote cellular activation with the blood cells. However, if this coating is not uniform (Fig. 1b), the binding process cannot be avoided as shown in Fig. 1e and g. Moreover, platelet aggregation is only seen in the grooved regions.

In addition, note that a higher concentration of platelet aggregation occurred in the noncovered material (Fig. 1d and f) than in the heparin-coated tubes (Fig. 1e and g). This is probably caused by the exposure of the lower grooves when the material was covered by heparin because it partially obstructs the binding among the cells. Nevertheless, when this coating is not regular, as in this case (grooves), the heparin efficiency avoiding the platelet aggregation is endangered because the platelets tend to bind exactly in those places (Fig. 1e and g). Thus, the importance of a complete recovering of the biomaterial to be used in extracorporeal circulation procedures seems clear.

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REFERENCES

1. Pekna M, Borowiec JW, Fagerhol MK, Venge P, Thelin S. Biocompatibility of heparin-coated circuits used in cardiopulmonary bypass. *Scand J Thorac Cardiovasc Surg* 1994;28: 5–11.
2. Kirklin JK, Westaby S, Blackstone EH, Kirklin JW, Chenoweth DE, Pacífico AD. Complement and the damaging effects of cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1983;86:845–57.
3. Borowiec JW, Bylock A, Vanderlinden J, Thelin S. Heparin coating reduces blood-cell adhesion to arterial filters during coronary bypass—A clinical study. *Ann Thorac Surg* 1993;55: 1540–5.
4. Borowiec JW, Thelin S, Bagge L, Hultman J, Hansson H-E. Decreased blood loss after cardiopulmonary bypass using heparin-coated circuit and 50% reduction of heparin dose. *Scand J Thorac Cardiovasc Surg* 1992;26:177–85.
5. Salzman EW, Rosenberg RD, Smith MH, Lindon JN, Favreau L. Effect of heparin fractions on platelet aggregation. *J Clin Invest* 1980;65:64–73.
6. Videm V, Mollnes TE, Garred P, Svenneving JL. Biocompatibility of extracorporeal circulation—In vitro comparison of heparin-coated and uncoated oxygenator circuits. *J Thorac Cardiovasc Surg* 1991;101:654–60.
7. Glauert AM. Fixation, dehydration, and embedding of biological specimens. In: Glauert AM ed., *Practical Methods in Electron Microscopy, Vol. 3.* Amsterdam: North-Holland, 1975:1–207.

ANEXO II

CELLULAR ADHESION ON UNCOATED AND DIFFERENT COATED PVC TUBES USED IN EXTRACORPOREAL CIRCULATION (ECC)

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Short title: Cell adhesion on different coated ECC tubes.

ABSTRACT

The greatest challenge of the extracorporeal circulation (ECC) procedure is to reduce the thrombus formation on the surface of the tubes and connectors that are components of its machinery. These connectors are normally made of PVC ((poly)vinyl chloride) and nowadays; one approach to reduce the blood response to a foreign surface has been the coating of these biomaterial surfaces with heparin and phospholipids. But it is important to understand and evaluate the interaction and reactivity of these different coatings with the blood elements in a comparative way once none has been made in this direction. Our study compares the effect of three different coatings on PVC tubes normally used in ECC procedures regarding cellular adhesion and proliferation. The results showed that the heparin, DMPC (phosphatidylcholine) and DMPE (phosphatidylethanolamine) coated PVC improved the biocompatibility of the PVC tubes reducing the cellular degeneration due to the probably decreased release of phthalates to the culture medium, although they do not totally prevent the cellular adhesion on their surface, except by the heparin coated PVC that was much more efficient avoiding it.

Key words: extracorporeal circulation (ECC), cellular adhesion and proliferation, heparin, phosphatidylcholine (DMPC), phosphatidylethanolamine (DMPE),

INTRODUCTION

Medical devices which come into direct contact with blood are still a challenge since their biocompatibility should be improved. When blood contacts a foreign surface, it triggers a sequence of events such as protein adsorption[1,2], followed by the platelet reactions as adhesion and aggregation[3,4], activation of the intrinsic coagulation as well as the participation of the fibrinolytic and complement systems[5,6].

One approach to reduce the blood response has been the coating of these biomaterial surfaces with heparin[7,8,9,10] and phospholipids[11,12]. The earlier is an anticoagulant normally used systemically during the extracorporeal circulation. However, its utilization may cause severe damages as hemorrhage[13,14]. Yet, the latter coating intends to mimic the membrane of blood cells and avoid recognition by the blood as foreign[12]. The most common phospholipids are the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) head groups, major components of erythrocyte and platelet membrane surfaces[15].

The compatibility analysis of a biomaterial involves several parameters. Although altering a biomaterial surface may reduce the blood response with respect to one parameter, it can increase the response with respect to another. For example, heparin enhances the contact activation, determined in terms of a factor XII-like activity in cellulose and polysulphone membranes[16]. That is why it is important to understand and evaluate the interaction and reactivity of these different coatings with the blood elements in a comparative way in order to establish some degrees of efficiency.

In this way, our study compares the effect of three different coatings on the poly (vinyl) chloride (PVC) tubes normally used in extracorporeal circulation (ECC) procedures regarding adhesion and proliferation of blood cells and fibroblastic-like cells in culture.

MATERIALS AND METHODS

Polymer coating preparation

The poly (vinyl) chloride (PVC) tubes were cut in small pieces of 0,25 cm^2 and coated by three different solutions in ethanol 70% of 800UI/ml. of Benzalkonium Chloride/Heparin (Sigma Chemical Co., St. Lois, MO, USA), 5mM of Phosphatidylcholine Dimyristoyl (DMPC-Sigma) and 5mM of Phosphatidylethanolamine Dimyristoyl (DMPE-Sigma) through the adsorption of these substances for 10 minutes. The uncoated samples were washed in methanol and deionized water just as a cleaning process. All the samples were sterilized in ethylene oxide. After sterilization, some samples were washed for 15 minutes three times in RPMI 1640 (Sigma) medium and added to the blood cell culture. The other samples were used for the cell adhesion assay.

Blood Cell Culture

Peripheric blood was obtained from healthy donor using heparinized syringe. Each culture bottle with 5 ml. of RPMI 1640 (Sigma) medium supplemented with 20% of bovine fetal serum (FCS, Nutricell Nutrientes Celulares Ltda., Campinas, SP, Brazil), 0.3 ml. of fitohemaglutinine (GIBCO BRL) and the heparin, phosphatidylcholine and phosphatidylethanolamine coated tubes and uncoated ones, received 1 ml. of the peripheric blood. The control tubes differed due to the lack of the PVC pieces coated or not and the presence of coverslips. The bottles were lightly agitated and incubated at 37°C. After 70 hours of incubation, the coated and uncoated samples as well as the glass coverslips were taken out of the culture and processed to Scanning Electron Microscopy (SEM). After the addition of colchicine (Sigma), the cellular suspension was hipotonized with a 0.075 M

KCl solution, fixed in methanol/ acetic acid 3:1 (v/v) and the material for analysis of the Mitotic Index was prepared by standard cytogenetics methods[17]. In order to establish the Mitotic Index (mitotic cell number/ total cell number) 1500 cells were counted for each coating. Lymphocytes in degeneration were also counted considering alterations in theirs morphology as non-homogeneous coloration of the cell nucleus as well as the nucleus splitting in many pieces, without damage of the plasma membrane.

Scanning Electron Microscopy (SEM)

The materials were fixed with Paraformoldehyde/Glutaraldehyde (Merck KgaA, Darmstadt, Germany) 2.5% in phosphate buffer 0.1 M pH 7.4 for 2 hours, then washed in phosphate buffer, post fixed in osmium tretroxide (Sigma) 1%, and dehydrated in ethanol series. The material was critical point dried (Balzers CPD030) and gold sputtered (Balzers SCD 050). The observations of the specimens were performed with JEOL JSM-5800 LV microscope.

Cell Adhesion Assay

For cell adhesion assay, 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) supplemented with 10% FCS (Nutricell) at 37°C. Vero cells are a lineage that is recommended for studies of cytotoxicity and for cell-substratum interactions with biomaterials research[18,19].

For this assay, it was used a modified procedure described by Murakami[20] et. al.., 1998. Briefly, the sterilized different coated and uncoated PVC tubes were inoculated on a 96 wells plate (Corning/Costar Corporation, Cambridge, MA, USA) in Ham F-10 medium (Sigma) without FCS for 24hs at 37°C. After this incubation time, 100µl of cell suspension (1.0×10^6 cell/ml) in Ham-F10 medium (Sigma) with 10% FCS (Nutricell) was inoculated in the wells with the different samples. The cells were cultured for 2h in Ham F-10 with 10% of FCS at 37°C, washed with 0.1M phosphate buffered saline (PBS) in pH 7.4, at 37°C, fixed in formaline 10% for 15 minutes, washed in PBS for the same time, stained by crystal violet 0.05% (in methanol 20%) for 15 minutes. The samples were then washed twice with 0.1M PBS for 30 minutes and incubated with sodium citrate 0.1M (in 50% ethanol at pH 4.2) for the same period. The plate with the remaining cells were read in Multiskan Bichromatic Version 1.06 microplate reader on 540nm wavelength. As a positive control the cultured plate itself (polypropylene) and as a negative control Teflon disks were used. We also read the absorbance of all experimental conditions (the coated and uncoated PVC tubes, negative or positive controls) without cells for dye staining control. All experiments were made in seven repetitions.

Comparison of continuous variables for all groups was done with the ANOVA. When a significant difference was found, the control group was compared with the uncoated and coated groups by using the Tukey's test. A P value of < 0.05 was considered statistically significant.

RESULTS

Analyzing the SEM micrographs of control cells in coverslips (Fig. 1A), the uncoated tubes (Fig. 1B) as well as the DMPE and DMPC coated tubes (Fig. 1C, 1D), we can notice considerable quantity of cellular agglomerates constituted mainly by platelets and red cells. These areas of cellular agglomeration are responsible for triggering the coagulation and thrombosis in patients who are submitted to ECC procedures. However, the heparin-coated tubes showed a better efficiency avoiding the adherence of those cells once it was really hard to find cellular agglomerations over them (Fig. 1E, 1F).

Nevertheless, all the coatings decreased the quantity of cells in division at about 50%, as it is demonstrated by the Mitotic Index shown in table I. They have also increased the degeneration ratio in about 60% compared with the control. Compared with the uncoated tubes, we can even state that the coatings contributed decreasing the quantity of cells in degeneration once the uncoated PVC presented two fold more cells in degenerative process.

Regarding adhesion, there were no significant differences found among the controls and the samples, except between the negative control (Teflon) and the DMPC coated PVC (Figure 2). The heparin and DMPE coated PVC showed a very similar adhesion result.

DISCUSSION

The greatest challenge of the extracorporeal circulation procedure is to reduce the thrombus formation on the surface of the tubes and connectors that are components of its machinery. These connectors are normally made of PVC and nowadays; most of them are

commercialized with a heparin coating, which has been proved to improve its hemocompatibility [8,10,13,21].

Hemocompatibility involves several characteristics as reduced activation of the complement system and cytotoxicity as well as reduced coagulation and fibrinolysis. These latter ones could be demonstrated by thrombus formation, which is, in fact, a consequence of the adsorption of some proteins that are responsible for the adhesion of blood cells and platelets [16].

Considering this, our scanning electron micrographies stresses that the heparin coating reduces the adhesion of blood cells and platelets (Fig. 1E, 1F). However, it also decreases the quantity of lymphocytes in division in about 47% compared to the control, what is relatively similar to the other coatings and the PVC itself (Table I). As the degenerative index increased in all the coatings and doubled in the uncoated PVC in comparison with the control, we can suggest that all of them cause cell degeneration with consequently death (data not shown). This could be due to the phthalates releasing. Phthalates are the plasticizers used in the majority of the PVC tubes and bags, which give a better flexibility to the material. The consequences of its releasing in the blood or in the culture medium in contact with the polymers have been reported by some authors as harmless[22,23] and by other as a serious problem, which should be considered[24,25,26,27].

Nevertheless, compared with the uncoated tubes, all the coated tubes were less effective in the degenerative process once they increased this index in about 60% and the former in about 100%. This could be happening because of the coatings, which are probably difficulting the releasing of phthalates to the culture medium. In this way, we can state that in spite of the increasing of the degenerative process, the different coatings presented a

protective effect under these circumstances, which can be considered as an improving of the biocompatibility.

Once the adhesion assay was performed with fibroblastic cell line that is dependent of anchorage, whereas blood cells are not, these results should be carefully analyzed, since the materials tested here are going to be used in contact with the blood. It was made the adhesion test with Vero cell line due to the fact that firstly, it is considered an international standard pattern for this kind of test[18,19], secondly it is a controlled assay in which we have a determined initial cell number and thirdly because since it is an adhesion assay, it would be better to use cells dependent of anchorage because if they do not adhere to the surface, they are going to die and in culture, the blood cells grow in suspension and can adhere or not to a surface, what can increase the possibility of errors.

As it is known, the ethylene oxide used in the sterilization process could cause the alkylation of hydroxyl, carboxyl, sulfhydryl and amino groups of the materials[28]. The DMPE´s alkylation causes an hydrofobicity on the material, what can decrease the cellular adhesion.. Furthermore, it has been shown by Marreco[29], 1999 that sterilized DMPC coated PVC improved its hydrophilic characteristic what also improves cellular adhesion[15] as it is demonstrated in this work (Figure 2).

CONCLUSION

The heparin, DMPC and DMPE coated PVC improved the biocompatibility of the PVC tubes reducing the cellular degeneration due to the probably decreased releasing of phthalates to the culture medium, although they do not totally prevent the cellular adhesion on their surface, except by the heparin coated PVC that was much more efficient avoiding it.

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REFERENCES

1. WENDEL, H.P.; WEBER, N.; ZIEMER, G., "Increased adsorption of high molecular weight kininogen to heparin-coated artificial surfaces and correlation to hemocompatibility". *Immunopharmacology,* **43,** 149-153 (1999).

2. BRASH, J.L., "Role of the plasma protein adsorption in the response of blood to foreign surfaces", in: *Blood Compatible Materials and Devices. Lancaster, PA:Technomic,* 1991, pp.3-24.

3. FORBES, C.D.; COURTNEY, J.M., "Thrombosis and artificial surfaces", in *Haemostasis and Thrombosis, Vol.2. Edinburgh: Churchill Livingstone,* 1994, pp.1301-1324.

4. COLMAN, R. W., "Platelet and Neutrophil activation in cardiopulmonary bypass". *Ann. Thorac. Surg.***49,** 32-34 (1990).

5. KAZATCHKINE, N.D.; CARRENO, M.P., "Activation of the complement system at the interface between blood and artificial surfaces". *Biomaterials,* **9,** 30-35 (1987).

6. CHENOWETH, D.E., "Complement activation produced by biomaterials". *Trans. Am. Soc. Artif. Intern. Organs,* **32,** 226-232 (1986).

7. NIIMI, Y.; ICHINOSE, F.; ISHIGURO, Y.; TERUI, K.; VEZONO, S.; MORITA, S.; YAMANE, S., "The effects of heparin coating of oxygenators fibers on platelet adhesion and protein adsorption". *Anesth. Analg.* **89**, 573-579 (1999).

8. te VELTHUIS, H.; BAUFRETON, C.; JANSEN, P.G.M.; THIJS, C.M.; HACK, C.E.; STURK, A.; WILDEVUUR, C.R.H.; LOISANCE, D.Y., "Heparin coating of

extracorporeal circuits inhibits contact activation during cardiac operations". *J.Thorac. Cardiovasc. Surg.* ,**114,** 117-122 (1997).

9. ØVRUM, E.; BROOSTAD, F.; ÅM HOLEN, E.; TANGEN, G.; ABDELNOOR, M., "Effects on coagulation and fibrinolysis with reduced versus full systemic heparinization and heparin-coated cardiopulmonary bypass". *Circulation,* **92(9),** 2579-2584 (1995).

10. PEKNA, M., BOROWIEC, J. W., FAGERHOL, M. K., VENGE, P., THELIN, S., "Biocompatibility of heparin-coated circuits used in cardiopulmonary bypass". *Scand. J. Thor. Cardiovasc. Surg.*, **28**, 5-11 (1994).

11. CHAPMAN, D.; CHARLES, S.A., "A coat of many lipids – in the clinic". *Chem. Br.*, **28**, 253-256 (1992).

12. DURRANI, A. A., MAYNARD, J. A., CHAPMAN, D. "Biomembranes as models for polymer surfaces II. The synthesis of reactive species for covalent coupling of phosphorylcholine to polymer surfaces". *Biomaterials* , **7**, 121-125 (1986).

13. BOROWIEC, J. W., THELIN, S., BAGGE, L. HULTMAN, J., HANSSON, H-E., "Decreased blood loss after cardiopulmonary bypass using heparin-coated circuit and 50 % reduction of heparin dose". *Scand. J. Thor. Cardiovasc. Surg.* , **26**, 177-185 (1992).

14. KIRKLIN, J. W., "Effects of protamine administration after cardiovascular bypass on complement, blood elements and the hemodynamic state". *Ann. Thorac. Surg.,* **41**, 193-198 (1986).

15. ALBERTS, B.; BRAY, D.; LEWIS, J., RAFT, M.; ROBERTS, K.; WATSON, J.D., "Membrane structure", in *Molecular Biology of the Cell, New York: Garland Publishing* 1994, pp.473-506.

16. COURTNEY, J.M.; LAMBA, N.M.K.; SUNDARAM, S.; FORBES, C.D., "Biomaterials for blood-contacting applications". *Biomaterials* , **15(10)**, 737-744 (1994).

17. DEITCH, A. D.; SAWICK, S.G., "Effects of cordycepin on microtubules of cultured mammalian cells". *Exp. Cell Res.*, **118**, 1-13 (1979).

18. ISO 10993-5. 1992(E). "Biological evaluation of medical devices - Part 5 - Tests for cytotoxicity: *in vitro* methods".

19. KIRKPATRICK C.J.. "Biological testing of materials and medical devices - A critical view of current and proposed methodologies for biocompatibility testing: cytotoxicity in vitro". *Regulatory Affairs* , **4**, 13-32 (1992).

20. MURAKAMI, N.; FUKUCHI, S.; TAKEUCHI, K.; HORI, T.; SHIBAMOTO, S.; ITO, F., "Antagonistic regulation of cell migration by epidermal growth factor and glucocorticoid in Human gastric carcinoma cells" *J. Cell. Physiol.* , **176**, 127-137 (1998).

21. MOREIRA, P.L.; WADA, M.L.F.; NOVELLO, W.P., "Importance of uniform heparin coating on biopolymers" . *Artif. Organs* **24(3)**, 209-211 (2000).

22. LABOW, R.S., CARD, R. T., ROCK, G., "The effect of the plasticizer Di (2-ethylhexyl)phthalate on Red Cells deformability". *Blood,* **70(1),** 319-323 (1987).

23. GUESS, W.L., HABERMAN, S., Toxicity profiles of vinyl and polyolefinic plastics and their additives. *J. Biomed. Mater. Res,.* **2,** 313-335 (1968).

24. HARRIS, C.A., HENTTU, P., PARKER, M.G., SUMPTER, J. P., The estrogenic activity of phthalate esters *in vitro. Environ. Health Perspect.,* **105(8)**, 802-811 (1997).

25. FRATANTONI, J.C., "Review: The platelet storage lesion: possible role of platicizers ?" *Blood Cells* **18**, 435-440 (1992).

26. BARRY, Y. A., LABOW, R.S., KEON, W. J., TOCCHI, M., ROCK, G., Perioperative exposure to plasticizers in patients undergoing cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg.*, **97,** 900-905 (1989).

27. SASAKAWA, S., MITOMI, Y., Di-2-ethylhexylphthalate (DEHP) content of blood or blood components stored in plastic bags. *Vox Sang,* **34**, 81-86 (1978).

28. RICHARDS, J.W. In: Introduction to Industrial Sterilization: New York: Academic Press 1968, pp. 166-171.

29. MARRECO, P.R., Estudo do recobrimento de cloreto de polivinila (PVC) com fosfatidilcolinas. *MSc Thesis – Chemical Engineering Faculty – Campinas State University, 1999 (in Portuguese).*

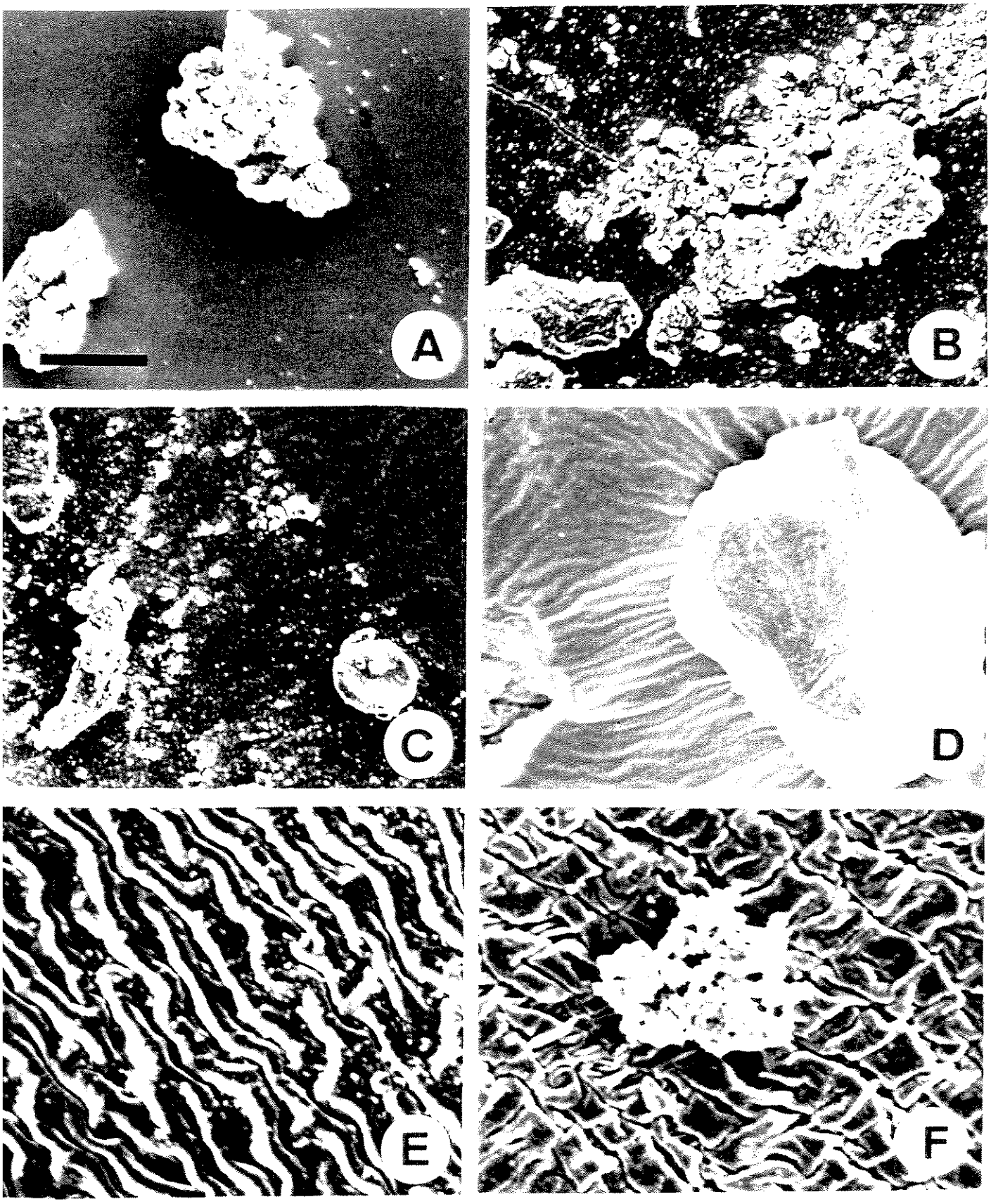
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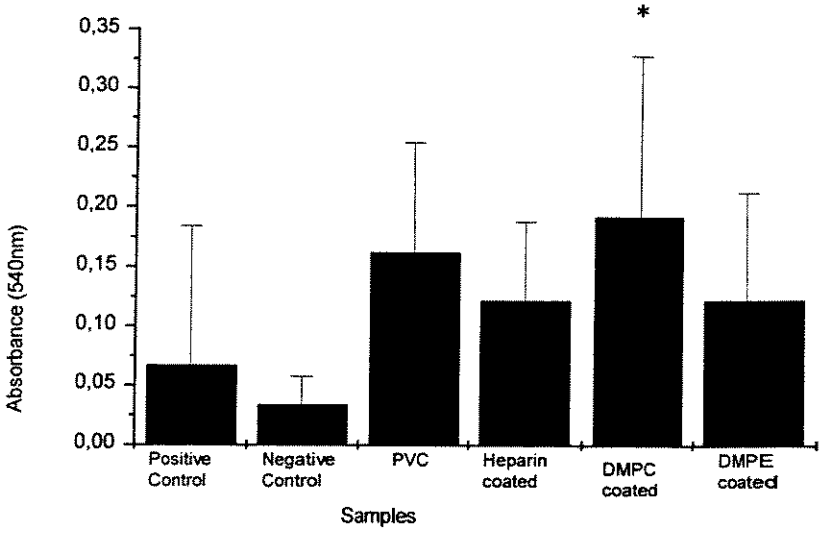
FIGURE 2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0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Absorbance (540nm)
Positive Control
Negative Control
PVC
Heparin coated
DMPC coated
DMPE coated
Samples

TABLE I

Mitotic and Degenerative Index of Lymphocytes Cultured in presence of Coated and Uncoated PVC tubes

Index	Control	Uncoated	Heparin-coated	DMPC - coated	DMPE – coated
Mitotic	2%	1.4%	1.06%	1.2%	1%
Degeneration	0.5%	1%	0.86%	0.86%	0.8%

Figure 1. Scanning electron microscopy showing blood cells adhered on uncoated and coated PVC tubes. Control cells on coverslips **(a),** Cells on uncoated PVC tubes **(b),** cells on DMPE coated PVC tubes **(c),** cells on DMPC coated PVC tubes **(d),** and Cells on heparin coated PVC tubes **(e)** and **(f).** Scale bar of 5μm for all pictures.

Figure 2. Adhesion assay on different PVC coated tubes. There was no significant difference among the controls (the plate as positive and the Teflon as negative) and heparin, DMPE coated tubes ($P < 0.05$). However, the negative control (Teflon) is significantly different from the DMPC coated tube. * Significantly greater than negative control.

ANEXO III

MORPHOLOGICAL ANALYSIS OF A CELL LINE CULTURED ON UNCOATED AND DIFFERENT COATED PVC TUBES USED IN EXTRACORPOREAL CIRCULATION (ECC)

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ABSTRACT

The initial response of the organism to the polymer must depend on its surface properties. Blood contact with artificial surfaces during cardiovascular bypass (CBP) and extracorporeal circulation (ECC) leads to activation of the complement and the coagulation cascade. One approach to minimize such effects is to improve the biocompatibility of the CBP and ECC circuits modifying their surfaces. Coating them with heparin or phospholipids can do this. This research investigated the adhesion and morphology characteristics of Vero cells cultured on uncoated PVC tubes as well as the heparin, phosphatidylcholine (DMPC) and phosphatidylethanolamine (DMPE) coated ones. Our scanning electron micrographs showed that cellular adhesion is a real problem in uncoated PVC materials, mainly over the grooves on the PVC surface that usually occur in its structure, which establishes the importance of an uniform coating independent of the substance used, since it should cover all the grooves on PVC tubes. We also realized that there was no effective cell adhesion to the coated-PVC surfaces. This fact supports the idea of the increased biocompatibility of PVC tubes using coating techniques for extracorporeal circulation. Finally, in all coated tubes, Vero cells presented a rounded morphology, which indicates that those cells do not spread on the surfaces. Since the cellular spreading is important to a good interaction with the substrate, the low interaction of those cells with the tubes could count as an improvement of the biocompatibility, decreasing the thrombus formation in ECC procedures.

ULTRAMINI-ABSTRACT

Blood contact with artificial surfaces triggers deleterious effects as complement activation and coagulation;

Improved biocompatibility of the Extracorporeal Circulation (ECC) circuits is obtained coating their surfaces with heparin, phosphatidylcholine (DMPC) and phosphatidylethanolamine (DMPE).;

Adhesion and morphology characteristics of Vero cells cultured on uncoated and coated PVC tubes.

phosphatidylcholine (DMPC), phosphatidylethanolamine (DMPE).

INTRODUCTION

Synthetic and naturally occurring polymers are important elements in new strategies for producing engineered tissue[1]. But to select appropriate polymers for tissue engineering, it is necessary to understand the influence of the polymer on cell viability, growth and function. Cell interactions with these polymers are usually studied using cell culture techniques.

Most tissue-derived cells are anchorage-dependent and require adhesion to a solid surface for viability and growth. As the cell adhesion to a surface precedes other events, like cell spreading, cell migration and often, differentiated cell function, this process is of fundamental interest in tissue engineering[2], being exclusively dependent on the characteristics of the substrate. Moreover, by maintaining the culture for longer periods, the influence of the substrate on cell viability and function can also be determined[2,3].

It is known that it is the surface of the polymer, which first comes into contact with the patient when it is placed in the body or in fresh blood. Therefore, the initial response of the organism to the polymer must depend on its surface properties. Blood contact with artificial surfaces during cardiovascular bypass (CBP) and extracorporeal circulation (ECC) leads to activation of the complement[4,5,6] and the coagulation cascate[7,8,9,10]. This inflammatory reaction can lead to life-threatening transient or permanent dysfunction of various organs[11]. One approach to minimize such effects is to improve the biocompatibility of the CBP and ECC circuits by modifying their surfaces. This can be achieved by coating them with heparin[6,12,13] or phospholipids[14,15]. The heparin is an anticoagulant used systemically during the ECC procedures despite of some eventual side effects as hemorrhage[16,17]. Yet, the phospholipids mimic the membrane of blood cells mainly constituted of phosphatidylcholine and phosphatidylethanolamine[18] which can avoid their recognition as foreign elements[15] by the blood.

This research investigated the adhesion and morphology characteristics of Vero cells cultured on uncoated PVC tubes as well as the heparin, phosphatidylcholine (DMPC) and phosphatidylethanolamine (DMPE) coated ones.

MATERIALS AND METHODS

Polymer coating preparation

The poly (vinyl) chloride (PVC) tubes were cut in small pieces of 0,25 cm^2 and were washed in methanol and deionized water just as a cleaning process. Then, some samples were coated by three different solutions in 70% ethanol: 800UI/ml. of Benzalkonium

Chloride/Heparin (Sigma Chemical Co., St. Lois, MO, USA), 5mM of Phosphatidylcholine Dimyristoyl (DMPC-Sigma) and 5mM of Phosphatidylethanolamine Dimyristoyl (DMPE-Sigma) through the adsorption of these substances for 10 minutes. All the samples were sterilized in ethylene oxide. After sterilization, the samples were washed for 15 minutes three times in Ham F-10 (Sigma) medium and added to the fibroblastic-like cell culture.

Cell Culture

The samples were used in the culture of 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. Vero cells are a lineage that is recommended for studies of cytotoxicity and for cell-substratum interactions with biomaterials research[2,3]. An initial cell suspension of 10^5cells/ml. in Ham-F10 medium (Sigma) supplemented with 10% FCS (Metrical) was inoculated on the different samples (DMPC, DMPE and Heparin-coated) as well as on cover slips. They were kept in culture at 37°C for 24, 48, 72 and 96 hours. During the experimental conditions, the culture was observed every day using a phase contrast microscopy (Olympus IX 50). Then, the samples were prepared for the scanning electron microscopy (SEM).

Scanning Electron Microscopy (SEM)

The materials were fixed with 2.5% paraformaldehyde/glutaraldehyde (Merck Kraal, Darmstadt, Germany) in 0.1 M phosphate buffer pH 7.4 for 2 hours, then washed in phosphate buffer, post fixed in 1% osmium tetroxide (Sigma), and dehydrated in an ethanol

series. The material was critical point dried (Balzers CPD030) and gold sputtered (Balzers SCD 050). The observations of the specimens were performed with a JEOL JSM-5800 LV microscope.

RESULTS

We could observe some cells over the polymers in the first 24 hours (Fig.1). But with scanning electron microscopy, we realized that there were no whole cells over their surface (Fig. 2e; 2g; 2i), except on the uncoated tubes (Fig.2c). On DMPE-coated PVC, we could not find cells even after 24 hours of culture (Fig. 1g). In fact, the cells observed presented a completely different morphology from the control ones (Fig.1a; 2a). Vero cells are normally flattened (Fig.1a; 1b) and not rounded as the ones on the coated-PVC surfaces (Fig. 1e – 1j; Fig. 2e –2j). In 96 hours of culture, besides the fact that we could see some cells on the PVC surfaces (Fig. 1d; 1f; 1h; 1j), the scanning analysis showed that no cells were really attached to their surfaces (Fig. 2d; 2f; 2h; 2j); this was much more evident on the uncoated PVC tubes (Fig. 2d). Again, we found pieces of cells in extremely reduced quantity (Fig. 2f) and not in all the coated-PVC surfaces (Fig.1h; 1j).

DISCUSSION AND CONCLUSIONS

The biggest challenge to improve the extracorporeal circulation (ECC) circuits resides in the platelet adhesion to theirs surfaces that can collaborate towards the thrombus formation and results in the activation of blood coagulation[10] . Our scanning electron micrographs showed that this is a common problem in the uncoated PVC materials (Fig. 2c), mainly

over the grooves on the PVC surface that are usually found on its structure[19]. This data corroborates with the importance of a uniform coating independent of the substance used, since it will cover all the grooves on PVC surfaces that favor cell adhesion[19].

However, even after coating the surfaces, we could still observe some cells on them, with phase contrast microscopy (Fig.1e-1j), but after preparing the coated tubes for scanning electron microscopy, we realized that the adherence of those cells on their surfaces was not strong enough to withstand the preparation procedures of this technique (Fig. 2e-2j). This suggests that there was no real cell adhesion to the coated-PVC surfaces. This fact supports the idea of increased biocompatibility of the PVC tubes used in extracorporeal circulation by coating techniques[5,6,7,14,16,20].

Moreover, after prolonged culture time, the cells on uncoated PVC tubes died (Fig.2d). This could be due to the phthalates released. Phthalates are the plasticizers used in the majority of the PVC tubes and bags, which give a better flexibility to the material. The consequences of its release in the blood or in the culture medium in contact with the polymers has been reported by some authors as harmless[21,22] and by others as a serious problem, which should be considered[23,24,25,26]. The coated-PVC tubes may diminish the phthalates release[27] also contributing to the increase of biocompatibility.

The morphology of Vero cells was described elsewhere[28] as flattened, which is a consequence of the cellular adhesion and spreading on the surface. In all the coated tubes, Vero cells presented a rounded morphology (Fig 1e-1j; Fig. 2e-2j), that indicates that they did not spread on the surfaces. Since the cellular spreading is important to a good interaction with the substrate, the low interaction of those cells with the tubes could account

for an improvement of the biocompatibility, decreasing the thrombus formation in ECC procedures.

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REFERENCES

1. HUBBELL, J.A. Biomaterials in tissue engineering, Biotechnology 13: 565-576, 1995.

2. ISO 10993-5. 1992(E). Biological evaluation of medical devices - Part 5 - Tests for cytotoxicity: *in vitro* methods.

3. KIRKPATRICK C.J.. Biological testing of materials and medical devices - A critical view of current and proposed methodologies for biocompatibility testing: cytotoxicity in vitro. Regulatory Affairs 4, 13-32 ,1992.

4. BOUMA, M.; MAESSEN, J.; WEERWIND, P.; DENTENER, M.; FRANSEN, E.; de JONG, D.; BUURMAN, W. Release of Lipopolysaccharide Toxicity-Modulating proteins in Patients undergoing Cardiopulmonary bypass using noncoated and Heparin-coated Extracorporeal Circuits – A Clinical Pilot Study. Chest 111, 577-583, 1997.

5. VIDEM, V., MOLLNES, T. E., GARRED, P., SVENNEVING, J. L., Biocompatibility of extracorporeal circulation-in vitro comparison of heparin-coated and uncoated oxygenator circuits. **J. Thorac. Cardiovasc. Surg.101**, 654-660, 1991.

6. PEKNA, M., BOROWIEC, J. W., FAGERHOL, M. K., VENGE, P., THELIN, S., Biocompatibililty of heparin-coated circuits used in cardiopulmonary bypass. *Scand. J.* **Thor. Cardiovasc. Surg. 28**, 5-11, 1994.

7. SALZMAN, E. W., ROSENBERG, R. D., SMITH, M. H., LINDON, J. N., FAVREAU, L., Effect of heparin and heparin fractions on platelet aggregation. **J. Clin. Invest. 65**, 64-73, 1980.

8. De SCHEERDER, I.; WANG, K.; WILCZEK, K.; MEULEMAN, D.; Van AMSTERDAM, R.; VOGEL, G.; PIESSENS, J.; Van de WERF, F. Experimental Study of Thrombogenicity and Foreign Body reaction induced by Heparin-coated Coronary Stents. **Circulation 95**, 1549-1553, 1997.

9. te VELTHUIS, H.; BAUFRETON, C.; JANSEN, P.G.M.; THIJS, C.M.; HACK, C.E.; STURK, A.; WILDEVUUR, C.R.H.; LOISANCE, D.Y., Heparin coating of extracorporeal circuits inhibits contact activation during cardiac operations. **J.Thorac. Cardiovasc. Surg. 114,** 117-122, 1997.

10. OEVEREN, W.V; WILDEVUUR, C.R.H.; KAZATCHKINE, M.D. Biocompatibility of extracorporeal circuits in heart surgery. **Transfus. Sci. 11**, 5-33, 1990.

11. KIRKLIN, J.K.; WESTABY, S.; BLACKSTONE, E.H.; KIRKLIN, J.W.; CHENOWETH, D.E.; PACIFICO, A.D. Complement and the damaging effects of cardiopulmonary bypass. **J. Thorac. Cardiovasc. Surg. 86**, 845-857, 1983.

12. NIIMI, Y.; ICHINOSE, F.; ISHIGURO, Y.; TERUI, K.; VEZONO, S.; MORITA, S.; YAMANE, S., The effects of heparin coating of oxygenators fibers on platelet adhesion and protein adsorption. **Anesth. Analg. 89**, 573-579, 1999.

13. ØVRUM, E.; BROOSTAD, F.; ÅM HOLEN, E.; TANGEN, G.; ABDELNOOR, M., Effects on coagulation and fibrinolysis with reduced versus full systemic heparinization and heparin-coated cardiopulmonary bypass. **Circulation 92,** 2579-2584, 1995.

14. CHAPMAN, D.; CHARLES, S.A., A coat of many lipids – in the clinic. **Chem. Br. 28**, 253-256, 1992.

15. DURRANI, A. A., MAYNARD, J. A., CHAPMAN, D. Biomembranes as models for polymer surfaces II. The synthesis of reactive species for covalent coupling of phosphorylcholine to polymer surfaces. **Biomaterials 7**, 121-125, 1986.

16. BOROWIEC, J. W., THELIN, S., BAGGE, L. HULTMAN, J., HANSSON, H-E., Decreased blood loss after cardiopulmonary bypass using heparin-coated circuit and 50 % reduction of heparin dose. **Scand. J. Thor. Cardiovasc. Surg. 26**, 177-185, 1992.

17. KIRKLIN, J. W., Effects of protamine administration after cardiovascular bypass on complement, blood elements and the hemodynamic state. **Ann. Thorac. Surg. 41**, 193-198, 1986.

18. ALBERTS, B.; BRAY, D.; LEWIS, J., RAFT, M.; ROBERTS, K.; WATSON, J.D., "Membrane structure", in **Molecular Biology of the Cell**, *New York: Garland Publishing* 1994, pp.473-506.

19. MOREIRA, P.L.; WADA, M.L.F.; NOVELLO, W.P., Importance of uniform heparin coating on biopolymers . **Artif. Organs 24(3)**, 209-211, 2000.

20. COURTNEY, J.M.; LAMBA, N.M.K.; SUNDARAM, S.; FORBES, C.D., Biomaterials for blood-contacting applications. **Biomaterials 15(10)**, 737-744, 1994.

21. LABOW, R.S., CARD, R. T., ROCK, G., The effect of the plasticizer Di (2-ethylhexyl)phthalate on Red Cells deformability. **Blood 70(1),** 319-323, 1987.

22. GUESS, W.L., HABERMAN, S., Toxicity profiles of vinyl and polyolefinic plastics and their additives. **J. Biomed. Mater. Res 2,** 313-335, 1968.

23. HARRIS, C.A., HENTTU, P., PARKER, M.G., SUMPTER, J. P., The estrogenic activity of phthalate esters *in vitro*. **Environ. Health Perspect. 105(8)**, 802-811, 1997.

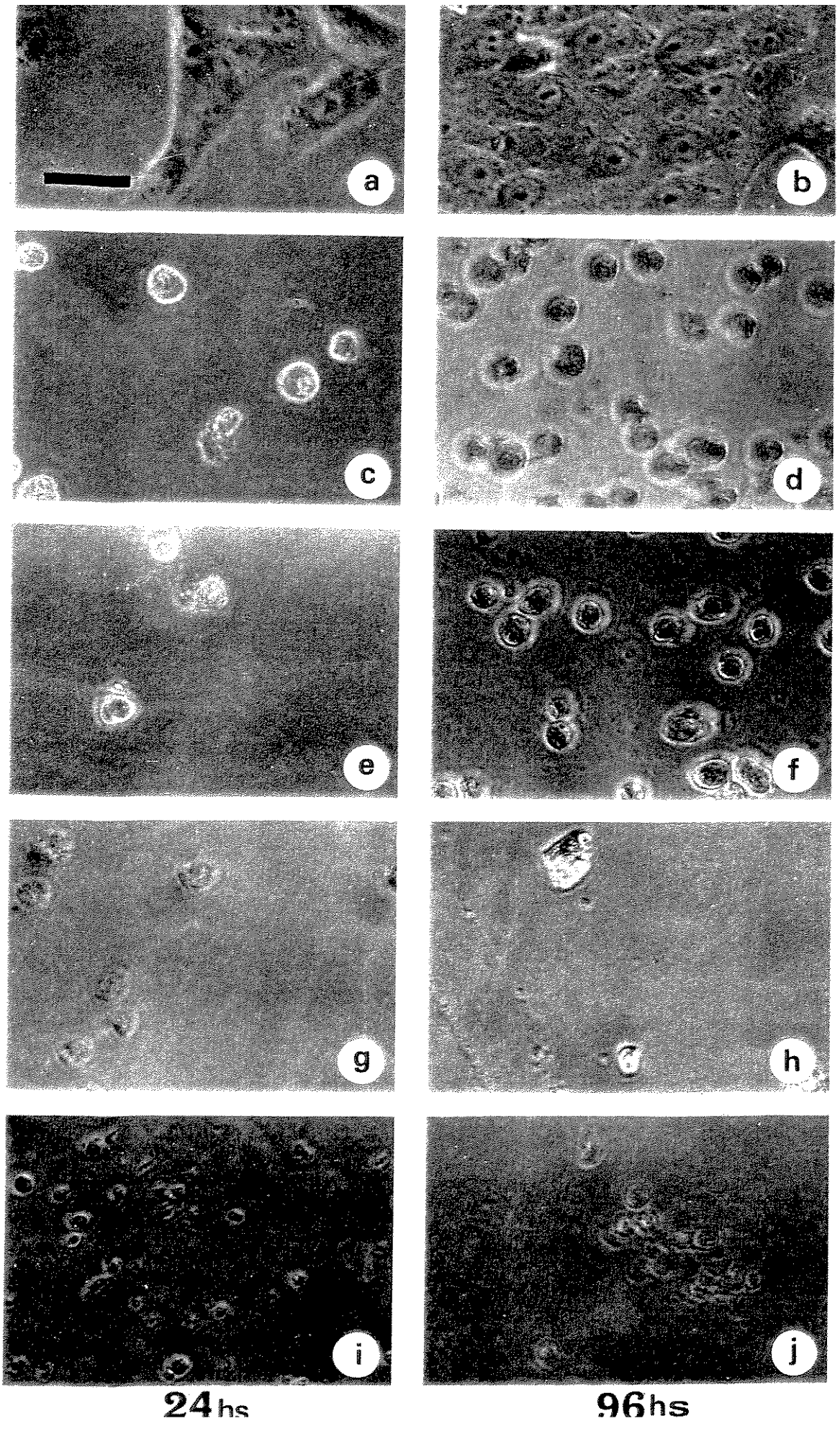
24. FRATANTONI, J.C., Review: The platelet storage lesion: possible role of platicizers ? **Blood Cells 18**, 435-440, 1992.

25. BARRY, Y. A., LABOW, R.S., KEON, W. J., TOCCHI, M., ROCK, G., Perioperative exposure to plasticizers in patients undergoing cardiopulmonary bypass. **J. Thorac. Cardiovasc. Surg. 97,** 900-905, 1989.

26. SASAKAWA, S., MITOMI, Y., Di-2-ethylhexylphthalate (DEHP) content of blood or blood components stored in plastic bags. **Vox Sang**, **34**, 81-86, 1978.

27. MOREIRA, P.L.; GENARI, S.C.; MORAES, A.M.; WADA, M.L.F. Cellular adhesion on uncoated and different coated PVC tubes used in Extracorporeal Circulation (ECC). **J. Biomed. Mat. Res. in preparation.**

28. GENARI, S.C., DOLDER, M. A. H., WADA, M. L. F., Scanning and transmition electron microscopy of transformed Vero cells, with altered *in vitro* growth characteristics. **J. Submicrosc. Cytol. Pathol. 28(4)**, 565-572, 1996.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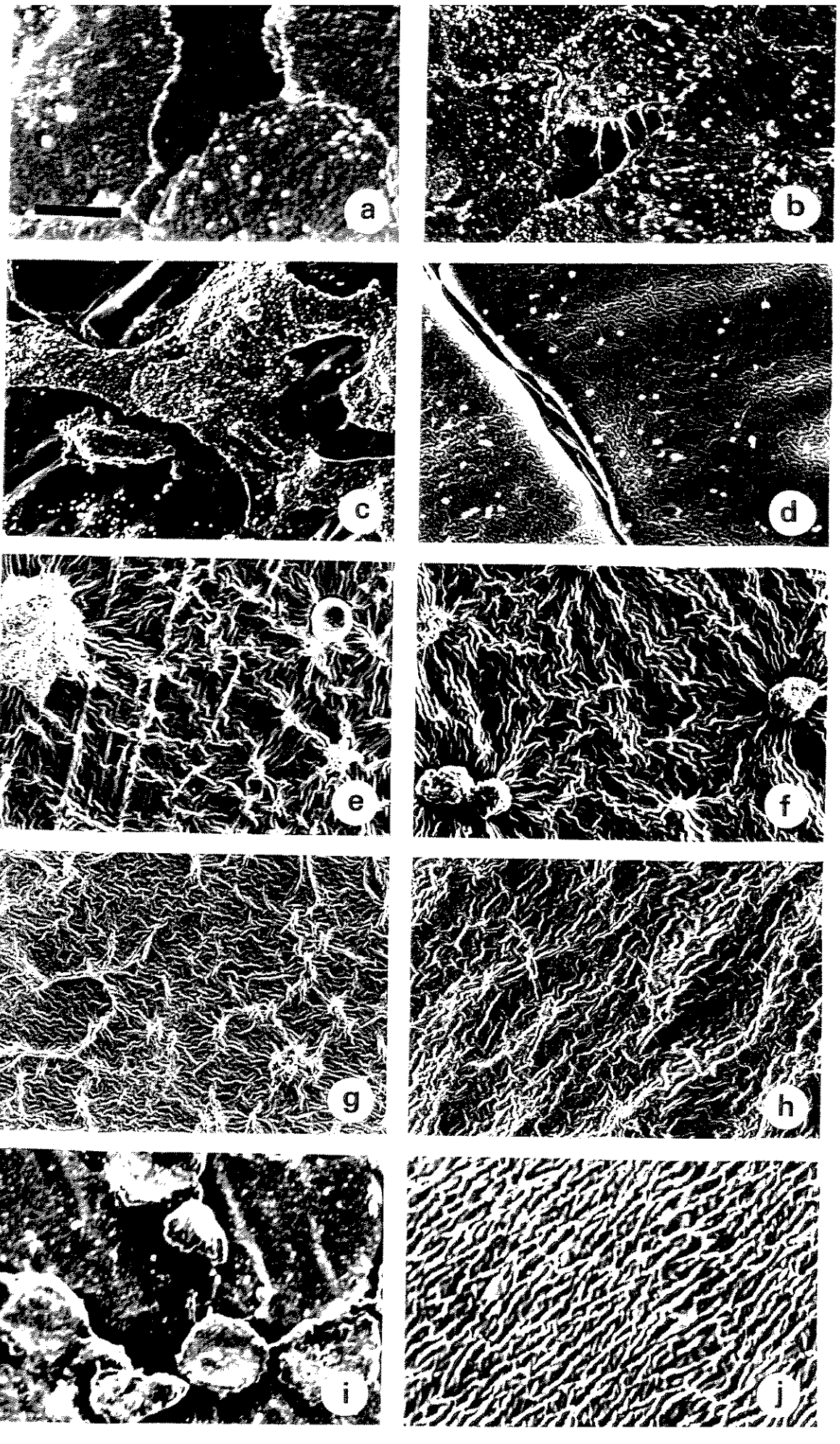
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Figure 1. Phase Contrast Micrographs of Vero cells. Control cells on coverslips after 24 hours **(a)** and 96 hours **(b)** of culture; cells on uncoated PVC tubes after 24 hours **(c)** and 96 hours **(d)** of culture; cells on DMPC coated PVC tubes after 24 hours **(e)** and 96 hours **(f)** of culture; cells on DMPE coated PVC tubes after 24 hours **(g)** and 96 hours **(h)** of culture and cells on Heparin coated PVC tubes after 24 hours **(i)** and 96 hours **(j)** of culture. Scale bar of 40 μm for all pictures.

Figure 2. Scanning Electron Micrographs of Vero cells. Control cells on coverslips after 24 hours **(a)** and 96 hours **(b)** of culture; cells on uncoated PVC tubes after 24 hours **(c)** and 96 hours **(d)** of culture; cells on DMPC coated PVC tubes after 24 hours **(e)** and 96 hours **(f)** of culture; cells on DMPE coated PVC tubes after 24 hours **(g)** and 96 hours **(h)** of culture and cells on heparin coated PVC tubes after 24 hours **(i)** and 96 hours **(j)** of culture. Scale bar of 5 μm for all pictures.

CONSIDERAÇÕES FINAIS

- ✓ O processo de esterilização utilizado sobre os materiais revestidos por heparina, DMPC e DMPE é muito importante, uma vez que podem causar falhas no recobrimento ou reagir quimicamente com o mesmo, favorecendo em ambos os casos a agregação celular, processo este que inicia a formação de trombos nos procedimentos de circulação extracorpórea.

- ✓ A esterilização com álcool causa falhas no recobrimento de heparina, o que favorece a adesão celular nestes sítios.

- ✓ Os tubos revestidos com DMPC apresentam um recobrimento mais uniforme, o que pode estar protegendo melhor a superfície do PVC contra possíveis alterações químicas causada pela esterilização com óxido de etileno. Este aspecto acaba contribuindo para um aumento da adesão celular sobre sua superfície.

- ✓ O teste de adesão utilizado neste trabalho apresentou algumas limitações com relação às diferentes espessuras do tubo de PVC utilizado, uma vez que estas podiam interferir na quantificação da absorbância, resultando em altas variâncias das medidas.

- ✓ Além disso, a microscopia de contraste de fase encontrou alguma dificuldade em focar células sobre os tubos de PVC recobertos ou não devido à característica dos próprios tubos que não apresentam grande translucidez.

- ✓ Apesar de algumas poucas células Vero aderirem à superfície dos polímeros revestidos, elas não se espalham sobre os mesmos, apresentando morfologia arredondada, impedindo sua proliferação.

- ✓ Os tubos de PVC apresentam uma citotoxicidade, provavelmente causada pela liberação de ftalatos, uma vez que os mesmos reduzem o número de linfócitos em degeneração quando comparado com os tubos não revestidos.
- ✓ O recobrimento de heparina apresentou boa compatibilidade e eficiência por impedir a adesão celular aos tubos, além de ser economicamente viável e facilmente obtido em escala industrial, devido ao seu baixo custo quando comparado com os recobrimentos de fosfolipídeos.

REFERÊNCIAS BIBLIOGRÁFICAS

ALVES, G.P. Recobrimento de superfícies de PVC com filmes de Langmuir-Blodgett usando fofatidiletanolamina.. *Dissertação de Mestrado- Faculdade de Engenharia Química – UNICAMP, 1999, 114p.*

ARNANDER, C., DRYJSKI, M., LARSON, R., OLSSON, P., SWEDENBORG, J., Thrombin uptake and inhibition on endothelium and surfaces with a stable heparin coating. *J. Biomed. Mat. Res. 1986:20:235-246.*

BOROWIEC, J. W., THELIN, S., BAGGE, L. HULTMAN, J., HANSSON, H-E., Decreased blood loss after cardiopulmonary bypass using heparin-coated circuit and 50 % reduction of heparin dose. *Scand. J. Thor. Cardiovasc. Surg. 1992:26:177-185.*

BRUINS, P.; te VELTHUIS, H.; EERENBERG-BELMER, A.J.M.; YAZDANBAKHSH, A.P.; de BEAUMONT, E.M.F.H.; EIJISMAN, L.; TROUWBORST, A.; HACK, C.E. Heparin-protamine Complexes and C-reative Protein induce activation of the classical complement pathway: studies in patients undergoing Cardiac surgery and in vitro. *Thromb. Haemost. 2000: 84: 237-243.*

BULL, B. S., KORPMAN, R. A., HUSE, W. M., BRIGGS, B. D., Heparin therapy during extracorporeal circulation. Problems inherent in existing heparin protocols. *J. Thorac. Cardiovasc. Surg 1975:69:674-682.*

BUTLER, J., ROCKER, G. M., WESTABY, S, Inflammatory response to cardiopulmonary bypass – *Ann. Thorac. Surg. 1993:55(2):552-559.*

CHAPMAN, D., DURRANI, A. A. ,Phospholipids acid esters, their preparation and their use for the preparation of biocompatible surfaces. European patent. London AI 0157469. 18.01.85.

COLMAN, R. W., Platelet and Neutrophil activation in cardiopulmonary bypass. . *Ann. Thorac. Surg 1990:49:32-34.*

DURRANI, A. A. , HAYWARD, J. A., CHAPMAN, D. Biomembranes as models for polymer surfaces II. The syntheses of reactive species for covalent coupling of phosphorylcholine to polymer surfaces. *Biomat. 1986:7:121-125.*

HOFFMAN, A.S., Modifications of materials surfaces to affect how interact with blood. *Annals of New York academy sciences 1987:516:96-101.*

KIRKLIN, J. W., Effects of protamine administration after cardiovascular bypass on complement, blood elements and the hemodynamic state. *Ann. Thorac. Surg 1986:41:193-198.*

MARRECO, P.R., Estudo do recobrimento de policloreto de vinila (PVC) com fosfatidilcolinas. *Dissertação de Mestrado - Faculdade de Engenharia Química - UNICAMP, 1999,151 p.*

MILLER, B., LEVY, J., The inflammatory response to cardiopulmonary bypass. *J. Cardiothorac. Vasc. Anest. 1997:11:355:366.*

MURIITHI, E.W.; BELCHER, P.R.; RAO, J.N.; CHAUDHRY, M.A.; NICOL, D.; WHEATLEY, D.J., The effects of heparin and extracorporeal circulation on platelet counts and platelet microaggregation during cardiopulmonary bypass. *J. Thorac. Cardiovasc. Surg. 2000:120(3):538-543.*

NIIMI, Y.; ICHINOSE, F.; ISHIGURO, Y.; TERUI, K.; VEZONO, S.; MORITA, S.; YAMANE, S., "The effects of heparin coating of oxygenators fibers on platelet adhesion and protein adsorption". *Anesth. Analg.1999: 89:* 573-579.

PEKNA, M., BOROWIEC, J. W., FAGERHOL, M. K., VENGE, P., THELIN, S., Biocompatibility of heparin-coated circuits used in cardiopulmonary bypass. *Scand. J. Thor. Cardiovasc. Surg 1994:28:5-11.*

ROBERTS, G., Langmuir-Blodgett Films. New York: Plenum Press, 1990.

SALZMAN, E. W., ROSENBERG, R. D., SMITH, M. H., LINDON, J. N., FAVREAU, L., Effect of heparin and heparin fractions on platelet aggregation. *J. Clin. Invest. 1980:65:64-73.*

SOUZA, M. H. L., ELIAS, D. O., Fundamentos da circulação extracorpórea, Centro editorial Alfa Rio, Rio de Janeiro, 1995

SVENNING, J.L., GEIRAN, O.R., KARLSEN, H., PEDERSEN, T., MOLLNES, T. E., KONGSGARD, U., FROYSAKER, T., Complement activation during extracorporeal circulation. *J. Thorac. Cardiovasc. Surg. 1993:6(3):466-472.*

TANAKA, K., et al. Alterations in coagulation and fibrinolysis associated with cardiopulmonary bypass during open heart surgery. . *J. Cardiothorac. Vasc. Anest. 1989:3(2):181-188.*

UEDA, T., ISHIMARA, K., NAKABAYASHI, N. Adsorption-desorption of proteins on phospholipid polymer surfaces evaluated by dynamic contact angle measurement. *J. Biomed. Mat. Res. 1995:29:381-387.*

UTLEY, J. R.,WACHTEL, C., CAIN, R. B., SPAW, E. A., et al. Effect of hypothermia, hemodilution and pump oxigenation on organ water content, blood flow and oxygen delivery and renal function. . *Ann. Thorac. Surg 1981:31:121.*

VIDEM, V., MOLLNES, T. E., GARRED, P., SVENNEVING, J. L., Biocompatibility of extracorporeal circulation-in vitro comparison of heparin-coated and uncoated oxygenator circuits. *J. Thorac. Cardiovasc. Surg. 1991:101:654-660.*

WENDEL, H.P.; WEBER, N.; ZIEMER, G., "Increased adsorption of high molecular weight kininogen to heparin-coated artificial surfaces and correlation to hemocompatibility". *Immunopharmacology, 1999:* ***43:*** 149-153.

YOUNG, B. R., PITT, W. G., COOPER, S. L., Protein Adsorption on Polymeric Biomaterials I. Asorption Isotherms. *J. Coll. Interfa. Scie. 1988:124(1):28-43.*