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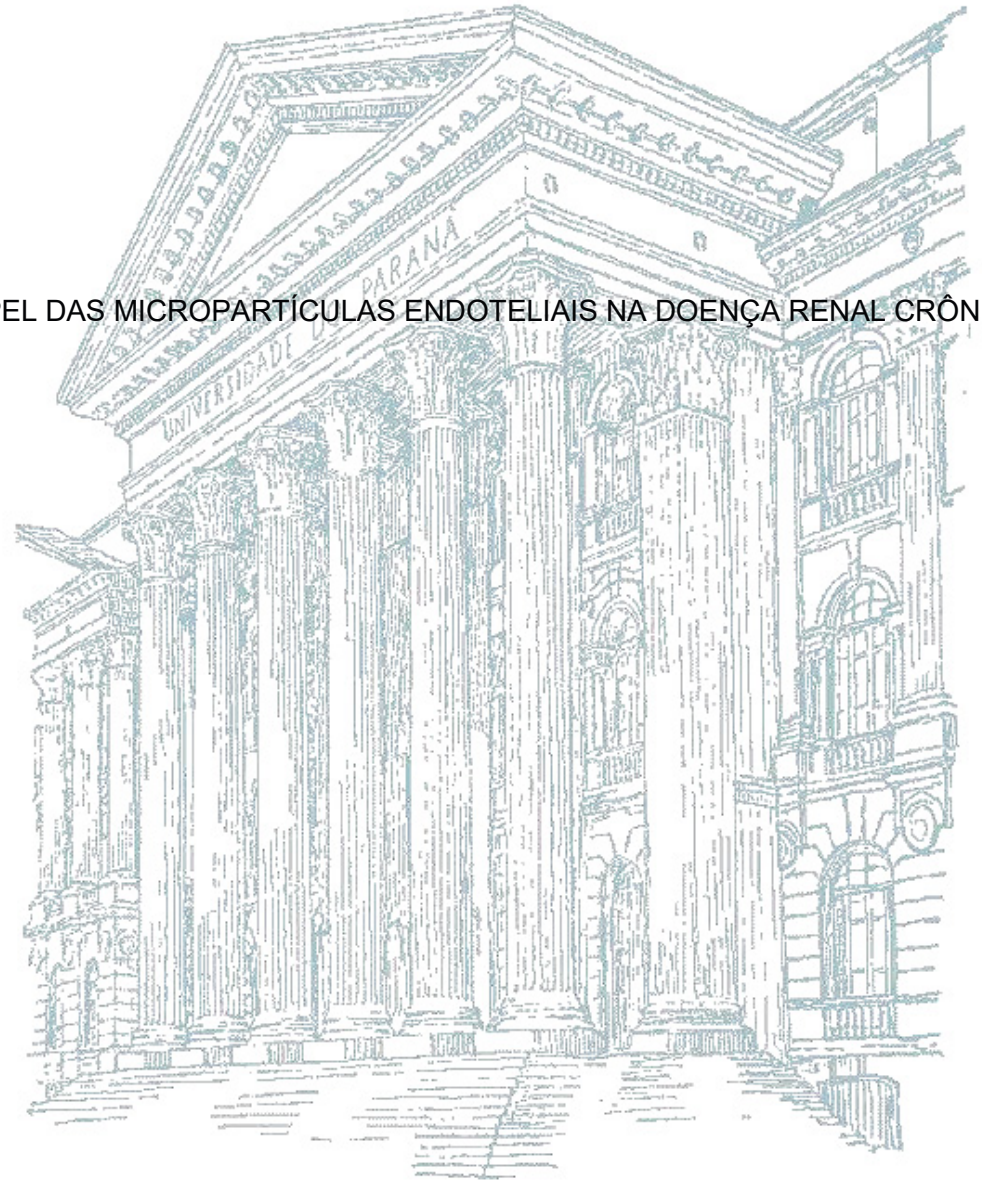

PAPEL DAS MICROPARTÍCULAS ENDOTELIAIS NA DOENÇA RENAL CRÔNICA

CURITIBA
2020

GIANE FAVRETTO

PAPEL DAS MICROPARTÍCULAS ENDOTELIAIS NA DOENÇA RENAL CRÔNICA

Tese apresentada como requisito parcial à obtenção do Grau de Doutora em Microbiologia, Parasitologia e Patologia, Departamento de Patologia Básica, Setor de Ciências Biológicas, Universidade Federal do Paraná.
Orientadora: Profa. Dra. Andréa Emília Marques Stinghen.
Coorientador: Profa. Dra. Maria Aparecida Dalboni.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação MICROBIOLOGIA, PARASITOLOGIA E PATOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **GIANE FAVRETTO** intitulada: **Papel das micropartículas endoteliais na doença renal crônica**, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 11 de Dezembro de 2020.

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DEDICATÓRIA

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O amor que me deu forças para chegar ao fim dessa jornada.

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos” (Isaac Newton).

RESUMO

Toxinas urêmicas, tais como *p*-cresil sulfato (PCS), indoxil sulfato (IS) e fosfato inorgânico (Pi), podem levar à liberação de micropartículas endoteliais (MPEs) em pacientes com doença renal crônica (DRC). As MPEs são importantes para a sinalização célula-célula, carreando informações genéticas e proteicas de sua célula de origem. O presente estudo visa compreender o mecanismo de formação de MPEs na uremia, através da exposição de células endoteliais às toxinas urêmicas PCS, IS e Pi, e a interação dessas MPEs na ativação endotelial e leucocitária. Nesse contexto, células endoteliais humanas foram cultivadas e tratadas com PCS, IS e Pi na concentração urêmica máxima (2,6 mg/L, 236 mg/L e 3 mM), expostas ainda a 2μM de ionóforo de cálcio (Ca^{2+}) para a indução da microvesiculação. A purificação das MPEs foi realizada por meio de centrifugação diferencial, enquanto a quantificação e caracterização foi por citometria de fluxo, NanoSigth e Microscopia Eletrônica de Varredura (MEV). A determinação da concentração de uso das MPEs in vitro foi realizada pelo ensaio de Alamar Blue. Avaliou-se o efeito das MPEs sobre as células endoteliais em relação à viabilidade pelo ensaio de MTT, à adesão a diferentes tipos de proteínas da matriz extracelular (MEC) pelo ensaio de adesão, à expressão de molécula inflamatória *vascular cell adhesion molecule 1* (VCAM-1) por imunocitoquímica e à produção de espécies reativas de oxigênio (EROs) com a sonda DCFH-DA. Para analisar a migração de células endoteliais e monócitos, foi realizado os ensaios de *Wound Healing* e migração celular através da câmara de Boyden, respectivamente. Através dos ensaios para a caracterização das MPEs foi possível identificar a morfologia, concentração e tamanho. A concentração de uso determinado após o ensaio de Alamar Blue foi de 10 μg/mL de MPEs. A adesão endotelial foi comprometida de forma significativa nas células endoteliais expostas a MPEs provenientes de PCS (PcsMPE) e Fosfato inorgânico (PiMPE) (***P*<0,01). A expressão de VCAM-1 pelas células endoteliais pode ser evidenciada nas células tratadas com MPEs provenientes de células controle (CtrlMPE), PCS (PcsMPE) e IS (IsMPE). Através do ensaio de *Wound Healing* foi possível evidenciar um aumento significativo na migração celular quando comparado células endoteliais tratadas com PcsMPE frente as tratadas com CtrlEMP (**P*<0,05). Não houve diferença significativa no ensaio de migração realizado para monócitos. Também foi possível observar que as MPEs não induzem a formação de EROS em células endoteliais. Nossos dados demonstraram que a exposição do endotélio a toxinas urêmicas é capaz de liberar MPEs com concentrações e morfologias diferentes dependendo do tipo de toxina que agrediu esse endotélio. Verificamos ainda que as MPEs podem agir diminuindo a expressão de integrinas no endotélio, dificultando a sua adesão na matriz. As MPE são capazes de induzir a expressão de VCAM-1 no endotélio, sugerindo o envolvimento no processo inflamatório. Células expostas a PcsEMP migraram mais que as células expostas aos outros tratamentos, indicando que PcsEMP podem estar correlacionadas com a ativação endotelial. Por fim, verificamos que as MPEs não possuem papel significativo no estresse oxidativo. Concluímos que as MPEs podem servir como biomarcadores de lesão endotelial e do processo inflamatório, entretanto estudos adicionais devem ser realizados para desvendar os mecanismos de formação e o papel das MPEs na uremia.

Palavras chaves: Micropartículas endoteliais, toxinas urêmicas, uremia, ativação leucocitária.

ABSTRACT

Uremic toxins, such as p-cresyl sulfate (PCS), indoxyl sulfate (IS), and inorganic phosphate (Pi), can lead to the release of endothelial microparticles (EMPs) in patients with chronic kidney disease (CKD). EMPs are important for cell-cell signaling, carrying genetic and protein information from their cell of origin. The present study aims to understand the mechanism of forming EMPs in uremia through the exposure of endothelial cells to uremic toxins PCS, IS, and Pi, and the interaction of these EMPs in endothelial and leukocyte activation. In this context, human endothelial cells were cultured and treated with PCS, IS, and Pi in the maximum uremic concentration (2.6 mg/L, 236 mg/L, and 3 mM), also exposed to 2 μM of calcium ionophore (Ca^{2+}) for the induction of microvesiculation. The purification of EMPs was performed by differential centrifugation, while quantification and characterization were by flow cytometry, NanoSigth, and Scanning Electron Microscopy (SEM). The determination of the concentration of use of EMPs in vitro was performed by the Alamar Blue assay. The effect of EMPs on endothelial cells concerning viability was evaluated by the MTT assay, adhesion to different types of matrix proteins by the adhesion assay, to the expression of vascular inflammatory molecule cell adhesion molecule 1 (VCAM-1) by immunocytochemistry, and the production of reactive oxygen species (ROS) with the DCFH-DA probe. Wound Healing and cell migration assay through the Boyden chamber were used to analyze the migration of endothelial cells and monocytes, respectively. Through the characterization of EMPs, it was possible to identify the morphology, concentration, and size. The concentration of use determined after the Alamar Blue test was 10 μg/mL of EMPs. Endothelial adhesion was significantly compromised in endothelial cells exposed to EMPs from PCS (PcsEMP) and inorganic phosphate (PiEMP) (**P<0.01). The VCAM-1 expression by endothelial cells can be seen in cells treated with MPEs from control cells (CtrlEMPs), PCS (PcsEMP), and IS (IsEMP). The Wound Healing assay demonstrated a significant increase in cell migration compared to endothelial cells treated with PcsEMP compared to those treated with CtrlEMP (*P<0.05). There was no significant difference in the migration assay performed for monocytes. It was also possible to observe that EMPs do not induce the formation of ROS in endothelial cells. Our data have shown that exposure of the endothelium to uremic toxins can release EMPs with different concentrations and morphologies depending on the type of toxin that attacked this endothelium. We also found that EMPs may act by reducing the expression of integrins in the endothelium, making it difficult for them to adhere to matrix protein. EMPs can also induce VCAM-1 expression in the endothelium, suggesting involvement in the inflammatory process. Cells exposed to PcsEMP migrated more than cells exposed to other treatments, indicating that PcsEMP may be correlated with endothelial activation. Finally, we found that EMPs do not play a significant role in oxidative stress. We conclude that EMPs may be a biomarker of endothelial injury and the inflammatory process. More studies should be carried out to unveil the mechanisms of formation and the role of EMPs in uremia.

Keywords: Endothelial microparticles, uremic toxins, uremia, leukocyte activation.

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LISTA DE ABREVIATURAS E SIGLAS

BMP-2 – Proteína morfogenética óssea-2

Ca^{2+} – Ionóforo de cálcio

CKD – Chronic renal dysfunction

CtrlMPE – Micropartículas endoteliais provenientes de células de controle.

DCV – Doença Cardiovascular

DM – diabetes mélitos

DRC – Doença renal crônica

EROs – Espécies reativas de oxigênio

ICAM-1 – *intercellular cell adhesion molecule-1*

IS – Indoxil sulfato

IsMPE – Micropartículas endoteliais provenientes de IS

MEC – Membrana extracelular

MEV – Microscopia eletrônica de Varredura

MPEs – Micropartículas endoteliais

MPs – Micropartículas

MV – microvesicles

PCS – *P*-cresil sulfato

PcsMPE – Micropartículas endoteliais provenientes de PCS

Pi – Fosfato Inorgânico

PiMPE – Micropartículas endoteliais provenientes de Pi

TNF-α – *Tumor necrosis factor alpha*

VCAM-1 – *Vascular cell adhesion molecule 1*

SUMÁRIO

1. INTRODUÇÃO

A doença renal crônica (DRC) é caracterizada pela perda da função progressiva dos rins, comprometendo a capacidade de filtração e remoção de solutos tóxicos provenientes da alimentação. Consequentemente, esses solutos, também denominados toxinas urêmicas, acumulam-se no organismo, desencadeando um estado chamado de uremia. As toxinas urêmicas, em conjunto com outros mecanismos da DRC, ocasionam a lesão constante ao endotélio, induzindo a disfunção endotelial, a ativação da cascata inflamatória, as alterações fenotípicas celulares e a liberação de exossomas, micropartículas (MPs) e corpos apoptóticos, além da consequente calcificação vascular (BARRETO et al., 2014; STINGHEN et al., 2011; VANHOLDER et al., 2018; FAURE et al., 2006).

A liberação de MPs tem um papel importante na sinalização célula-célula. As MPs são capazes de transferir receptores e outras moléculas bioativas, como microRNAs, entre os diversos tipos celulares, propriedades que estão correlacionadas com o carreamento de parte da membrana e citoplasma de sua célula de origem. As MPs interagem com as membranas das células receptoras, processo que ocorre por meio da interação ligante-receptor e/ou adesão à célula com subsequente internalização das MPs (FAVRETTO et al., 2019; CHIRONI et al., 2009). Essas características são responsáveis pela sinalização de longo alcance. Nesse contexto, as MPs são investigadas como possíveis biomarcadores para diversas patologias, incluindo a DRC e a doença cardiovascular (DCV), bem como de lesões vasculares e condições pró-trombóticas e pró-inflamatórias (MALLAT et al., 2000; LU et al., 2015; BURTON et al., 2013).

Os níveis de MPs circulantes variam de acordo com o tipo de patologia. O aumento insidioso de MPs pode ser observado em doenças como DRC, diabetes, pré-eclâmpsia e hipertensão. Evidências recentes sugerem que as MPs não são simplesmente uma consequência da doença, mas que a formação delas pode contribuir para a evolução do processo patológico. Podem, portanto, ser consideradas um grupo de armazenamento disseminado de efetores bioativos, que particularmente são bem evidenciados em doenças vasculares, inflamação e resposta do sistema

imune. Dessa forma, o endotélio vascular assim como as diversas células do organismo quando sofrem estímulos podem liberar micropartículas contendo em sua superfície proteínas, como por exemplo as moléculas de adesão específicas para células endoteliais, tais como, *intercelular adhesion molecule-1* (ICAM-1), E-selectina, P-selectina e *platelet endothelial cell adhesion molecule-1* (PECAM-1) (CHIRONI et al., 2009; DIGNAT-GEORGE; BOULANGER.; 2011).

A liberação de MPs está correlacionada com a calcificação vascular. Estudos sugerem que o fator de necrose tumural (TNF-α) é responsável pela estimulação das células endoteliais e que, esse estímulo é responsável por causar um aumento na expressão da proteína morfogenética óssea-2 (BMP-2) e a produção de micropartículas endoteliais (MPEs) (BUENDIA et al. 2015).

As toxinas urêmicas tais como indoxyl sulfato (IS), indoxil-3-acetato ácido (IAA), *p*-cresol e *p*-cresil sulfato (PCS) são capazes de induzir através da disfunção endotelial a formação e liberação de MPEs *in vitro* (FAURE et al., 2006; MEIJERS et al., 2009; GAO et al., 2015). Considerando a importância das micropartículas na comunicação celular, o presente estudo teve como objetivo isolar e caracterizar MPEs provenientes de células expostas às toxinas urêmicas PCS, IS e fosfato inorgânico (Pi), bem como investigar o seu efeito sobre as células endoteliais e os monócitos. A elucidação desses mecanismos fisiopatológicos da lesão endotelial induzido pela toxicidade urêmica poderão contribuir para o desenvolvimento de novos alvos terapêuticos na DRC.

2. OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar os mecanismos envolvidos na disfunção endotelial e formação de MPEs em células endoteliais humanas tratadas com toxinas urêmicas (PCS, IS e Pi).

2.2. OBJETIVOS ESPECÍFICOS

- Induzir a formação de MPEs em células endoteliais humanas tratadas com PCS, IS e Pi, caracterizá-las e purificá-las;
- Determinar a concentração de uso das MPEs;
- Avaliar a viabilidade, adesão e migração celular das células endoteliais tratadas com MPEs;
- Avaliar a ação das MPEs na inflamação celular e expressão de VCAM-1, ativação e migração de monócitos;
- Avaliar a ação das MPEs no estresse oxidativo através da produção de ROS.

3. REVISÃO DE LITERATURA

3.1. CONCEITO GERAL DE MICROPARTÍCULAS

Em estado fisiológico, as células possuem a capacidade de liberar uma grande variedade de vesículas extracelulares, responsáveis pela comunicação intercelular e intracelular. Essas vesículas variam de 30 a 5.000 nm de tamanho e podem ser do tipo exossomos, micropartículas (MPs) e corpos apoptóticos (BURGER et al., 2013, 2016; MOREL et al., 2011). São diferenciadas pelo seu tamanho e mecanismo de biogênese. Os exossomos são provenientes de corpos multivesiculares medindo entre 30 a 100 nm liberadas do meio intracelular e que se fundem a membrana plasmática da célula de origem. As MPs são estruturas arredondadas, vesiculares que se formam a partir do remodelamento da membrana celular e são consideradas produtos da brotação exocítica, possuindo um tamanho entre 0,1 a 1,0 μm de tamanho. As maiores vesículas extracelulares encontradas no organismo são os corpos apoptóticos que medem entre 1 a 5 μm de tamanho e são encontrados durante os estágios finais do apoptose celular (KARPMAN; STÅHL; ARVIDSSON, 2017; LEMOINNE et al., 2014). As principais características e mecanismos de formação de vesículas extracelulares estão descritas na tabela 1.

TABELA 1 – PRINCIPAIS CARACTERÍSTICAS DE EXOSSOMOS, MICROVESÍCULAS E CORPOS APOPTÓTICOS

	Exossomos	**Micropartículas**	**Corpos apoptóticos**
Tamanho	30–100 nm	100–1000 nm	1–5 μm
Origem	Vesículas intraluminais de corpos multivesiculares	Membrana plasmática e conteúdo celular	Membrana plasmática e fragmentos celulares
Mecanismo de formação	Fusão de corpos multivesiculares com a membrana plasmática	Borbulhamento externo da membrana plasmática	Encolhimento celular e morte celular programada

Liberação	Constitutivo e/ou ativação celular	Constitutivo e/ou ativação celular	Apoptose
Tempo de liberação	Dez minutos ou mais	Segundos	–
Vias	Dependente de ESCRT, de tetraspanina, de ceramida, de estímulos	Dependente de Ca^{2+}, de estímulo e da célula	Relacionado à apoptose
Composição da membrana lipídica	Enriquecido de colesterol e ceramida, exposição de fosfatidilserina na face externa da membrana, contém balsas lipídicas	Enriquecido de colesterol e diacilglicerol, exposição de fosfatidilserina na face externa da membrana, contém balsas lipídicas	–
Conteúdo	Proteínas, RNAm, miRNA, lipídios	Proteínas, RNAm, miRNA, lipídios	Organelas, proteínas, frações nucleares, DNA, RNAs codificantes e não codificantes, lipídios

FONTE: Adaptado de Stahl et al. (2017).

As vesículas são liberadas por praticamente todas as células em resposta à lesão, apoptose ou ativação celular e estão intimamente correlacionadas com processos fisiológicos, tais como participação na manutenção da homeostase corporal, ou podem iniciar um processo deletério em caso de excesso de vesículas circulantes ou ao transportar constituintes patogênicos (CHIRONI et al 2009; FREYSSINET et al 2015; LEMOINNE et al 2014; BURGER et al., 2017).

Os mecanismos que levam a formação de vesículas extracelulares bem como a origem sub celular e o conteúdo determinam a atividade fisiológicas. A transferência do conteúdo das MPs pode afetar o fenótipo da célula-alvo, uma vez que combinam múltiplos mediadores em sua superfície sendo consideradas um meio mais efetivo de comunicação intercelular, do que a transferência de moléculas individuais (LING et al., 2011). As MPs são constituídas de componentes citoplasmáticos, proteínas, RNAs, microRNAs, fosfolipídeo fosfatideilserina aniônica (PhtdSer), organelas e de

antígenos expressos na membrana celular (UTA and THU et al 2015; HEIJNEN et al 1999; LEMOINNE et al. 2014).

A membrana celular é caracterizada por sua plasticidade e pela organização lateral de balsas lipídicas que lhe conferem alta resistência mecânica à membrana. As balsas lipídicas são enriquecidas com colesterol e lipídios, com caráter mais rígido que o restante da membrana. São regiões especializadas que têm a função de organizar as proteínas citoplasmáticas em transporte via caveolinas, ou ainda em microdomínios de transdução de sinal. A organização dessas balsas, dependendo dos estímulos recebidos pelas células podem gerar respostas específicas e únicas, resultando na exclusão ou inclusão de proteínas e lipídeos, dando origem a MPs com composição diferente da mesma origem celular (HUGEL 2005).

As MPs identificadas na circulação sanguínea podem ser provenientes de eritrócitos, de plaquetas, de leucócitos e de células endoteliais. As micropartículas endoteliais (MPEs) podem carrear em sua superfície moléculas de adesão e proteínas inflamatória provenientes do endotélio vascular que a deu origem (CHIRONI et al. 2009; BOULANGER 2010b). Estudos demonstraram que células endoteliais humanas (HUVECs) estimuladas por TNF-α aumentam a produção de MPE, sendo positivas para anexina-V, cálcio e altos níveis de BPM-2, estando correlacionadas com a calcificação vascular e diferenciação osteogênica (BUENDÍA et al., 2015).

Em condições patológicas a concentração de MPEs no sangue é aumentada e está correlacionada com processos inflamatórios, angiogênese e contribuem de forma direta e indireta na cascata de coagulação sanguínea através do fator tecidual (TF) (CURTIS et al., 2013; MARKIEWICZ et al., 2013; SIERKO; SOKÓŁ; WOJTUKIEWICZ, 2015).

3.2. MECANISMOS DE FORMAÇÃO DE MICROPARTÍCULAS

A membrana celular é composta por uma bicamada fosfolipídica é caracterizada pela distribuição de esfingomielina e fosfatidilcolina externamente e, fosfatidilserina e fosfatidilatanolamina na sua camada interna. A assimetria da membrana se dá pelo envolvimento de proteases como as caspases, calpaína e o aumento do cálcio intracelular (MIYOSHI et al., 1996). Os níveis intracelulares de

cálcio ativam enzimas chamadas de scramblase, flipase e flopase que, por sua vez, modificam a distribuição assimétrica dos fosfolipídios da membrana. Com o aumento significativo do cálcio intracelular, a translocase é inibida e a scramblase é ativada, resultando na translocação da fosfatidilserina da parte interna da membrana da célula para a parte externa. Com esse mecanismo de externalização, as MPs carreiam em sua superfície grandes quantidades de fosfatidilserina e são ricas em balsas lipídicas associadas a proteínas provenientes da bicamada lipídica (CAMUSSI et al., 2010; FAVRETTO et al., 2019).

A presença de fosfatidilserina no folheto externo da MPs é facilmente identificada, uma vez que se liga facilmente a anexina V. As vias intracelulares começam a ativar a reorganização do citoesqueleto celular, são capazes de induzir o desprendimento de protusões da membrana através da calpaína, uma protease sensível aos altos níveis de cálcio e com capacidade de desprendimento de proteínas da membrana do citoesqueleto intracelular. A ativação da calpaína faz com que a gelsolina também seja ativada e ligada aos filamentos de actina, favorecendo o remodelamento da membrana celular. Contudo, o mecanismo de formação e a origem da MP vai conferir características próprias de cada MPs, como o tamanho e a composição molecular. Durante a eliminação as MPs podem expressar em sua superfície receptores ligeiramente diferentes de sua célula de origem, assim como o conteúdo citoplasmático pode ser modificado através de um processo seletivo durante sua liberação. Entretanto, MPs liberadas durante os processos de apoptose ou de células em repouso expressam receptores diferentes de MPs liberadas por células ativadas (CAMUSSI et al., 2010; STÅHL et al., 2017).

3.3. CARACTERÍSTICAS DAS MICROPARTÍCULAS ENDOTELIAIS (MPEs)

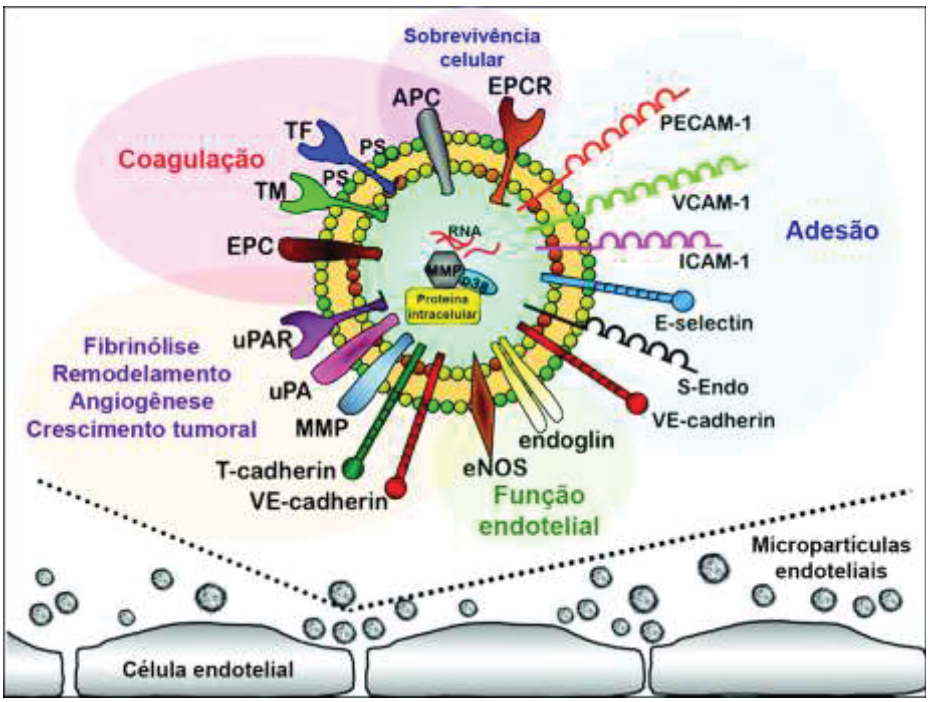
As MPEs são liberadas por meio da ativação das células endoteliais ou ainda no processo de apoptose. Mediadores inflamatórios como o fator de necrose tumoral (TNF-α) induzem a formação de MPEs, e são expressos na membrana dessas vesículas (DIGNAT-GEORGE; BOULANGER, 2011).

Como todas as MPs as MPEs são vesículas que não possuem núcleo e são formadas por meio do mecanismo de remodelamento do citoesqueleto e da

membrana plasmática, medindo em torno de 100 a 1000 nm (DENG; WANG; ZHANG, 2017). Proteínas da membrana plasmática do endotélio são responsáveis pela formação e empacotamento das MPEs, que incluem ICAM-1, PECAM, E-selectina, VE-caderina, αvβ3, VEGFR-2 e eNOS (DIGNAT-GEORGE; BOULANGER, 2011; LEOPOLD, 2013).

As MPEs estão envolvidas em vários processos que perturbam a homeostase vascular, atuam nas vias da inflamação, coagulação, angiogênese e na disfunção endotelial. Podem promover estresse oxidativo e inflamação vascular, contribuindo para a progressão de doenças cardiovasculares. Indutores como a angiotensina II, peróxido de hidrogênio e lipopolissacarídeos, desencadeiam a liberação de MPEs na circulação, levando a progressão da aterosclerose. Entretanto, existem diversas vias fisiológicas para a formação de MPEs, como a via Rho quinase e as proteínas ativadas por mitogênio, e a formação de espécies reativas de oxigênio (ROS) derivado do NADPH oxidase (DIGNAT-GEORGE; BOULANGER, 2011; PAUDEL; PANTH; KIM, 2016). A figura 1 esquematiza as principais moléculas encontradas nas MPEs e suas respectivas funções biológicas no organismo.

FIGURA 1 – REPRESENTAÇÃO DAS MOLÉCULAS CARREADAS PELAS MPEs E SEUS EFEITOS BIOLÓGICOS ASSOCIADOS



Nota: A representação para EPC indica a proteína C endotelial e a EPCR indica o receptor de proteína C endotelial. As moléculas de adesão expressas pelo endotélio são externalizadas pelas MPEs, tais como PCAM-1, VCAM-1, E-selectina, S-Endo, VE-caderina. As moléculas de função e ativação endotelial como a glicoproteína endoglina e eNOS também são representadas. Moléculas responsáveis pela angiogênese, remodelamento da membrana e fibrinólise também podem ser encontradas, tais como T-caderina e VE caderina. E por fim as moléculas presentes no processo de coagulação sanguínea ativador do plasminogênio da uroquinase (uPA), receptor do ativador do plasminogênio da uroquinase (uPA) e as metaloproteases (MMP).

FONTE: Adaptado de Dignat-George and Boulanger (2011).

3.4. O PAPEL DAS MPEs NA DISFUNÇÃO ENDOTELIAL E DRC

O estado do endotélio vascular pode ser refletido pela correlação do número de MPEs circulantes e os níveis de marcadores inflamatórios como, por exemplo, a interleucina-6 (IL-6). Os níveis séricos de MPEs podem estar intimamente associadas ao risco do desenvolvimento de trombos, por apresentarem durante a formação componentes citoplasmáticos e fosfolipídios carregados negativamente e com potencial pró-coagulante. As MPEs carreiam o fator de Von Willebrand, responsável por promover a agregação plaquetária, além de possuir receptores em sua superfície que podem atuar sobre células pró-inflamatórias e plaquetas intensificando o processo de coagulação (MALLAT et al., 1999; SHANTSILA, 2009).

O aumento, composição e a atividade biológica das MPEs são alteradas devido a alta exposição à glicose, sendo diretamente associadas ao aumento de complicações cardiovasculares (BURGER et al., 2017). Em pacientes portadores de diabetes mellitus (DM) foi observado que o nível plasmático de MPEs é significativamente mais alto do que na população saudável e está relacionado com complicações provenientes da disfunção endotelial e doenças vasculares associadas ao DM (DENG; WANG; ZHANG, 2017).

Na doença renal crônica (DRC) os níveis de MPEs aumentados está fortemente correlacionado com a ativação da via do complemento e as lesões vasculares e renais, que podem desencadear doenças inflamatórias agudas como a síndrome hemolítica urêmica atípica. A produção de altos níveis de MPEs ativadoras do sistema complemento em pacientes renais pode estar associado a inflamação crônica na DRC (JALAL et al., 2018).

O nível de ativação endotelial das DRC e na DCV está relacionado com um alto nível de liberação de MPEs e podem refletir em disfunção ou dano ao endotélio. Em concordância com esses eventos os elevados níveis de MPEs estão associadas ao risco de eventos cardiovasculares e morte. O efeito biológico das MPE pode contribuir o desenvolvimento de eventos trombóticos em pacientes com DRC e pode ser um indicador importante entre a disfunção endotelial e os eventos pró-trombóticos nesses indivíduos (MÖRTBERG et al., 2019; SINAURIDZE et al., 2007).

3.5 INDUÇÃO DA FORMAÇÃO DE MPEs POR TOXINAS URÊMICAS

As toxinas urêmicas são responsáveis por induzir a formação de MPEs, que podem levar a ativação e disfunção de células endoteliais adjacentes ou de outros tipos celulares e modular a expressão de microRNAs, que desempenham um papel de grande relevância na regulação de processos celulares (DA CUNHA et al., 2020).

A toxina urêmica indoxil sulfato (IS) aumenta a liberação de MPEs. Essas MPEs derivadas da exposição ao IS (IsMPE) estão envolvidas na alteração do reparo do endotélio em pacientes com DRC. As IsMPE além de compartilhar as moléculas de suas células progenitoras, possuem em sua membrana externa um aumento de marcadores de adesão endotelial, ligação à anexina V e PECAM-1 (CARMONA et al., 2017).

Faure et al. (2006), em uma combinação de estudos *in vivo* e *in vitro*, demonstraram níveis circulantes aumentados de MPEs em pacientes em DRC pré-dialítica e em hemodiálise. Ainda, observaram que células endoteliais humanas quando em contato com PCS e IS, elevam a produção de MPEs, sugerindo que estas duas toxinas estão envolvidas a disfunção endotelial associada a toxicidade urêmica (FAURE et al., 2006).

Há evidências que as IsMPE influenciam de forma significativa a hiperplasia da camada neoíntima e a proliferação de células do tecido do músculo liso, através da fosforilação de moléculas como TGF-β e moléculas de junções em células de músculo liso vasculares (VSMCs) (JH; PARK; SJ, 2017). Mais recentemente estudos evidenciaram que células endoteliais humanas de veia umbilical (HUVECs) expostas a IsMPE sofrem senescência e promovem a calcificação em VSMCs, representando

um novo mediador de calcificação vascular no mecanismo de transição osteogênica, estando envolvidas na resposta inflamatória das VSMCs antes do desenvolvimento da calcificação vascular (ALIQUE et al., 2020).

Outras toxinas urêmicas são responsáveis pela indução da formação de microvesiculação de células endoteliais. Já foi relatado que micropartículas provenientes da ativação endotelial por *p*-cresil sulfato (PcsMPE) contribuem para a disfunção endotelial e progressão da doença cardiovascular (MEIJERS et al., 2009). Outros estudos demonstraram que o precursor de *p*-cresil sulfato (PCS), o *p*-cresol, está envolvido no aumento de micropartículas endoteliais (PcMPE), provocando a desregulação da homeostase vascular e reparo endotelial. A capacidade migratória das células é diminuída quando em contato com as PcMPE, a formação de novos vasos é comprometida, aumentando a senescência das células endoteliais maduras (GUERRERO et al., 2020).

O fosfato inorgânico em altas concentrações possui a capacidade de indução de microvesiculação endotelial (PiMPE) em artérias coronárias e células endoteliais de linhagem EA.hy 926. Uma maior quantidade de micropartículas positivas para anexina II também foi encontrada sob alta concentração de fosfato (DI MARCO et al., 2012). Pacientes com DRC avançada e hiperfosfatemia apresentam MPEs circulantes pró-coagulantes elevadas, o que leva a um estado pró-trombótico, que pode contribuir para eventos oclusivos agudos (ABBASIAN et al., 2015).

4. JUSTIFICATIVA

Na DRC, a exposição constante do endotélio às toxinas urêmicas tais como PCS, IS e Pi, pode levar a mudanças no fenótipo celular e na expressão de moléculas inflamatórias, com rápida degeneração do sistema cardiovascular e indução de MPEs. Dados da literatura já demonstraram que o PCS, IS e o Pi estão intimamente associados à mortalidade nestes pacientes, principalmente em decorrência de problemas cardiovasculares. As MPEs formadas a partir da indução de toxinas urêmicas vem sendo estudadas como potenciais biomarcadores de disfunção endotelial e complicações cardíacas. O mecanismo de formação de MPEs no ambiente urêmico ainda não foi completamente elucidado e neste contexto observou-se a necessidade de estudos que avaliem os mecanismos de formação de MPEs por toxinas urêmicas e a interação com leucócitos no processo de disfunção endotelial. Nosso estudo poderá contribuir para a elucidação dos processos da biogênese das MPEs induzidas pelo ambiente urêmico, bem como para a descoberta de biomarcadores de disfunção endotelial e ativação leucocitária, e consequentemente pode resultar na descoberta de novos alvos terapêuticos para a DRC.

5. CAPÍTULO 1: ARTIGO DE REVISÃO

toxins

MDPI

Review

Endothelial Microparticles in Uremia: Biomarkers and Potential Therapeutic Targets

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Abstract: Endothelial microparticles (EMPs) are vesicles derived from cell membranes, which contain outsourced phosphatidylserine and express adhesion molecules, such as cadherin, intercellular cell adhesion molecule-1 (ICAM-1), E-selectin, and integrins. EMPs are expressed under physiological conditions and continue circulating in the plasma. However, in pathologic conditions their levels increase, and they assume a pro-inflammatory and pro-coagulant role via interactions with monocytes; these effects are related to the development of atherosclerosis. Chronic kidney dysfunction (CKD) characterizes this dysfunctional scenario through the accumulation of uremic solutes in the circulating plasma, whose toxicity is related to the development of cardiovascular diseases. Therefore, this review aims to discuss the formation of EMPs and their biological effects in the uremic environment. Data from previous research demonstrate that uremic toxins are closely associated with the activation of inflammatory biomarkers, cardiovascular dysfunction processes, and the release of EMPs. The impact of a decrease in circulating EMPs in clinical studies has not yet been evaluated. Thus, whether MPs are biochemical markers and/or therapeutic targets has yet to be established.

Keywords: Endothelial microparticles; cardiovascular disease; uremia

Key Contribution: EMPs have an important role in mediating intercellular communication in the uremic environment. Therefore, this review addresses the current knowledge of EMP formation and internalization processes as well as its potential as a biochemical marker or therapeutic target in CKD.

1. General Concept of Microparticles

Microparticles (MPs) were first described by Wolf in 1967 [1], when he observed a halo of debris surrounding activated platelets that he termed 'platelet dust'. Since then, the available techniques for the detection of MPs have improved, and currently include flow cytometry, dynamic light scattering, nanoparticle tracking analysis, fluorescence correlation spectroscopy, immune blotting, mass spectrometry, transmission electron microscopy, and atomic force microscopy [2,3]. Unfortunately,

the lack of uniformity with regards to the nomenclature of MPs has in some way hindered a better understanding of their role in pathophysiologic processes. Actually, different terms, such as nanoparticles, MPs, exosome-like vesicles, liposomes, and prostasomes, have been used depending on the sample source or the protocol used to isolate the MPs. Besides, MPs are often described as exosomes, smaller (40–100 nm) particles of endocytic origin, microparticles also known as microvesicles (100–1000 nm), from the reorganization of the plasma membrane, and apoptotic blebs (50–5000 nm) released by dying cells [2,4].

Cells release a variety of extracellular vesicles, including exosomes, MPs, and apoptotic bodies. MPs are found in the plasma and in other biological fluids of healthy individuals and their levels are altered in a pathological state. MPs present in the plasma are derived from several types of cells, such as endothelial cells, platelets, monocytes, neutrophils, and T-cells. They are vesicles derived from plasma membrane remodeling, virtually released by all cells in response to injury, apoptosis, or cellular activation. MPs are closely correlated with physiological processes. They may participate in the intercellular communication that helps in the maintenance of homeostasis under physiological conditions, or may initiate a deleterious process, e.g., an immune response, in the event of infection or in the presence of pathogens by transporting pathogenic constituents [5–9].

In general, MPs expose specific molecules to the parental cell. Depending on their origin, they may contain signaling molecules, including receptors, cytokines, mRNA, micro-RNA, and bioactive lipids [8,10]. The plasma membrane has a lateral organization of lipid raft domains that confers plasticity to the membrane. These lipid rafts are specialized regions of the cell membrane enriched with lipids and cholesterol, which allows them to be more rigid than the rest of the membrane. Lipid rafts also have the function of organizing the proteins into microdomains, transduction of signals, and transport via caveolin. This organization promotes the generation of unique responses resulting in the inclusion or exclusion of specific proteins and lipid species in cells, thereby explaining the different intravesicular compositions of MPs of the same cellular origin [6,11].

The release of MPs may induce cell signaling or may lead to the transfer of receptors between different cell types, since they carry parts of their cells of origin in their own membrane. This process occurs through the binding of the MPs to the membrane of the recipient cells; this binding may be of two types: (i) a ligand-receptor or (ii) a cell-adhesion interaction with subsequent MP internalization. These characteristics allow MPs to be able to mediate long-range signaling, which explains why they have been considered as emerging biomarkers for the diagnosis of various pathologies [5,10,12]. It has been demonstrated that circulating red cell MPs may be a potential blood biomarker for the differentiation between acute graft-versus-host disease and infection or sepsis after hematopoietic stem cell transplantation [13,14]. In recent years, the MPs from endothelial cells (EMPs) have been studied as endothelial injury markers, mainly in patients with cardiovascular disease, including the ones with chronic kidney disease (CKD) [15].

When exposed to a stimulus, endothelial cells can release MPs; endothelium-specific proteins, including endothelial cell-specific adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, E-selectin, P-selectin, and platelet endothelial cell adhesion molecule, are found in these MPs [7,12]. Experimental data have shown that TNF-α stimulates human endothelial cells to increase the production of MPs expressing annexin-V, contain calcium and high levels of bone morphogenic protein-2, and are correlated with vascular calcification and osteogenic differentiation [16]. Burger et al. [17,18] showed that EMPs induce cell-to-cell signaling responses leading to inflammation, oxidative stress, and apoptosis [17,18]. High blood EMP concentrations found in some pathological conditions have been associated with inflammation and angiogenesis. EMPs may also contribute directly and indirectly to the blood coagulation cascade through the tissue factor (TF) [19–21].

2. Mechanisms of EMP Formation

Studies have shown that several compounds, such as TNF-α, glucose (high concentrations), thrombin, angiotensin II, uremic toxins (Figure 1), and others [22–24], are capable of inducing MP

formation by endothelial cells. In addition, physical aspects such as shear stress can also lead to the release of MPs [25]. The lipid bilayer of the cell membrane is asymmetric due to the activity of transmembrane proteins such as flippases and floppases. The interior of the lipid bilayer is rich in phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PhtdSer), while the outer side is rich in phosphatidylcholine and sphingomyelin. Cell activation from a stimulus or apoptosis leads to the loss of membrane asymmetry and cytoskeleton rearrangement, a process mediated by the increase in the intracellular levels of calcium released from the endoplasmic reticulum [7].

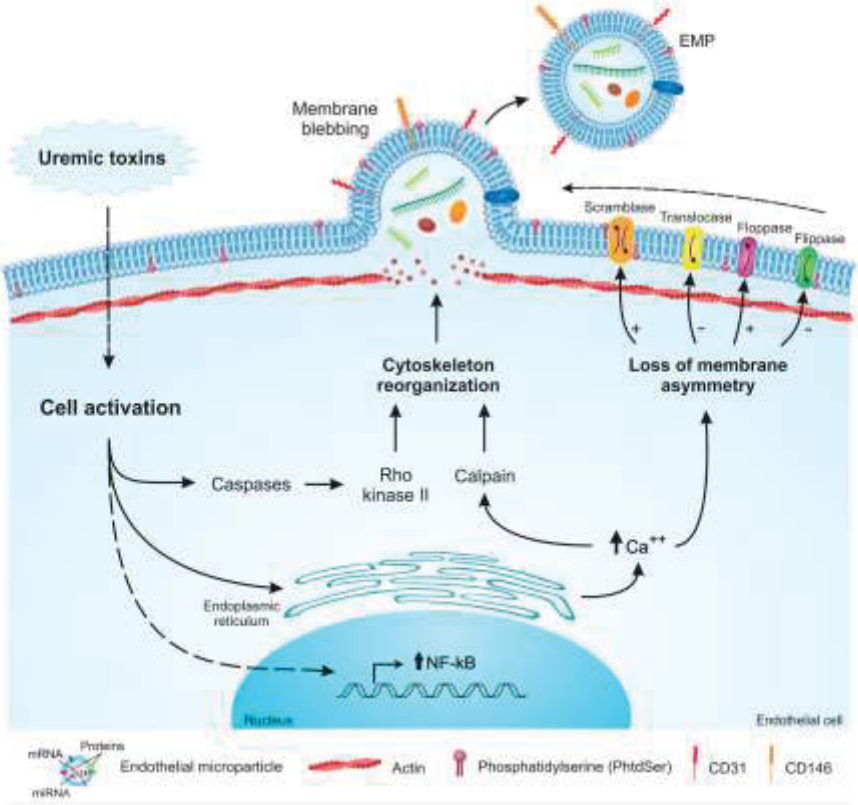

Figure 1. Schematic mechanisms of endothelial microparticle (EMP) formation induced by uremic toxins. Uremic toxins induce endothelial cell activation. This process activates the caspases, and consequently, rho kinase II, which leads to the reorganization of the cytoskeleton. Cell activation induces the release of calcium from the endoplasmic reticulum. The intracellular increase of calcium activates calpain, which in turn, induces the reorganization of the cytoskeleton. Calcium also leads to the activation or inhibition of proteins responsible for the maintenance of membrane asymmetry, causing the loss of this asymmetry. Cell activation elevates NF-κB expression. These processes cooperatively promote membrane blebbing and EMP formation.

The increase in intracellular calcium deposition leads to the activation of receptors, which induces the release of calcium from the endoplasmic reticulum and the activation of caspases. The calcium flux activates calpain and phospholipase A2 [26]. Calcium is responsible for modulating the activity of two enzymes present in the plasma membrane, scramblase and aminophospholipid translocase,

which results in the externalization of PhtdSer and activates cytosolic enzymes such as calpain [7,27,28]. The activation of caspases triggers Rho kinase II and MAP kinase signaling pathways, which contribute to cytoskeletal remodeling, enabling the formation of MPs [22,23].

Laminar shear stress also leads to the formation of EMPs. Atheroprone low shear stress conditions seem to increase the activity of Rho kinases and the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), resulting in increased MP generation, while the inhibition of these pathways reduces the formation of EMPs. In contrast, endothelial cells exposed to atheroprotective high shear stress conditions produce nitric oxide (NO), which downregulates the expression of ABCA1 flippase, a protein that modulates the membrane distribution of PhtdSer, and therefore, limits the formation of EMPs [25].

3. Characterization of EMPs

3.1. EMP Characterization by Flow Cytometry

MPs can be isolated from blood circulation or cultured cells, such as endothelial cells (Figure 2). Flow cytometry is the most widely used method for characterizing MPs; however, some functional assays for MP analysis and characterization based on coagulation activation are also available [29]. Although flow cytometry provides useful information, some limitations of this method, such as low threshold for particle size detection, need for standardized instrument settings, and the requirement for appropriate antibodies against cell-associated antigens that are not expressed by other cell lineages, should be considered. For these reasons, the isolation of MPs from whole blood is a multi-step procedure, and many different process-dependent variables have been shown to affect the characterization and analysis of MPs. The outer leaflet of the MP membrane may express PhtdSer, which is a procoagulant phospholipid recognized by annexin-specific receptors [5]. The annexin binds to PhtdSer on the surface of the MPs [30]. Additionally, endothelial cells show a high expression of $CD31^+$ and $CD144^+$ [31]. Together, $CD31^+$, $CD144^+$ and annexin V^+ expression have been described to enable the characterization of EMPs by flow cytometry.

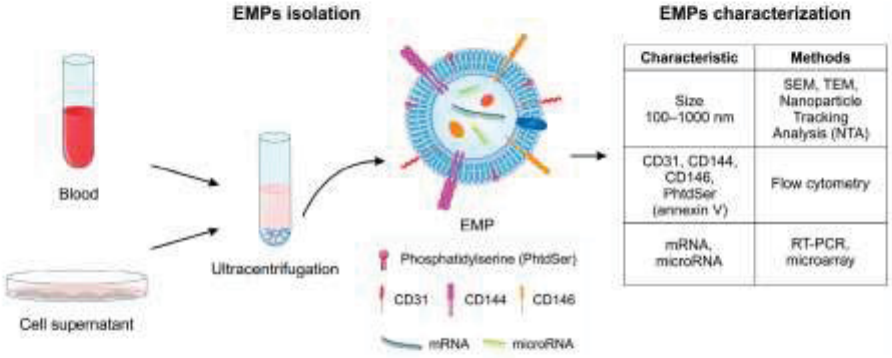

Figure 2. Schematic mechanism of the isolation and characterization of endothelial microparticles (EMPs). EMPs can be isolated from the blood or cell supernatants by ultracentrifugation. The EMPs can be characterized by their size (100–1000 nm), presence of PhtdSer in the membrane, and protein and nucleic acid compositions.

Platelet-poor-plasma (PPP) obtained from citrated whole blood must be first centrifuged for 15 min at 500× *g* in order to obtain platelet-rich plasma (PRP); then this PRP must be centrifuged for 5 min at 14,000× *g* to pellet the platelets and obtain PPP. Then 50 µL from PPP must be incubated with 4 µL (the concentrations have been optimized by the titration of each reagent) of monoclonal antibodies against $CD31^+$ and $CD144^+$, followed by incubation with the annexin kit reagents, according to the manufacturer's instructions, for 30 min in the dark at room temperature. After incubation, 450 µL of

buffered saline solution (HBS; 20 mM HEPES, 150 mM NaCl, 2.5 mM calcium) must be added into the tube. The isotype antibodies must be used as negative controls.

It is important to note that this method is common to characterize EMP. However, to obtain purified apoptotic bodies marked by Annexin V from EMP, it is necessary to use flow cytometric-based cell sorting. This methodology has the benefit of selecting individual particles of interest [32].

3.2. Sorting of EMP

Cell sorting allows the investigator to analyze quantitatively several fluorescence and light scattering parameters of individual particles and to purify those events with the desired characteristics for further study. No other technology can separate a heterogeneous cell suspension into purified fractions containing a single cell type with the speed and accuracy of high-speed cell sorters. For the sorting of EMPs, 250 µL PPP must be stained with $CD31^+$, $CD144^+$, and Annexin V^+ and the corresponding isotype and negative controls. Stained plasma must be incubated for 45 min in the dark at room temperature according to the manufacturer's suggestions. To sort EMPs, it is necessary to use flow cytometry cell sorting equipment. Vesicles between 100–1000 nm in diameter and stained with $CD31^+$, $CD144^+$, and Annexin V^+ must be gated for sorting.

3.3. EMP Characterization by Electron Microscopy

Electron microscopy (EM) is currently used as a gold standard to characterize the morphology of microparticles, allowing the identification and measures of size of all different classes of extracellular microparticles. EM has a resolution around 0.5 nm, smaller than the size of the exosomes (40–100 nm), allowing a detailed visualization of the structural information of the MPs. EM provides semiquantitative information of the sample and it is not suitable for phenotyping. Another disadvantages are the changes in the characteristics of the MPs caused by vacuum procedures and standard dehydration procedures in EM, [3,10,33]. Variants of electron microscopy have been used to study the ultrastructure of MPs.

Scanning electron microscopy (SEM) uses beams to scan the entire surface of the sample, generating topographic information. Sampling for SEM is simple when compared to other microscopy techniques. Samples should be fixed and followed by gradual dehydration with alcohol, followed by spraying a thin gold conductive layer to generate the images [33]. SEM imaging is carried out using 5 kV acceleration and a secondary electron (SE) detector. For SEM imaging, several randomly selected frames from each sample are captured for morphological observation and statistical purposes [34]. Figure 3 demonstrates EMP SEM.

Transmission electron microscopy (TEM) has the ability to characterize a single MP, providing information of biochemical and surface protein properties of the sample, and it is used to detect particles with 1 nm image resolution. It is the most widely used instrument to monitor the quality and purity of MPs-containing samples [35,36]. The disadvantages of TEM and SEM methods are that the preparation of samples is time-consuming, the MPs visualization can only be distinguished by their size and morphology, and by surface protein biochemical properties [37].

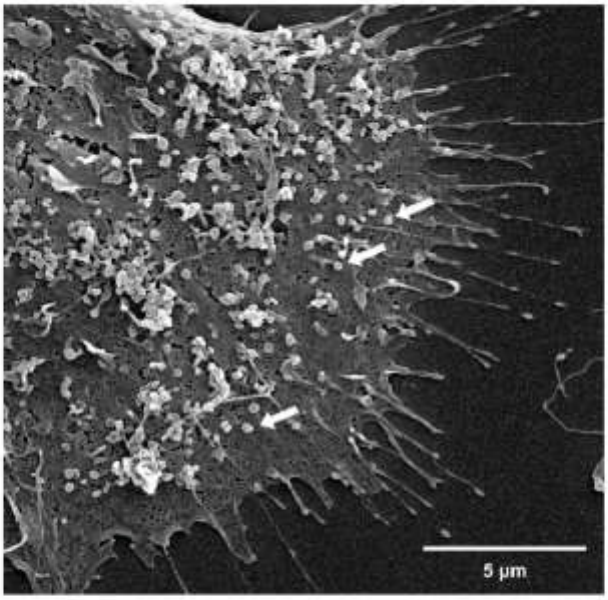

Figure 3. Scanning electron microscopy (SEM) showing endothelial cell microparticles on its surface. Arrows show microparticles. Magnitude: 15,000×.

3.4. Nanoparticle Tracking Analysis (NTA)

Another commonly used method to directly detect MPs is NTA, a real-time method visualization that analyzes the nanoparticles in liquids in order to determine the size of MPs by light scattering using a light microscope. This method is used for sizing particles from 30 to 1000 nm. Brownian motion of the microparticles is individually screened and is dependent of the diffraction index of the submicron vesicles. Several videos are recorded, from which NTA software calculates, according to the Stokes–Einstein equation (Einstein relation), the size and total concentration of MPs present in the sample [3,38].

NTA has been gaining popularity in the MPs retraction analysis. Recently, Wang et al. [39] described novel methods to purify and detect MPs shed from endothelial cells and endothelial progenitor cells by combining microbeads with fluorescence quantum dots (Q-dots) coupled with nanoparticle tracking analysis (NTA). These novel methods provide ideal approaches for functional analysis and biomarker discovery [39]. Another study developed by Dragovic et al. [40] demonstrated that NTA is able to analyze total cellular MPs in human plasma using a fluorescent quantum dot-labeled cell tracker peptide [40]. Weber et al. [41] demonstrated that the characterization of individual MPs present in human whole blood showed a high level of reproducibility by fluorescence-based nanoparticle tracing analysis when compared to other methods and could be adjusted for characterization of MPs from cell culture supernatants [41].

4. Internalization and Signaling Pathways Induced by EMPs

EMPs interact with target cells (recipient) through membrane proteins and phospholipids [42]. This interaction allows the activation of membrane receptors and the internalization of the EMPs, which entails the transfer of active biomolecules and other contents into a recipient cell. In these cells, EMPs can lead to the activation of signaling pathways that result in changes of the cellular phenotype. In fact, studies have demonstrated that EMPs induce biological effects on recipient cells, such as endothelial

cells, vascular smooth muscle cells (VSMCs), fibroblasts, monocytes, and others [11,43–45]. Among these biological effects, endothelial dysfunction induced by EMPs from endothelial cells exposed to indoxyl sulfate, a protein-bound uremic toxin, may be highlighted [43]. Although MPs have been considered an emerging topic in recent years, only a few studies have been devoted to EMPs [2,20].

MPs may possibly use more than one pathway to enter the recipient cells; however, studies have suggested that endocytic pathways are the main mechanisms of MP uptake. Thus, endocytosis inhibitors may be used to reverse or attenuate the biological effects induced by EMPs [46]. The internalization of MPs can occur by different mechanisms, such as caveolin-, clathrin-, or lipid raft-dependent endocytosis, phagocytosis, macropinocytosis, and direct membrane fusion (Figure 4). Each of these internalization mechanisms occur in a variety of different ways, particularly with regards to the proteins involved in each process. Caveolin-dependent endocytosis involves small invaginations in the membrane called caveolae; the protein caveolin-1 plays a crucial role in this process. It was observed that EMPs released by activated cells had an attenuated effect on caveolin-1 knockout endothelial cells or after treatment with dynasore, an inhibitor of caveola formation, compared to the case in wild-type endothelial cells [11]. Clathrin-dependent endocytosis is a process that involves the invagination of the membrane by accessory proteins, followed by the formation of clathrin-coated pits [46]. Similarly, lipid raft-dependent endocytosis involves membrane invaginations mediated by clathrin, caveolin, or other proteins in small regions rich in cholesterol, protein receptors, and sphingolipids [47]. Furthermore, phagocytosis is characterized by the formation of membrane extensions that surround the MPs to be internalized [48]. Macropinocytosis results from the rearrangement of the cytoskeleton, thus leading to the formation of membrane ruffles and allowing the internalization of extracellular materials such as MPs [49]. All these endocytic mechanisms result in the formation of a vesicle that merges with an intracellular compartment, in which membrane fusion and the release of the contents from MPs into the recipient cell could occur [50]. The mechanism by which this membrane fusion occurs is unclear, but an acid environment seems to contribute with this process [51]. Direct fusion of the MP membrane with the recipient cell is not so frequent; studies suggested that pH of the microenvironment may also contribute to this fusion [51–53].

Studies have identified several proteins that are important for the internalization of EMPs. However, the complete protein content of MPs remains difficult to be established. More than 300 proteins have been reported by proteomics, some of which are cytosolic and some membranous, and are dependent of the cell type [54]. The MP phenotypes vary according to cellular origin and parental cell response to stimulus [55,56]. Proteomic analysis of EMPs from starved endothelial cells demonstrated the presence of annexin I, an intracellular protein that is translocated to regions in the membrane that are rich in PhtdSer following apoptotic stimuli. Annexin I interacts with the PhtdSer receptor (PSR) expressed on endothelial cells, allowing the entry of EMPs into the target cell. The silencing of annexin I or PSR with small interference RNA significantly reduced the incorporation of EMPs by the recipient cells [30]. Another study demonstrated that the internalization of EMPs by endothelial cells involves proteins such as $\alpha_v\beta_3$ integrin and lactadherin [42]. In addition, EMPs can bind to components of the extracellular matrix, including fibronectin, to which it binds through interaction with the α_v integrin. EMPs also activate matrix metalloproteinase-2, whose activity is important for vascular matrix remodeling [57].

The activation and incorporation of EMPs leads to a response by the recipient cells, such as inflammation and oxidative stress, leading ultimately to cellular dysfunction. EMPs released by TNF-α-treated endothelial cells induce the activation of the nuclear-factor kappa-b (NF-κB), a transcription factor involved in the expression of several pro-inflammatory molecules. An increased phosphorylation of the p65 fraction of NF-κB by lung endothelial cells after 30 min of exposure to EMPs from TNF-α-treated cells has been described. The expression of genes regulated by NF-κB, such as intercellular adhesion molecule-1 (ICAM-1), increased. An increase in the phosphorylation of epidermal growth factor receptor (EGFR), whose inhibition attenuated the activation of NF-κB, was observed [11]. EGFR also participates in the response of endothelial cells exposed to EMPs

generated from angiotensin II-treated cells. In this case, both a higher production of reactive oxygen species (ROS) and expression of inflammatory molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and CD31, were observed [58]. Moreover, EMPs produced after treatment with high glucose concentrations promoted the expression of von Wille-brand factor on the surface of endothelial cells; this contributed to a greater interaction between platelets and the endothelium [42]. Finally, a greater level of phosphorylation of ERK1/2 and Src has been reported in endothelial cells exposed to EMPs, suggesting that the activation of these pathways may also play a part in the response of cells to EMPs [18].

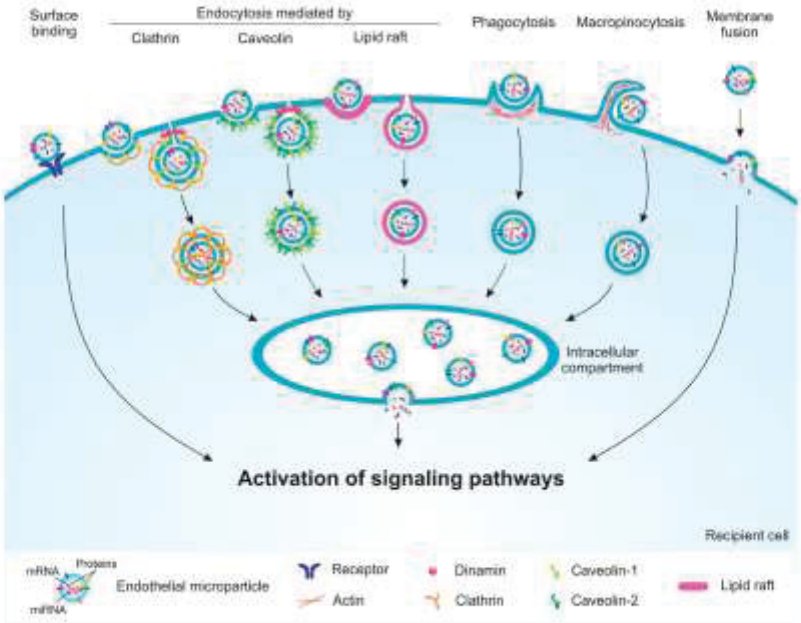

Figure 4. Schematic mechanisms of the internalization of endothelial microparticles (EMPs) by recipient cells. EMPs can interact with the surface of recipient cells through receptors, leading to the activation of signaling pathways. The uptake of the EMPs by the recipient cell can occur through clathrin-, caveolin-, and lipid raft-mediated endocytosis, phagocytosis, and macropinocytosis. These processes lead to the formation of vesicles that fuse with an intracellular compartment and induce the activation of various signaling pathways. The direct fusion between the membranes of the EMPs and the recipient cell may also occur.

The type of stimulus that leads to EMP production seems to be important for the activation of signaling pathways in the recipient cell. In fact, in vitro analyses have demonstrated that EMPs could attenuate the endothelial inflammation induced by TNF-α. In this case, EMPs derived from starved cells transfer into a recipient cell, a functional microRNA-222 that can reduce the ICAM-1 expression. Interestingly, EMPs derived from cells treated with high concentrations of glucose contain lower amounts of microRNA-222, and therefore do not affect the ICAM-1 expression [59].

The role of EMPs on endothelial dysfunction and vascular inflammation has been studied in vivo. For this purpose, EMPs released from cells exposed to high glucose concentrations were administrated to apolipoprotein-E knockout mice in order to simulate diabetes. It was demonstrated that ICAM-1 and VCAM-1 expression was induced, resulting in increased macrophage infiltration in the vessel walls, which is a well-recognized factor linked to the pathogenesis of atherosclerosis. In agreement with these

findings, in vitro studies demonstrated increased ROS production and the increased phosphorylation of p38, a signaling protein involved in endothelial activation [60]. Other studies have also shown that EMPs induce ROS production in endothelial cells [18,60–62]. This increase is explained, at least in part, by the activation of the NADPH oxidase pathway [18]. Moreover, the production and the bioavailability of NO is reduced, which has an effect on endothelium-dependent vasorelaxation [18,61]. Otherwise, it was recently described that EMPs from TNF-α-treated cells may have a protective effect against palmitate-induced oxidative stress in endothelial cells [63]. These findings suggest that EMPs could have an ambivalent role depending on the microenvironment.

EMPs can also modulate immune response processes such as monocytic activation. It has been demonstrated that EMPs, released from quiescent cells, have the miR-10a transferred to the monocytes, which results in the inhibition of the NF-κB pathway in this cell type [64]. This shows that the bioactive molecule composition of the EMPs is important for the activation or suppression of signaling pathways. In vitro studies have also shown that EMPs derived from TNF- α-treated endothelial cells are capable of increasing the production of the tissue factor-dependent procoagulant activity in monocytes [45]. EMPs may also influence the activation of plasmacytoid dendritic cells, by increasing the expression of the inflammatory cytokines interleukins-6 (IL-6) and IL-8 [65]. Moreover, Wheway et al. [66] demonstrated that EMPs interact by releasing their content into $CD4^+$ and $CD8^+$ cells, increasing cell proliferation. This study also showed that EMPs express important molecules for the activation of T cells, such as CD40, ICOSL, and MHC II, which suggests that EMPs could modulate the cellular response [66].

5. Uremia and EMPs

In CKD settings, it has been shown that the generation of MPs may not only be a consequence of this disorder, but also be a major cause of the onset of pathological processes [16,67]. The constant injury of the endothelium promoted by inflammation, uremic toxins, and other mechanisms induce endothelial dysfunction, being responsible for the production of MPs and consequent vascular calcification [68,69]. In fact, several studies have demonstrated that uremic toxins such as phosphate, *p*-cresol, *p*-cresyl sulfate, indoxyl sulfate, and homocysteine are closely correlated with the induction of MPs [69–74].

Calcium and phosphate demonstrated to be present in uremia-related endothelial dysfunction in CKD patients, and are also directly associated with high circulating levels of EMPs [69]. Hyperphosphatemia caused by the intracellular accumulation of phosphate in patients with CKD is considered a crucial factor for the development of cardiovascular disease; it may lead to a greater increase of circulating MP levels [72,73]. Intracellular accumulation of phosphate mediated by PiT1/slc20a1 transporters resulted in increased membrane blebbing and an increase in the release of MPs. Pi-induced MPs have procoagulant properties and are involved in vascular and thrombotic events [72].

P-cresyl sulfate increases endothelial permeability and is closely correlated with the Rho kinase protein, which is responsible for the reorganization of the cytoskeleton and consequent MP formation [27,74,75]. *p*-Cresol and *p*-cresyl sulfate are associated with endothelial dysfunction and the release of MPs, with serum *p*-cresol levels being independent of the number of circulating MPs in patients with CKD and patients undergoing hemodialysis. In contrast, *p*-cresyl sulfate (0.1, 0.5, and 1 mmol/L) induces dose-dependent MP formation in vitro [69]. Endothelial cells treated with p-cresol (40 μg/mL) and indoxyl sulfate (256 μg/mL) in the free fraction and protein-bound in the presence of human serum albumin (HAS) have an important impact on endothelial activation and the in the release of EMPs [68].

Indoxyl sulfate is capable of activating the endothelium, thus inducing MPs in the circulation, it also alters the endothelial repair process in patients with CKD, and pathologies associated with endothelial dysfunction, such as CKD and antiphospholipid syndrome. Indoxyl sulfate-induced (256 μg/mL) MPs have miRNA and other endothelium-characteristic molecules that participate in signaling pathways involved in oxidative stress and cellular apoptosis, hindering endothelial regeneration [43]. Indoxyl sulfate-induced EMPs influence the neointimal hyperplasia and smooth muscle cell proliferation

through phosphorylation of TGF-β downstream molecules in VSMCs. Therefore IS-induced (250 μg/mL) EMPs have a critical role in not only stimulating the TGF-β signaling pathway in VSMCs, but also in neointimal formation [76].

EMPs released by cells exposed to indoxyl sulfate (250 μg/mL) promoted functional loss in endothelial progenitor cells, which are bone marrow-derived cells with angiogenic properties that are important for endothelial repair. These EMPs can modulate the classic endothelial roles of progenitor cells as colony forming units and form new vessels, as well as increase the expression of NF-κB and p53 by the endothelial progenitor cells in vitro. Indoxyl sulfate is capable of inducing endothelial vesiculation with different membrane characteristics, miRNAs and other molecules, which makes maintaining of vascular homeostasis of endothelial progenitor cells not fully functional [43]. Clinical studies have also associated the increase of EMPs with the reduction of endothelial progenitor cells in patients with CKD [44,77].

High levels of EMPs in chronic uremic renal patients (CRI) in non-dialyzed or hemodialyzed (HD) patients. Elevation of EMPs can be compared to other vascular pathologies mediated by uremia and consequent endothelial dysfunction [68]. EMPs seem to participate in the disruption of vascular homeostasis promoted by uremic toxicity, which could contribute to uremia-related cardiovascular disease. Actually, EMPs isolated from CKD patients induced the expression of osteocalcin, an osteogenic protein linked to vascular calcification, in endothelial progenitor cells, VSMCs, and fibroblasts [44].

6. Microparticles and Cardiovascular Disease

Endothelial cells play an important role in the development of cardiovascular diseases in response to activation and release of MPs. Several studies have demonstrated the association between high circulating levels of MPs and cardiovascular events inflammation-related. MPs are involved in thrombosis, angiogenesis, autophagy, cell survival, and apoptosis, which are important events in homeostasis and in progression of cardiovascular diseases [12,78]. Indeed, it was demonstrated that MPs are capable of mediating long-range signaling, acting on different targets from those of their own cellular origin. Depending on their cellular origin and signaling, MPs may exert different stimuli on vascular endothelial cell [67,73,78]. As a matter of fact, Faure et al. [68] demonstrated that patients who do not have a history of cardiovascular disease have relatively similar levels of EMPs compared to patients with a history of cardiovascular disease. The study did not exclude the possibility that high levels of EMPs in patients with uremia is a result of vascular diseases, since it is already well established that patients with CKD have an accelerated progression of atherosclerosis related to endothelial dysfunction [68].

Patients with age-related cardiovascular diseases such as congestive heart failure, coronary artery disease, peripheral vascular disease, and cerebral ischemia have an increased number of circulating MPs [21]. In atherothrombotic cardiovascular diseases, increased levels of MPs derived from platelets, endothelial cells, monocytes, granulocytes, and red blood cells may be detected [27].

MPs originating from monocytes, platelets, and lymphocytes induce endothelial dysfunction by reducing NO levels and increasing oxidative stress, activate proinflammatory cytokines such as IL-1, IL-6, and IL-8, and may interact with adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 [79]. EMPs selectively affect the signal transduction pathway and the release of NO by endothelial cells, which leads to the generation of cyclical guanosine monophosphate (cGMP) in VSMCs; such cellular processes are important for endothelium-dependent vascular relaxation. Circulating EMPs may act as specific inhibitors of the synthesis of NO by endothelial cells, through negatively affecting the acetylcholine-induced release of cGMP, an important compound for the biosynthesis of NO [67].

Elevated levels of circulating MPs have been detected in cardiovascular and immune-mediated diseases. MPs of patients with myocardial infarction induce endothelial dysfunction through the impairment of the NO pathway in endothelial cells, but without altering the expression of endothelial NO synthase (eNOS). However, this effect seems to depend on the cellular source of MPs. T cell-derived MPs may induce endothelial dysfunction by altering the expression of the eNOS and caveolin-1 genes.

In addition, MPs may promote the expression of proinflammatory proteins involved in changes in vascular contractility [80].

7. Therapeutic Interventions and Modulation of the Levels of MPs

Clinical and experimental evidence suggests the use of MPs as biochemical markers or therapeutic targets [81]. Although the amount of consistent data from clinical trials is relatively small, interesting studies have suggested the use of polyphenolic compounds, simvastatin, kidney transplantation, immunosuppressant drugs, and convective hemodialysis as potential strategies for MP modulation [82,83].

Ammollo et al. [82] investigated the influence of polyphenolic compounds (flavonoids, phenolic acid, resveratrol) on MPs from whole blood. For 3 weeks, grapes (5 g/kg/day) or placebo were offered to 20 and 10 healthy volunteers, respectively. After 3 weeks, grape consumption was associated with a decrease of thrombin generation, decreasing the number and activity of procoagulant MPs. The antithrombotic effect was sustained after 4 weeks of washout time. Based on these findings, it was suggested that the grape compounds sustained anticoagulant and profibrinolytic effects through the modulation of procoagulant MPs [82].

Almquist et al. [84] tested the effects of simvastatin alone or with ezetimibe on MPs from platelets, monocytes, and endothelial cells for 8–10 weeks, in a cohort of 39 patients with diabetes mellitus with or without CKD. In 18 patients who had diabetes mellitus and CKD stages 3–4 the levels of almost all types of MPs tested were elevated, when compared to diabetic patients without CKD. Administration of simvastatin (40 mg daily) was associated with the reduction of the procoagulant effects of MPs in the diabetic CKD patients, while the combination of simvastatin and ezetimibe had no further effect on the levels of MPs. These results suggest that simvastatin might have beneficial action on hypercoagulability in this high-risk population by modulating the MP concentration [84].

Kidney transplantation and immunosuppressive therapy also seem to play a role in regulating the levels of MPs. Al-Massarani et al. [85] studied the effects of two different immunosuppressive regimens on endothelial biomarkers, including EMPs, in 52 patients who underwent kidney transplantation, in reference to 50 healthy subjects as the controls. They found a favorable impact of kidney transplantation after 12 months on endothelial markers, expressed among other factors, through the reduction of the levels of EMPs, which reached normal values. Of note, cyclosporine microemulsion/azathioprine seems to have more pronounced positive effects on the concentration of EMPs, when compared to tacrolimus/mycophenolate mofetil [85].

Ramirez et al. [86] investigated whether on-line hemofiltration would be an efficient strategy to remove uremic toxins, including EMPs in comparison to high-flux hemodialysis. In this study, 15 stable patients on high-flux hemodialysis were switched to on-line hemofiltration for 4 months and thereafter switched back to high-flux hemodialysis. A decrease in the numbers of endothelial MPs and endothelial progenitor cells was observed during the on-line hemofiltration treatment period, which signals the attenuation of endothelial damage. Interestingly, after returning to the previous therapy, their levels increased their basal values. These observations are consistent with those of a prospective crossover study by Ariza et al. [83], which demonstrated lower levels of apoptotic EMP, when comparing post-dilution high convective transport techniques and high-flux hemodialysis.

8. Conclusions

MP formation has been widely studied in several physiological and pathological conditions such as coagulation disturbances, diabetes, hypertension, cardiovascular diseases, uremia, and others. Their formation is complex and involves an increase in the intracellular calcium deposition, which in turn increases the release of MPs from several types of cells, including endothelial cells. Endocytic pathways are the main mechanisms of MP uptake by a variety of other recipient cells; it is possible that MPs use more than one pathway to enter the recipient cells. In the intracellular space, MPs activate several inflammatory and oxidative stress pathways, ultimately leading to cellular dysfunction.

In uremic patients, a constant attack on the endothelium by uremic toxins, such as the protein-bound p-cresyl sulfate and indoxyl sulfate and inorganic phosphate, induces the release of EMPs from endothelial cells; however, the details of this mechanism have not been fully elucidated. Current evidence, though limited, has shed some light on the possible strategies to reduce EMP release, which seems to share the common pathway of ameliorating the uremia-related pro-inflammatory state. It is noteworthy that the impact of reducing the levels of EMPs on clinical outcomes has not yet been evaluated. Whether MPs are biochemical markers or therapeutic targets remains to be established.

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6. CAPÍTULO 2: ARTIGO EXPERIMENTAL

Formation of endothelial microparticles by uremic toxins, p-cresyl sulfate, Indoxyl sulfate, inorganic phosphate and their role in endothelial dysfunction

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OBS: Este trabalho ainda passará por revisão de inglês

Abstract

p-Cresyl sulfate (PCS), indoxyl sulfate (IS), and inorganic phosphate (Pi) are common uremic retention solutes found in chronic kidney disease (CKD). PCS, IS, and Pi are closely associated with the activation of inflammatory biomarkers, cardiovascular dysfunction, and the release of endothelial microparticles (EMPs). EMPs are vesicles derived from cell membranes, which in homeostatic disorders increase and assume a proinflammatory and procoagulant role related to atherosclerosis development. The present study aims to understand the mechanisms of formation and internalization of EMPs in uremia through the exposure of human endothelial cells to PCS, IS, and Pi. In this scenario, human endothelial cells were treated with PCS, IS, and Pi in pre-provided uremic and kinetic recommendations. EMPs were characterized by electron microscopy (SEM), flow cytometry, and Nanosight assays. To establish a concentration of use, the Alamar Blue assay was performed, and the MTT assay gave the cell viability. Cell adhesion to extracellular matrix proteins (EMC) was analyzed for cell adhesion assay. Vascular cell adhesion molecule (VCAM-1) was analyzed by immunocytochemistry and the production of reactive oxygen species (ROS) by DCFH-DA probe. Endothelial cells and monocytes migration were investigated by Wound Healing and cell migration (Boyden chamber) assays, respectively. Our data showed that endothelial cells stimulated with uremic toxins can form EMPs of different sizes, quantities, and concentrations depending on the uremic toxin. With the Alamar Blue test, it was possible to standardize the work concentration of 10 µg/mL of EMPs. Cell adhesion was compromised to form in cells exposed to microparticles from PCS (PcsEMP) and inorganic phosphate (PiEMP). VCAM-1 expression was evident in cells treated with EMPs from control cells (CtrlEMP), PcEMP, and IS (IsEMP). Through the Wound Healing assay, it was possible to observe an endothelial cell increased migration in cells treated with PcsEMP compared to CtrlEMP cells (**P*<0.05). There was no significant difference in the migration test performed for monocytes. Finally, we found that EMPs do not play a significant role in oxidative stress. We conclude that EMPs may be a biomarker of endothelial injury and the inflammatory process. More studies should be carried out to unveil the mechanisms of formation and the role of EMPs in uremia.

Keywords: Endothelial microparticles, uremic toxins, uremia, leukocyte activation.

1. INTRODUCTION

There has been a greater interest in elucidating the negative impact of uremic toxicity in patients with CKD in recent years. The elimination of endogenous and exogenous substances by the kidney is essential for maintaining the organism's homeostasis. Kidney excretion of these substances is closely linked with physiological events, such as filtration, secretion, and absorption (Barreto et al. 2014). The uremic syndrome results from the malfunction of the body's systems due to the retention of organic compounds, which would be excreted or metabolized by the kidneys under normal conditions. These biologically active compounds are referred to as uremic toxins (Vanholder et al., 2018). In CKD, the constant exposure of the endothelium to high concentrations of uremic toxins such as PCS, IS, and Pi is believed to lead to changes in cell phenotype, increased generation of reactive oxygen species (ROS), expression of inflammatory molecules, with rapid degeneration of the cardiovascular system and, besides, the increase in the number of circulating endothelial microparticles (EMPs) associated with vascular calcification and cardiovascular disorders (Faure et al. 2006; Buendía et al. 2015; Cunha et al. 2020).

Like most cells, endothelial cells, in response to vascular activation or apoptosis, release different types of membrane vesicles, including microparticles (MP) and exosomes. These different vesicles are distinguished from each other based on their cellular origin, size, content, and formation mechanism. EMPs, specifically, have a size ranging from 0.1 μM to 1 μM, most express phosphatidylserine and carry endothelial proteins such as cadherin, platelet endothelial adhesion molecule, intercellular cell adhesion molecule-1 (ICAM-1), E-selectin, integrins, among others (Favretto et al., 2019). EMPs are complex vesicular structures of activated or apoptotic endothelial cells containing cell surface proteins and cytoplasmic elements and express specific surface markers of the cells they originate. They play a notable role in coagulation, inflammation, endothelial function, and angiogenesis, thereby disturbing vascular homeostasis, contributing to vascular diseases' progression (Curtis et al., 2013; Markiewicz et al., 2013). Currently, EMPs have been used as circulating markers to represent different endothelial cell injuries (Favretto et al., 2019).

Increased circulating microparticles, mainly originating from endothelial cells and platelets, have been associated with cellular dysfunctions (Guerrero et al., 2020).

MP of various cellular (i.e., platelet, leukocyte, and endothelial) origins are found in healthy individuals' circulating plasma and may be increased under pathological conditions (Paudel et al., 2016; Carmona et al., 2017a). EMPs represent a small part of total circulating MPs; however, they believe that the EMPs behave as diffusible vectors of biological activities, participating in exchanges of information between circulating cells and the endothelium. Currently, EMPs not only constitutes an emerging marker of endothelial dysfunction but also plays an essential role in inflammation, vascular injury, angiogenesis, and thrombosis, with elevated levels of EMPs being found in pathologies such as disorders vascular and hypercoagulability, diabetes, antiphospholipid syndrome, and cardiovascular diseases (Chironi et al. 2009; Dignat-George and Boulanger 2011; Favretto et al., 2019).

The circulating MPs stimulate procoagulant activity, directly linked to the formation of coagulation complexes and consequently associated with coronary syndromes' development. It was demonstrated that patients in the final stage of CKD have high plasma levels of circulating EMPs compared to healthy individuals (Carmona et al., 2017a; Jalal et al., 2018). Faure et al. (2006), in their research, demonstrated increased circulating levels of EMPs in patients on predialytic CKD and hemodialysis. Furthermore, they observed that human endothelial cells, when in contact with PCS and IS, increase the formation of EMPs, suggesting that these two toxins are involved in endothelial dysfunction associated with uremic toxicity (Faure et al., 2006). In addition to these findings, Sabatier et al. (2002) demonstrated that tumor necrosis factor (TNF)-stimulated EMPs binds to monocytes (THP-1 cells) and induces a procoagulant state in these cells; such activity involves the interaction between ICAM-1 on the surface of EMPs and integrins on the surface of monocytes (Sabatier et al., 2002).

EMPs play an essential role in cellular communication, mediating physiological and pathological processes, including developing cardiovascular diseases that commonly affect patients with CKD. However, little is known about the role of EMPs in uremic conditions and their impact on the systemic endothelium and monocytes in CKD. Therefore, the present study aims to isolate and characterize EMPs from cells treated with PCS, IS, and Pi uremic toxins and evaluate their effect on endothelial dysfunction and leukocyte activation. A better understanding of the mechanisms

involved in forming EMPs in uremic conditions and their cellular effects may contribute to discovering new biomarkers and therapeutic targets in CKD.

2. MATERIALS AND METHODS

2.1. Endothelial Cell Culturing

An immortalized human endothelial cell line EA.hy926 (CRL-2922, ATCC, Manassas, VA, USA) was used. This cell line is derived from a fusion of a human vein endothelial cell (HUVEC) with an A549 tumor epithelial cell resulting in a hybridoma (Edgell et al. 1983). This cell line retains critical features of differentiated endothelium and is considered an authentic model for examining systemic endothelial dysfunction. Cells were cultured in DMEM (Life Technologies, New York, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA) and 10 mg/mL of penicillin/streptomycin (Gibco, Grand Island, USA) and maintained at 37 ºC in a humidified atmosphere containing 5% CO_2.

2.2. Uremic toxins preparation

For the concentrations used in our experiments, we referred to the list of uremic toxins provided by the European Uremic Toxin Work Group (EuTox- http://eutoxdb.odeesoft.com/index.php). Therefore, we studied PCS, IS, and Pi, respectively, at maximum uremic concentrations (2.6 mg/L, 236 mg/L, and 3 mM).

2.3. Induction of EMPs formation with uremic toxins

Endothelial cells were washed with HHBS buffer (10 mM HEPES, 140 mM NaCl, pH 7.4) twice and incubated for 3 hours in MEM 199 medium without phenol red and SFB at 37 °C in an oven containing 5% CO_2. In this same medium were added 2 µM calcium ionophore (Control), and the induction of EMPs occurred in the presence of different concentrations of uremic toxins described above. After this period, EMPs purification was performed.

2.4. Purification of EMPs by differential centrifugation

After the treatment of the endothelial cells, the EMPs were obtained by differential centrifugation. First, the cells were centrifuged at 500 g for 5 min. The supernatant was removed to a new tube and centrifuged twice at 6.000 g, for 30 min at each centrifugation to remove cell debris. Purification of EMPs was performed by supernatant centrifuging at 100,000 g for 1 h and 30 min. The pellet containing the microparticles was resuspended in HEPES buffer and subjected to the Bradford method's total protein dosing with the Bio-Rad Protein Assay kit (Bio-Rad, California, USA). After dosing, the EMPs were stored at - 80°C.

2.5. Quantification of EMPs by Flow Cytometry Analysis

Endothelial cell microparticles (EMPs) were incubated with monoclonal antibodies conjugated to their respective fluorochromes: CD31 Phycoeritrin (PE), CD144 AlexaFluor 647 (AF 647), CD41 Fluorescein isothiocyanate (FITC) and Annexin V FITC in 100 µl of PBS/$CaCl_2$, for 35 minutes, at 4 ºC in the dark. After incubation, 200 µl of PBS/$CaCl_2$ were added. Quantification and characterization were performed by flow cytometry (FacsCanto - BD Biosciences, San Diego, USA) immediately or within 2 h after the markings. In this condition, the samples remained refrigerated and in the dark.

2.6. Characterization of EMPs by Flow cytometry analysis

A frontal scatters plot (FSC) versus lateral scatter plot (SSC) was used, where the EMPs were selected by size ranging from 100 to 200 nm and labeled by Annexin V $^{+}$ -FITC (gate-P1). EMPs were quantified and characterized by double labeling of $CD31^{+}$ and $CD144^{+}$ antibodies. From this selection, the autofluorescence regions (background) of the EMPs without the labeling of monoclonal antibodies were defined to be eliminated from the analyzes. For EMPs acquisition, a condensation adjustment of the samples was performed. This procedure was performed to minimize the interference of the light emission captured by the different fluorochromes used in the analysis. From the SSC versus FSC plot, 30,000 events were acquired for quantification and characterization of EMPs. The analysis was

performed in FacsDiva software (BD, USA), using dot plot type graphs. The EMPs characterization was expressed as a percentage (%).

2.7. Characterization of EMPs by the NanoSigth assay

EMPs were characterized using the NanoSight NTA 3.0 (NTA technology - Nanoparticle Tracking Analysis, Amesbury, United Kingdom) and an sCMOS camera. The data were collected at 20 ºC and analyzed 26.13 frames per second with measurements every 30 seconds. Using the property of light scattering and Brownian motion, the particle size distribution and its concentration in particles/mL were determined. EMPs were suspended in HBSS solution and applied to the apparatus with a 1 mL syringe aid. For control (white), the HBSS was used, and the samples were read in triplicate. The particle size distribution determined the final result as a function of concentration.

2.8. Scanning Electron Microscopy (SEM)

For SEM, 10^5 cells previously treated with uremic toxins PCS, IS, and Pi at maximum uremic concentrations (2.6 mg / L, 236 mg / L, and 3 mM) and 2 µM Ca_2 were fixed on microscope slides for 1 h in Karnovski's solution (2% glutaraldehyde, 4% paraformaldehyde, 1 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer) and post-fixed with 1% osmium tetroxide for 1 h The cells were dehydrated by increasing concentrations of ethanol (30%, 50%, 70%, 90% and twice with 100%) and subsequently subjected to the critical point in CPD 010 (Critical Point Dryer), gold plated in the SCD 030 device (Balzers Union, Liechtenstein). The images were obtained with a scanning electron microscope JEOL JSM 6360 - LV (Jeol USA Inc., Peabody, USA). Three independent experiments were carried out in triplicate.

2.9. Determination of EMP concentration by Alamar Blue assay

To determine the concentration of the EMPs used in the treatments, the Alamar Blue cytotoxicity test was performed, whose active ingredient is resazurin (7-hydroxy-10-oxophenoxazin-10-ium-3-one) (Invitrogen, Carlsbad, California, EUA). Resazurin has a non-fluorescent blue color when reduced by cellular metabolism. However, resofurin has a fluorescent pink color and does not precipitate. Initially,

endothelial cells were plated in 96-well plates at a density of 10^4 cells per well. After 24 h of incubation, the medium was replaced, and the cells were treated with 5 µg, 10 µg, and 15 µg of CtrlEMP, PcsEMP, IsEMP, and PiEMP. After 24 h of incubation with the EMPs, the treatment was removed, the cells were washed with PBS, and 50 µL of the Alamar Blue reagent solution was added to a well at a concentration of 10% about the volume of the MEM-ALFA and DMEM media present in the wells (v/v), according to the manufacturer's recommendations (Alamar Blue® Cell Viability Reagent). Then it was kept in an incubator with 5% CO_2 for 4 h at 37 °C. After this period, the presence of viable cells was detected through fluorescence filters with 570 nm (emission) and 600 nm (excitation) in a microplate reader (Microplate Reader F5, Molecular Probes).

2.10. Cell viability assay

Cell viability was assessed using the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, USA) assay, according to Mosmann (1983). For this, endothelial cells were plated in a 96-well culture plate at a density of 10^4 cells per well. After 24 hours of incubation, the medium was replaced, and cells were treated with 10 µg EMPs derived from treatment with uremic toxins. Culture medium was then replaced with a fresh medium (100 µL/well), and 10 µL of MTT solution (5 mg/mL) was added to each well. The plate was left in the incubator for another 4 h. The culture medium was subsequently removed and replaced by dimethylsulfoxide (DMSO) to dissolve the crystals of reduced formazan (700 rpm, 15 min), and absorbance was measured at 570 nm (Bio-Rad, CA, USA).

2.11. Cell adhesion assay

Endothelial cells were treated with CtrlEMP; PcsEMP; IsEMP and PiEMP after 24 hours of exposure were imposed on the cell adhesion assay. For that, different extracellular membrane proteins (MEC), such as fibronectin (FN), Laminin (LN) and Vitronectin (VN), were used at a concentration of 10 µg/mL, adding 50 µL per well. As a positive control, adhesion to the polymer it self (substrate of the 96-well plate) was

used. As a negative control, bovine albumin (1% BSA) was used. The plate remained for 2h in the incubator with 5% CO_2 at 37ºC. Subsequently, the plate was washed with sterile PBS, and the blocking of specific sites was performed with 1% BSA for 1 h in the incubator with 5% CO_2 at 37ºC. After blocking, the treated cells were plated at a concentration of $2x10^4$ cells per well and were kept for 2 h in the incubator with 5% CO_2 at 37ºC. After 2 h of incubation, the cells were fixed with methanol for 10 min and stained in the sequence with 0.8% violet crystal dissolved in 20% methanol for 20 min. The plate was washed with 1x PBS and the violet crystal was eluted with 0.1 M sodium citrate in 50% methanol for 30 min. The absorbance reading was performed in a plate reader with a 540 nm filter. The test was carried out in three independent experiments in triplicate.

2.12. VCAM-1 immunostaining assay

For VCAM-1 immunostaining assay, 3x104 cells/well were plated on coverslips in a 24-wells plate. When the cells reached about 80% confluence, they were treated with CtrlEMP; PcsEMP; IsEMP, and PiEMP at a concentration of 10 µg for 24 h. Afterward, the cells were fixed with 2% paraformaldehyde for 20 minutes, permeabilized with Triton X-100 for 5 minutes, and incubated with 3% hydrogen peroxide for 30 minutes (to suppress endogenous peroxidase activity). The blockade was performed with 0.5% BSA, incubation with the primary antibody (Primary R&D Systems, Minneapolis, MN, USA) at a 1:100 dilution for 24 hours at 4 °C and then with the secondary antibody (anti-sheep) labeled with peroxidase (HRP) at 1:150 dilution for 1 h at 25ºC. The reaction was developed with the Vector Red kit (Vector Laboratories, CA, USA). The coverslips were mounted on microscope slides with Entellan (Millipore Merck, Darmstadt, Germany). The images were made in light microscopy (AxioImager Z2, Carl Zeiss, Jena, Germany) coupled with an automated slide scanner (Metasystems, Altlussheim, DE) 100X magnification. Three experiments were carried out in triplicate.

2.13. Wound Healing Assay

The wound healing test is used to evaluate cell migration and validate molecules that may interfere with the migration process. This test was carried out to analyze

an action by EMPs on the migration of endothelial cells. For that, the endothelial cells were trypsinized, counted, and added $5{,}10^4$ cells per well in 24-well plates. Upon reaching the 70% confluence, cells were treated with 10 µg metformin for 24 hours to interrupt the cell cycle. After the 24-hour incubation of the wells, they were washed with PBS 1X, and the wound was made (streak), using 10 µg tips with the aid of a ruler. The wells were washed once more with PBS, and the treatments (10 µg/mL de CtrlEMP, PcsEMP, IsEMP, and PiEMP) were added in DMEM medium. In the controls, only DMEM medium was appropriate. The plates were photographed at 0 h and 24 h, or until the wound was closed entirely.

2.14. Cell Migration Assay

The cell migration assay was performed in a Boyden chamber with a polycarbonate filter free of polyvinylpyrrolidone with a porosity of 5 µm, which was used to separate the upper and lower compartments (GE Healthcare Life Sciences do Brasil Ltda.) (Leavesley et al., 1993; Li; Zhu, 1999). The chemoattractive effect under monocytes (U937) was evaluated from the deposition of culture medium containing endothelial microparticles from CtrlEMP, PcsEMP, IsEMP, and PiEMP at a concentration of 10 µg. A standard solution of 50 ng/mL MCP-1 was used as a positive control. The monitoring of monocytes' spontaneous movement (negative control) was performed with the DMEM medium's addition in the lower compartment of the chamber. Subsequently, the chamber's upper compartment was filled with 200 µL of monocyte suspension at 10^6 cells/mL on the filter. The chambers were then incubated in an oven at 37 ºC and 5% CO_2 for 90 minutes. The chemoattractive capacity of the supernatant (migration) was measured by counting the number of cells in the lower compartment with the Neubauer chamber's aid (Perretti et al., 2002). Five independent experiments were carried out.

2.15. ROS Measurement

ROS was measured using a 2', 7'-dichlorofluorescein diacetate (DCFH-DA) probe. The endothelial cells were transferred to 96-well plates (2.5×10^3). After 24 h of culture, they were treated with PcsEMP, IsEMP, and PiEMP at a concentration of 10 µg, with or without N-acetyl-L-cysteine (NAC, 2 mM), an antioxidant is known to reduce ROS

levels for 72 h. Subsequently, the cells were washed with 1x PBS and marked with 1 μM DCFH-DA for 30 min at 37 ° C. The cells were again washed twice with PBS, and fluorescence was measured immediately using a ROS spectrophotometer (λEx 504 nm, λEm at 524 nm). Data were expressed, showing the percentage increase in fluorescence compared to untreated cells (control).

2.16. Data analysis

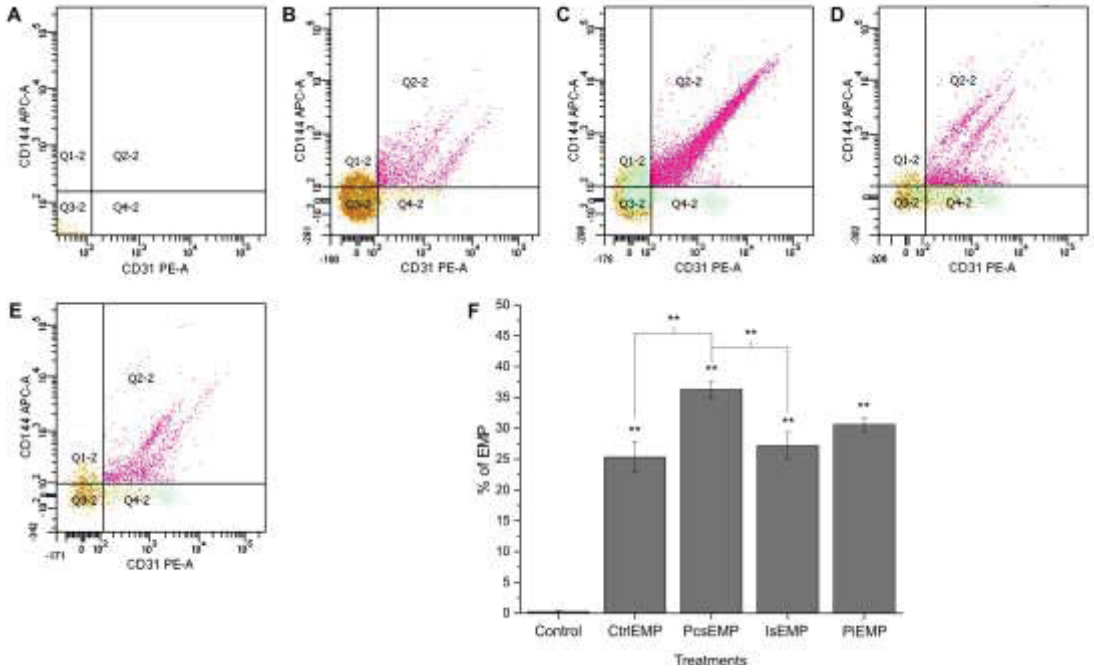
Statistical analyses were performed using the statistical packages JMP (version 8.0; SAS Institute Inc., Cary, N.C., USA) and SigmaStat (version 3.5; Systat Software Inc., Erkrath, Germany). Determination of significant differences was performed by Student's *t*-test or analysis of variance (ANOVA) for paired data and Mann-Whitney and ANOVA on Rank's for unpaired data. Values were expressed as mean ± standard error of the mean (SEM) of three or five independent experiments. $P<0.05$ was considered statistically significant.

3. RESULTS

3.1. EMPs quantification by flow cytometry

The quantification of the EMPs attainment was evaluated by flow cytometry. It was observed that all fluorescent events marked with CD144 - Alexa Fluor + and CD31 - PE + (markers that characterize EMPs) corresponded to the quadrant Q2-2 (Figure 1).

Figure 1 – Quantification of EMPs by flow cytometry

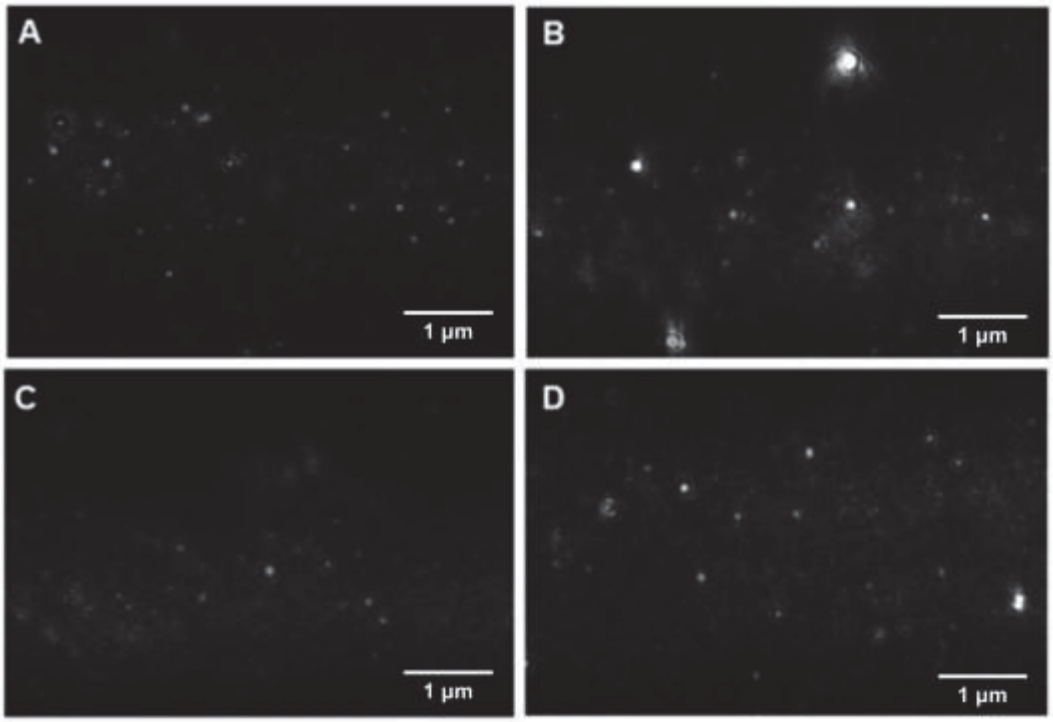


Note: Percentage of EMPs characterized by fluorescence markers with CD144 - Alexa Fluor + and CD31 - PE + and induced by 2 μM Ca^{2+} and uremic toxins at maximum uremic concentration. (a) negative control (Ctrl) (EMP from endothelial cells without Ca^{2+} induction), (b) CtrlEMP (EMPs from endothelial cells with Ca^{2+} induction), (c) PcsEMP (EMPs from PCS treatment (2.6 mg/L) and Ca^{2+} induction), (d) IsEMP (EMPs from treatment with IS (236 mg/L) and Ca^{2+} induction), (e) PiEMP (EMPs from treatment with Pi (3 mM) after Ca^{2+} induction), (f) Graph relative to% of obtaining EMPs. ** *P*<0.01: Control vs CtrlEMP, PcsEMP, IsEMP, PiEMP; PcsEMP vs CtrlEMP, IsEMP.

3.2. Characterization of EMPs by NanoSigth

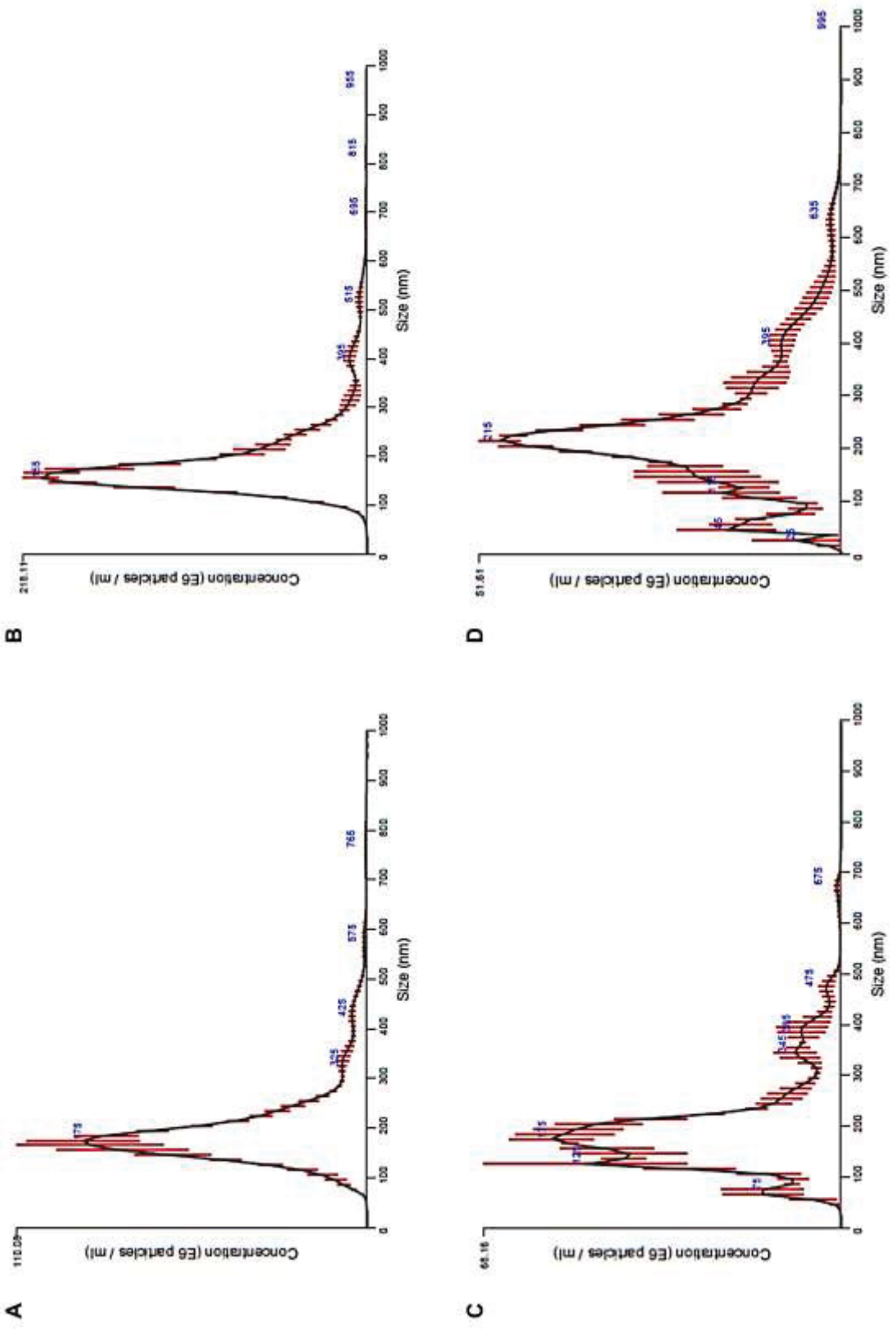
For the characterization and identification of the EMPs concentration, the nanosight test was performed. Figure 2 corresponds to one of the frames registered to obtain the size of the EMPs using the NTA 3.0 software coupled to the microscope. The average diameter of EMPs was 175 nm for CtrlEMP, 155 nm for PcsEMP, 175 nm for IsEMP, and 215 nm for PiEMP. The analysis of size distribution and concentration of EMPs are shown in Figure 3. The concentration of purified microparticles obtained for CtrlEMP were 110.08 x 10^6 particles/mL, PcsEMP 436.22 x 10^6 particles/mL; IsEMP 136.32 x 10^6 particles/mL, and PiEMP 103.22 x 10^6 particles/mL.

Figure 2 – Characterization of EMPs by NanoSigth



Note: Frames extracted from the recorded video for the EMPs analyzes. (a) CtrlEMP (EMPs from endothelial cells after Ca^{2+} induction), (b) PcsEMP (EMPs from PCS treatment (2.6 mg/L) and Ca^{2+} induction), (c) IsEMP (EMPs from treatment with IS (236 mg/L) and Ca^{2+} induction), (d) PiEMP (EMPs from treatment with Pi (3 mM) and induction Ca^{2+}).

Figure 3 – Distribution of EMPs size



Note: Distribution of EMPs size as a function of concentration. The analyzes were performed at 24ºC, totaling n = 3. Treatments with toxins have a dilution correction factor of 2. (a) CtrlEMP (EMPs from endothelial cells with Ca^{2+} induction), (b) PcsEMP (EMPs from PCS treatment (2.6 mg/L) and Ca^{2+} induction), (c) IsEMP (EMPs from treatment with IS (236 mg/L) and Ca^{2+} induction), (d) PiEMP (EMPs from treatment with Pi (3 mM) and induction Ca^{2+}).

3.3. EMPs and endothelial cells ultrastructural analysis by SEM

Figure 4 shows electromicrographs 1, 2, 3, 4, 5, and 6 control cells maintained in the cell culture only in the presence of culture medium, for a period of 3 h. In the higher magnitude images 4, 5, and 6, it was observed that the control cells produce and secrete EMPs. It was followed by a predominance of EMPs over the substrate; in image 5, the variation of the diameter of these is well evident. These cells secrete EMPs also by the membrane projections observed in image 6.

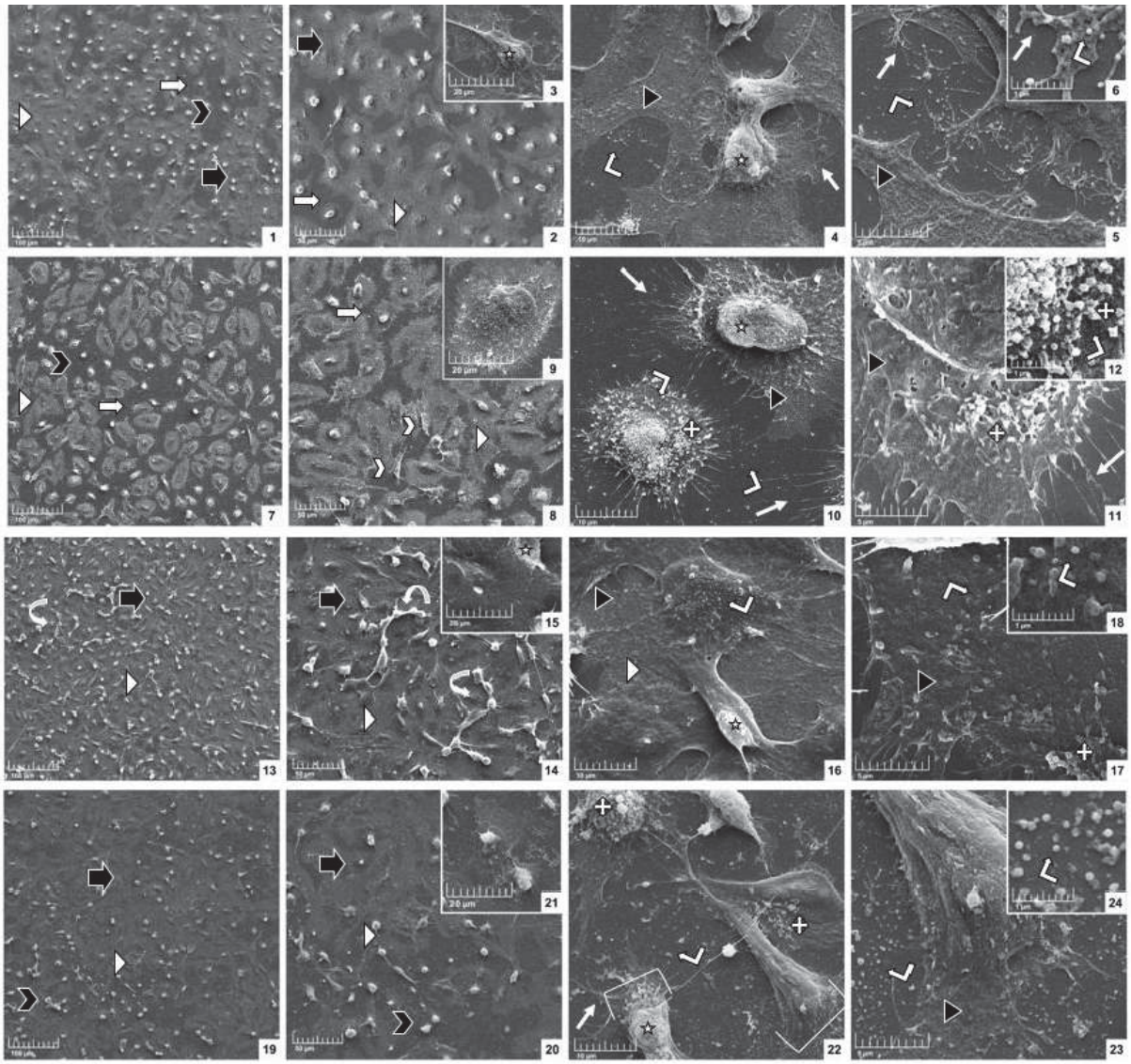
In the panoramic, image 7 showed cells treated with PCS. This treatment induced a precise morphological alteration. Two different morphological patterns are observed. In a larger magnitude 8, 9, and 10 images, it was observed that some of these cells present an evident detachment of the peripheral portion of the cell body. In images 10, 11, and 12, it is evident the increase of particulate material present on the body of these cells, possibly blabes, EMPs, and exosomes (images 10 and 12). On the substrate are seen micro-small molecules which are being secreted by the cell body.

Images 13, 14, 15, 16, and 17 show cells exposed to IS. In the panoramic image 13, it was observed that these cells are smaller and alter the cell culture pattern; there seems to be the largest number of cells. Image 14 and 16 show that these cells, even when adhered and sprayed, are looser in the substrate and emit projections of the cell body, presenting well-distant morphologies. In larger magnitude16, 17, and 18 images, EMPs appear to be smaller in size than the control cell and exposure to PCS, possibly exosomes. These EMPs may be viewed in larger magnitude image 18 being secreted from the cell body.

Images 20, 21, 22, 23, and 24 show cells exposed to Pi. In the panoramic image, the visualization of morphological and ultrastructural alterations is not clear when compared with the control cells. In larger magnitude images 21, 22, and 23,

the ultrastructural changes become evident; these cells present morphologically, more elongated. This treatment by these images 23, 24, and 25 induced greater production of microvesicles of different diameters. There is a large number of microvesicles above the cell body, above the membrane projections, and secreted into the substrate.

Figure 4 – Morphological and ultrastructural analysis of endothelial cells exposed to uremic toxins



Note: Morphological and ultrastructural analysis of human endothelial cells, controls, and exposure to uremic toxins for 3 h in scanning electron microscopy. Control cells were maintained only in culture medium images 1, 2, 3, 4, 5, and 6. PCS (2.6 mg/L) and 2 μM Ca^{2+} exposed cells, images 7, 8, 9, 10, 11 and 12. Cells exposed to IS (236 mg/L) and 2 μM Ca^{2+}, 13, 14, 15, 16, 17 and 18. Cells exposed to Pi 3 mM and 2 μM Ca^{2+}, images 19, 20, 21, 22, 23 and 24. The panoramic images 1,7,13, and 19 are in the 1,000X magnification, the images 2, 8, 14, and 19, increase of 3,000X, respective highlights 3, 9, 15, and 21, increase of 5,000X. Images 4, 10, 16, and 21 increase by 10,000X. The images 5, 11, 17and 23, increase of 20,000X, respective highlights 6, 12, 18 and 24, increase of 60,000X. Six- sample tests.

This arrowhead identifies cells in confluence.

This arrow identifies cells in subconfluence.

This arrow identifies adhered, spiked cells with contact inhibition, with monolayer formation.

This arrow identifies cells with distinct morphologies, with significant expansion of the cell body, well evident and prominent nucleus (*).

This arrowhead identifies rounded cells detaching from the characteristic cell substrate with loss of cell viability.

This arrow shows characteristic branched, arborescent membrane projections, the filopodia, structures of perception.

This arrow shows EMPs over the substrate, with a variation of the diameter. It is also present in the membrane expansions.

This arrow indicates cells with detachment from the peripheral portion of the cell body.

This arrow indicates an increase in particulate matter over the cell body, possibly blabes, EMPs, and exosomes.

This arrow indicates juxtaposed cells forming a monolayer with contact inhibition. It is observed a more significant number of cells, suggesting cellular proliferation.

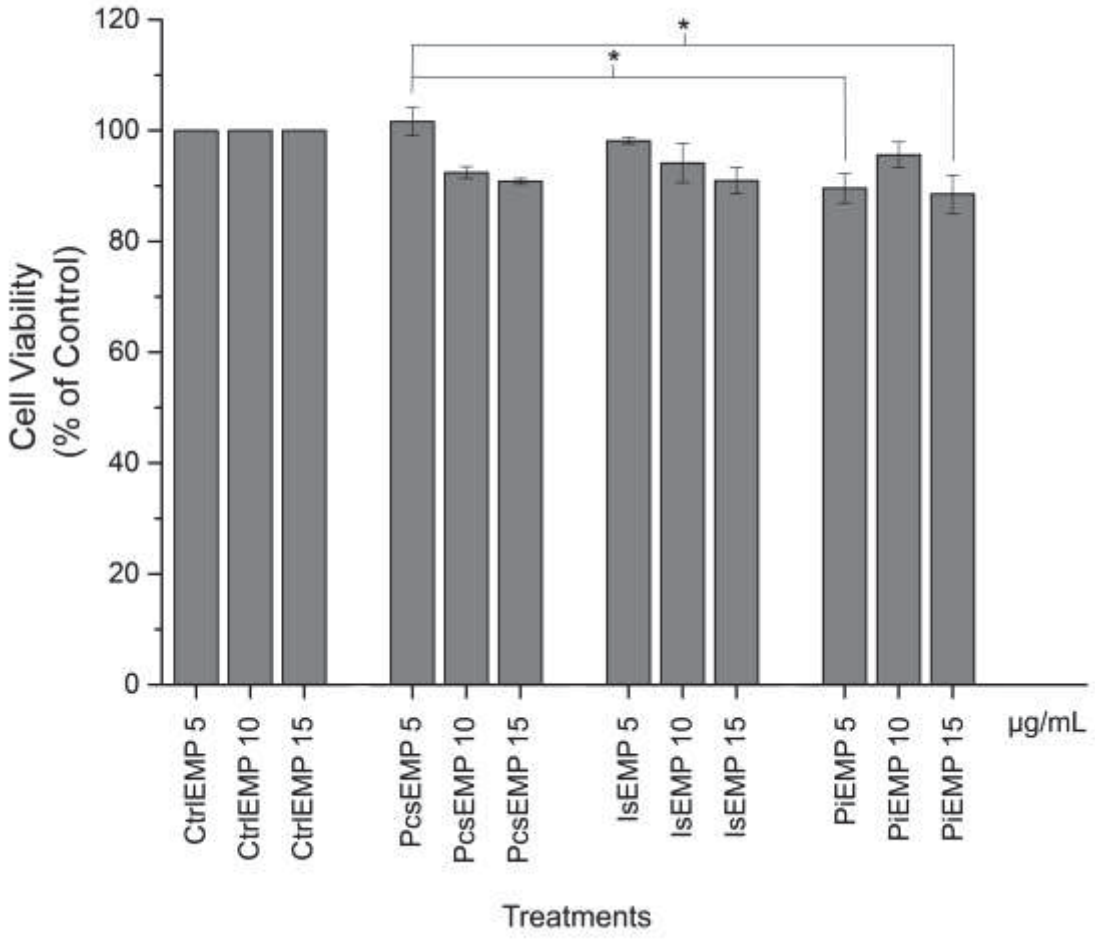
We evidenced characteristic lamellipodium, visible structure when that in migratory cells

3.4. Determination of EMPs concentration by Alamar Blue Assay

Alamar Blue assay was used to obtain the concentration of EMPs to be used to treat the endothelial cells. This test measures the mitochondrial metabolic activity and the ability to treat to react with EMPs and reduce resazurin and resorufin (Figure 5). Endothelial cells treated with CtrlEMP were considered as control, expressed as 100%. Cells treated with 5 μg/mL, 10 μg/mL, and 15 μg/mL did not suffer a reduction in mitochondrial metabolic activity compared to control cells.

However, the metabolic activity of cells exposed to PcsEMP 5 µg/mL was completed greater than cells exposed to PiEMP in the configurations of 5 µg/mL and 15 µg/mL (P<0.05).

Figure 5 – Effect of EMPs on endothelial cells mitochondrial metabolic activity

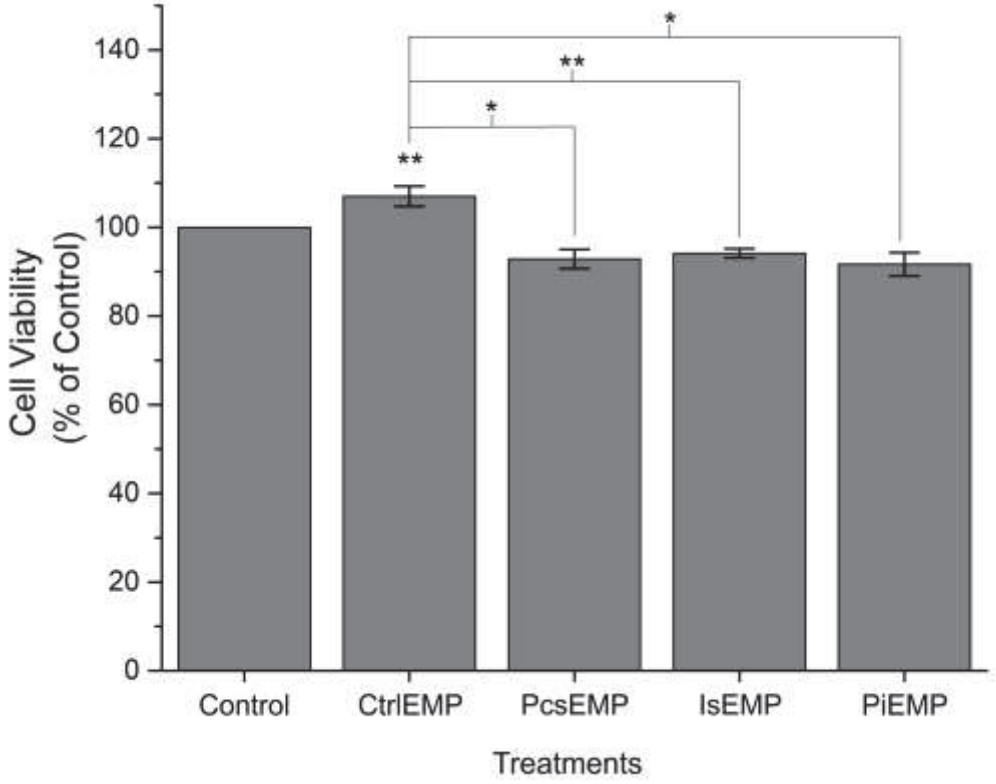


Note: Control: cells treated with 5 µg/mL, 10 µg/mL and 15 µg/mL of CtrlEMPs as cells were treated with control induced by EMPs; PcsEMP 5 µg/mL, 10 µg/mL and 15 µg/mL: the cells were treated with PCS induced by EMPs; IsEMP 5 µg/mL, 10 µg/mL and 15 µg/mL: the cells were treated with IS induced by EMPs; PiEMP 5 µg/mL, 10 µg/mL and 15 µg/mL: the cells were treated with Pi induced by EMPs. *P<0.05: PcsEMP 5 µg/mL *vs* PiEMP 5 µg/mL, PcsEMP 5 µg/mL *vs* PiEMP 15 µg/mL.

3.5. Cell viability assay

The viability of cells treated with culture media (control) was considered to be 100%. Data were expressed as SEM from 5 independent experiments. There was an increase in cell viability when the cells were exposed to CtrlEMP (***P*<0.01), however, there was a significant decrease in cell viability in cells treated with PcsEMP, IsEMP, and PiEMP (**P*<0.05) when compared to CtrlEMP (Figure 6).

Figure 6 – Effect of EMPs in cell viability

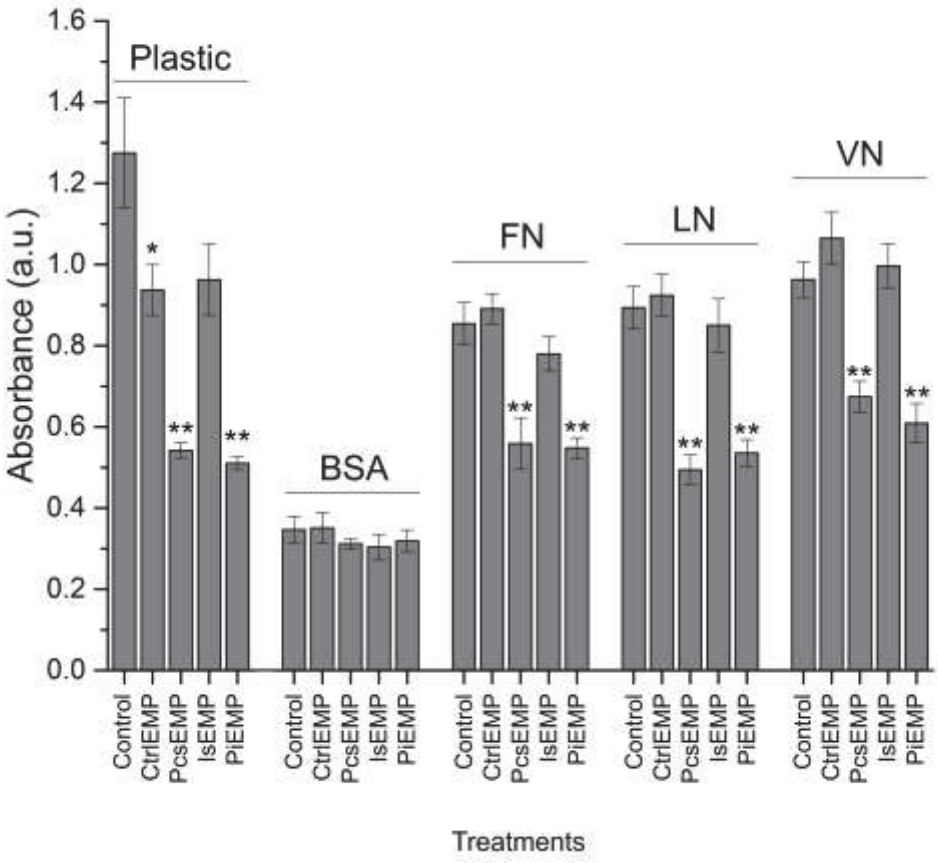


Note: Effect of EMPs on endothelial cell viability. Endothelial cells were cultured with EMPs for 24 h and then treated with MTT for 3 h. Cell viability was determined by measuring the absorbance at 570 nm. Viability of untreated control cells was taken as 100%. The expressed as mean ± SEM of 6 independent experiments. Control cells treated with culture media; CtrlEMP (EMPs from endothelial cells with Ca^{2+} induction), PcsEMP (EMPs from PCS treatment (2.6 mg/L) and Ca^{2+} induction), IsEMP (EMPs from treatment with IS (236 mg/L) and Ca^{2+} induction), PiEMP (EMPs from treatment with Pi (3 mM) and induction Ca^{2+}). ***P*<0.01: Control vs CtrlEMP; CtrlEMP *vs* IsEMP. **P*<0,05: CtrlEMP *vs* PcsEMP, PiEMP.

3.6. Cell adhesion assay

Endothelial cells previously treated with CtrlEMP, PcsEMP, IsEMP, and PiEMP at a concentration of 10 µg/mL, were cultured in 96-well plates containing 10 µg/mL of cellular matrix proteins (fibronectin (FN), laminin (LN) and vitronectin (VN)), BSA (negative control) and directly on the plastic (plate polymer) (positive control). There was a decrease in cell adhesion in cells treated with PcsEMP and PiEMP in all plaque characteristics (**P<0.01) (Figure 7).

Figure 7 – Effect of EMPS on endothelial cell adhesion



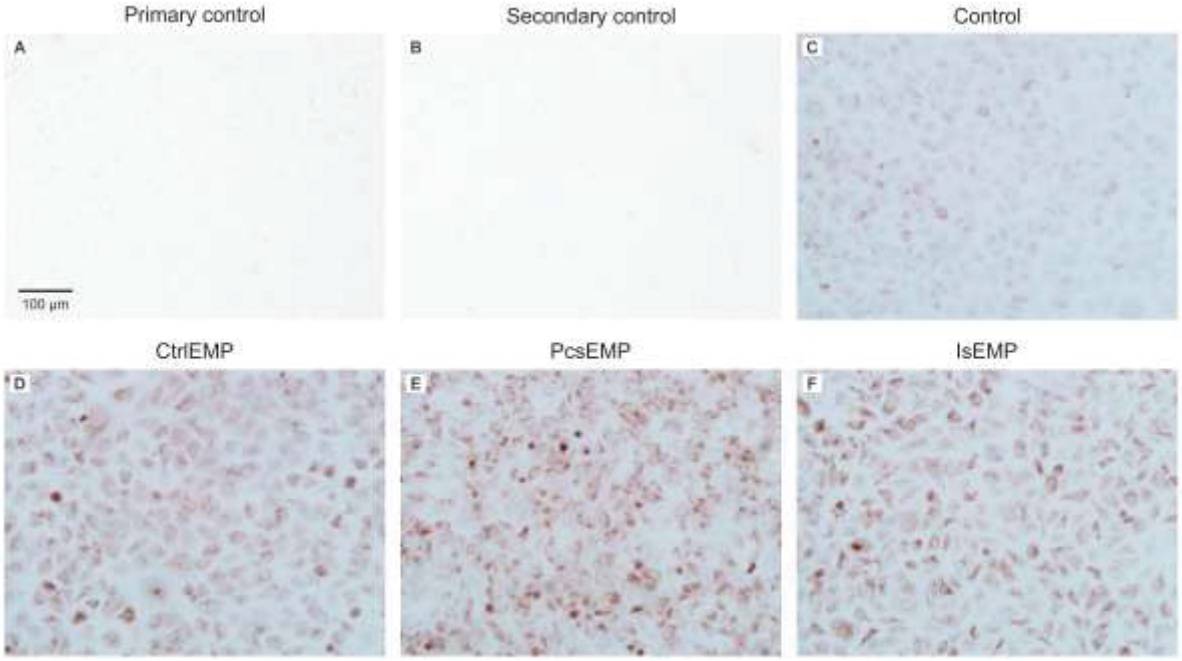
Note: Adhesion of endothelial cells treated with MPEs to extracellular matrix proteins. Endothelial cells were exposed to 10 µg/mL MPEs (CtrlEMP, PcsEMP, IsEMP, and PiEMP) and subsequently were plated in wells pretreated with matrix proteins (fibronectin (FN), laminin (LM) and vitronectin (VN)), directly in the plated polymer (plastic) and with BSA and a cell adhesion capacity is evaluated. Three tests were performed in triplicate; the values were expressed ± SEM. **P<0.01: Control

Plastic *vs* PcsEMP, PiEMP; Control FN *vs* PcsEMP, PiEMP; Control LN *vs* PcsEMP, PiEMP; Control VN *vs* PcsEMP, PiEMP. **P*<0,05: Control Plastic *vs* CtrlEMP.

3.7. VCAM-1 immunostaining

VCAM-1 is basal expressed in the healthy endothelium. It is assumed that an expression of VCAM-1 may result from endothelial activation and monocyte adhesion. Human endothelial cells were treated with 10 µg/mL of EMPs (CtrlEMP, PcsEMP, IsEMP, and PiEMP) and stained. As shown in Figure 8, positive staining was visualized in cells treated with EMPs. Endothelial cells treated with medium only were considered negative control.

Figure 8 – Effect of EMPs on VCAM-1 endothelial expression

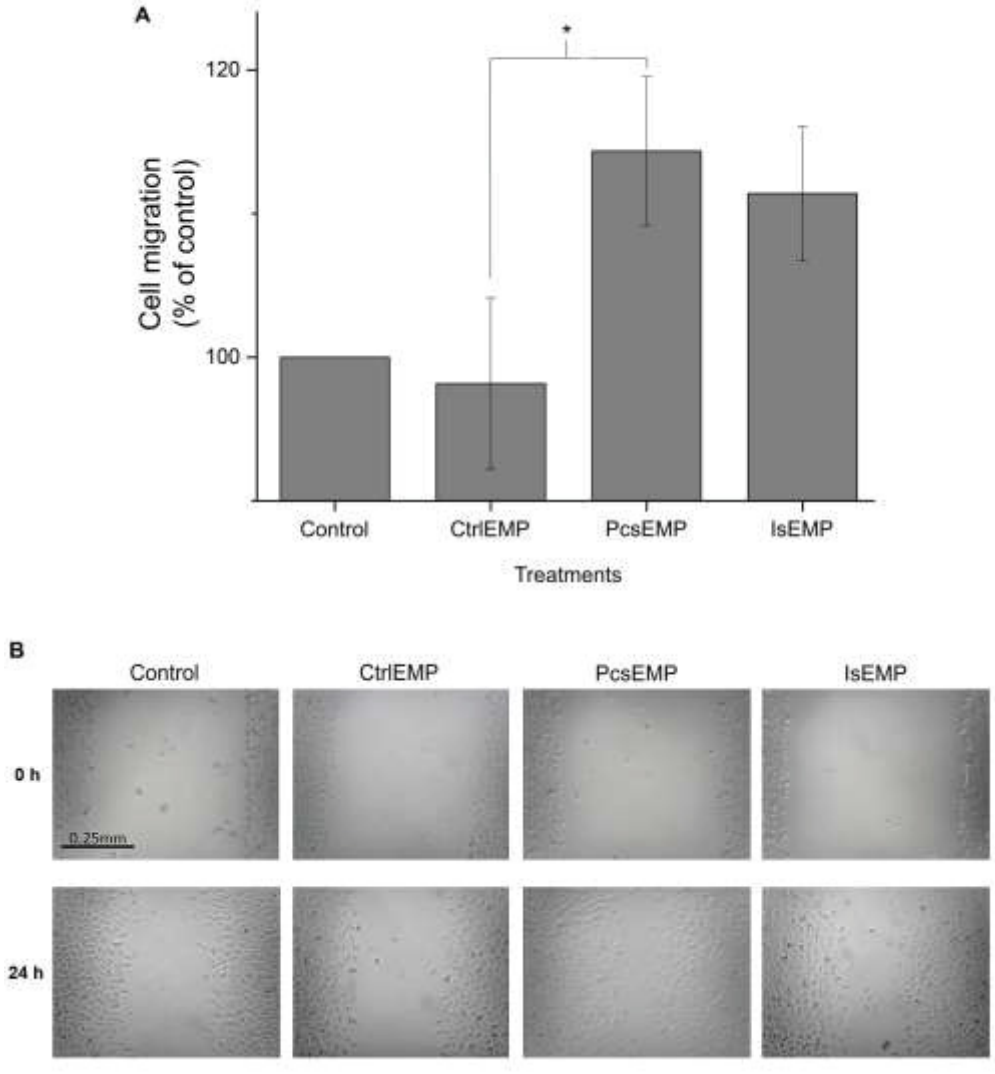


Note: Endothelial cells were treated with EMPs (10 µg) (control) for 24h and immunostained for VCAM-1 (A) control (primary antibody); (B) control (secondary antibody only); (C) control (untreated cells); (D) CtrlEMP; (E) PcsEMP; (F) IsEMP. 200x magnification. Three experiments were carried out in triplicate.

3.8. Wound Healing Assay

The wound-healing assay was performed on endothelial cells treated with EMPs for 24h. Untreated cells (control) were considered to be 100%. Data were expressed as from triplicate independent experiments (Figure 9).

Figure 9 – Endothelial cell migration after exposure to EMPs



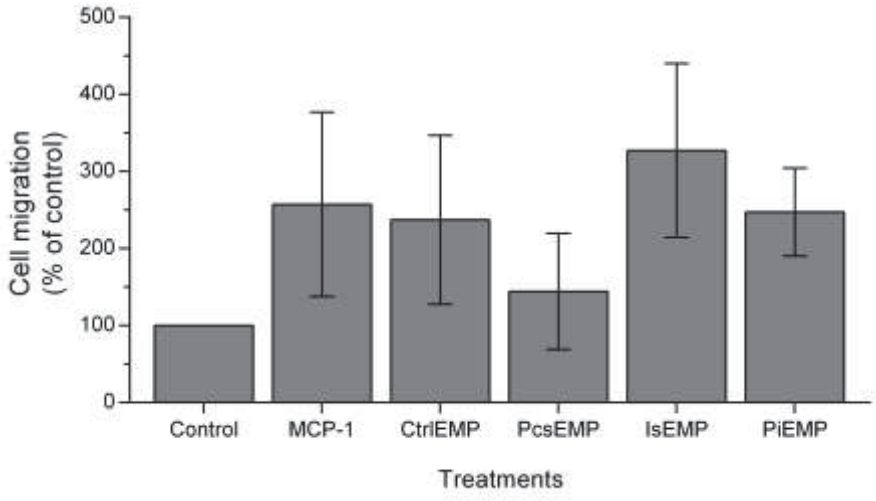
Note: A. The graph shows the percentage of cell migration of cells treated with 10 µg/mL EMPs (CtrlEMP, PcsEMP, and IsEMP). B. Images of wound closure at 0h and 24h. Control: endothelial cells treated with culture medium. CtrlEMP (EMPs from endothelial cells with Ca^{2+} induction). PcsEMP (EMPs from PCS treatment (2.6 mg/L) and Ca^{2+} induction). IsEMP (EMPs from treatment

with IS (236 mg/L) and Ca^{2+} induction). The values of % of cell migration were given by subtracting the area occupied by cells at 24h time by the area occupied by cells at time 0h. We evaluated by ANOVA post hoc Tukey the assay analysis was performed using the SafeHealing program. *P<0.05: CtrlEMP vs PcsEMP.

3.9. Monocytes migration assay

Figure 10 demonstrates the effect of with 10 µg/mL EMPs (CtrlEMP, PcsEMP and IsEMP) on monocyte migration. The culture medium was used as a negative control to evaluate the spontaneous monocyte movement, and a solution of MCP-1 at 50 ng/mL was used as a positive control for all solutions. After 90 min of incubation, there was no significant increase between treatments.

Figure 10 – Effect of EMPs on monocyte migration

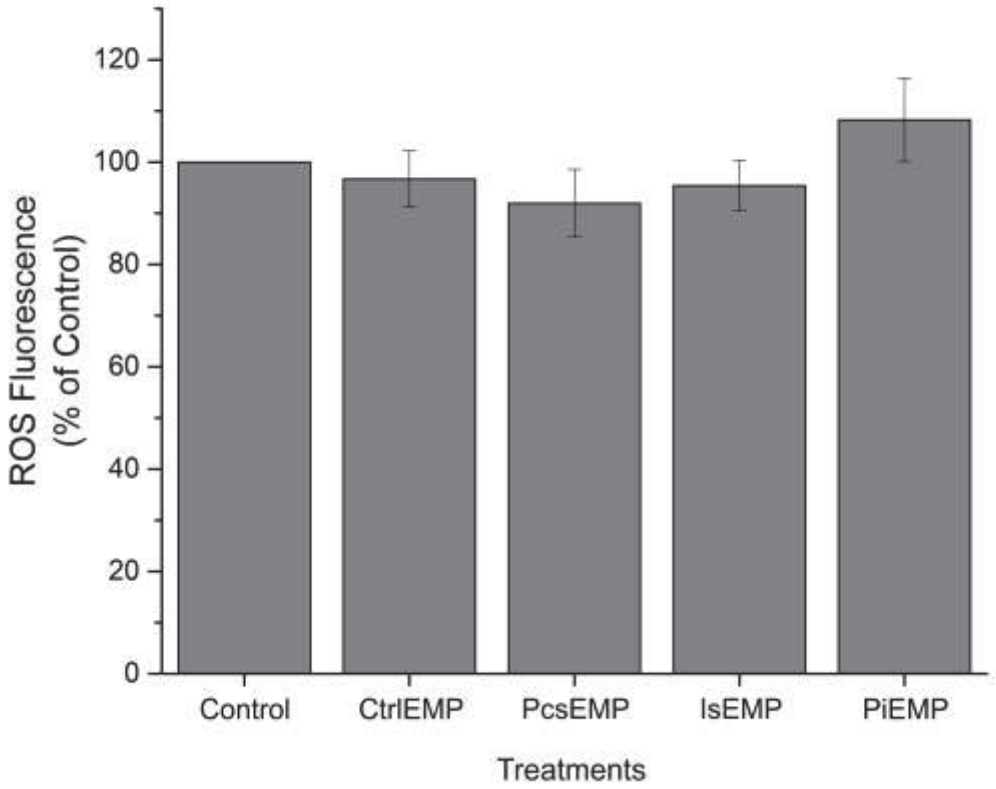


Note: Effect of endothelial cell supernatants, treated with EMPs after treatment with 10 µg/mL EMPs (CtrlEMP, PcsEMP, IsEMP, and PiEMP). Data were expressed as mean ± SEM of 5 independent experiments.

3.10. Measurement of ROS

To investigate the effect of EMPs on oxidative stress in endothelial cells, intracellular ROS levels were evaluated using a DCFH-DA probe. Figure 11 shows that treatments with CtrlIEMP, PcsEMP, IsEMP, and PiEMP, did not have ROS levels increased in endothelial cells.

Figure 11 – Effect of EMPs on ROS production in endothelial cells



Note: Effect of EMPs on the production of ROS in endothelial cells. Endothelial cells were incubated with 1 μM DCFH-DA in PBS at 37 °C for 30 min and then treated with CtrlEMP, PcsEMP, IsEMP, and PiEMP. ROS levels were determined immediately by measuring fluorescence (λEx 504 nm, λIn 524 nm). The production of ROS in untreated control cells was evaluated at 100%. Data are expressed as mean ± SEM of four independent experiments. There was no statistically experimental result.

4. DISCUSSION

EMPs formed in the uremic environment may contribute to endothelial dysfunction and leukocyte activation in CKD. To elucidate the mechanisms involved in this process, the main findings of the present study are: (I) Uremic toxins such as PCS, IS, and Pi induces the formation of EMPs in human endothelial cells; (II) EMPs can have different sizes, present phosphatidylserine and express CD31+ and CD144+; (III) PcsEMP and PiEMP decrease cell adhesion capacity; (IV)

CtrlEMP, PcsEMP and IsEMP induce VCAM-1 expression in endothelial cells; and (V) PcsEMP might induce the migration of endothelial cells.

PCS, IS, and Pi are closely related to pathological processes, such as endothelial dysfunction, formation, induction, and increase of circulating EMPs, which are associated with various circulatory, thrombotic, apoptotic disorders and the development of CVD in CKD patients (Faure et al. 2006; Ryu et al. 2017; Guerrero et al. 2020). We observed the release of EMPs by endothelial cells treated with uremic toxins in the electromicrographs obtained by SEM. EMPs are characterized according to the membrane glycoproteins they carry from their progenitor cell (Deng et al., 2017). Our data demonstrated that EMPs isolated from the microparticulation of cells exposed to PCS, IS, and Pi in their maximum uremic concentrations positive for CD31+ (PECAM, present in endothelial junctions) and CD144+ (VE-cadherin, expressed only in endothelial cells and which are part of the adherent junctions), epitopes used as positive markers of endothelial cells. Higher amounts of EMPs were also found in CKD patients than healthy subjects (Faure et al. 2006; Carmona et al. 2017a; Jalal et al. 2018; Mörtberg et al. 2019). Similarly, a more significant amount of CD144+ EMPs was observed in nephrectomized rats (CKD model) treated with a phosphorus-rich diet (Abbasian et al. 2020b). In vitro, Carmona et al. (2017b) found that EMPs derived from IS-treated cells showed greater expression of CD31, CD144, and ICAM-1 than EMPs from untreated cells (Carmona et al. 2017b). In addition to proteins, the authors have also shown that IsEMPs have higher levels of miR-181a-5p, miR-4454, miR-150-5p, and hsa-let-7i-5p, which are involved in processes of inflammation, senescence, and apoptosis (Carmona et al. 2017b).

Through the NanoSigth assay, we observed that homogeneous or heterogeneous EMPs might occur depending on the toxin to which the cells are exposed. EMPs from cells treated with PCS (PcsEMP) had an average diameter of 155 nm and homogeneous distribution. On the other hand, EMPs from IS (IsEMP) and Pi (PiEMP) a heterogeneous distribution, with averages ranging from 75–175 nm and 25–215 nm, respectively. Corroborating with our findings, in vitro studies have shown that endothelial cells incubated with 2.5 mM of Pi for 90 min led to an increase in the number of EMPs, which were characterized by a diameter ranging

from 100–200 nm and positive labeling for CD144 as well as phosphatidylserine (Di Marco et al. 2013; Abbasian et al. 2015, 2020a). Recently, Abbasian et al. (2020) found that Pi (2.5 mM) induced cytoskeleton disruption of endothelial cells, with the participation of DAPK-1 and TPM proteins, contributing to the greater formation of EMPs (Abbasian et al. 2020a).

To analyze the effects of EMPs in vitro, we chose a concentration of 10 μg/mL that did not significantly affect cellular metabolic activity, determined by the AlamarBlue assay. Moreover, we observed that the viability of endothelial cells exposed to CtrlEMPs increased compared to the control (without treatment), while PcsEMP, IsEMP, and PiEMP did not alter the viability. Interestingly, EMPs derived from cells exposed to uremic toxin þ-cresol induced endothelial cell senescence compared to untreated cells (Guerrero et al. 2020).

Our data demonstrated that PcsEMP and PiEMP decreased the ability of endothelial cells to adhere to plastic and cellular matrix proteins, such as fibronectin, laminin, and vitronectin. In contrast, we found no difference in adhesion between untreated cells and cells exposed to CtrlEMP and IsEMP. The interaction between endothelial cells and cellular matrix proteins is mediated mainly by the integrins, such as αvβ1 and αvβ3 (Gonzalez et al. 2002; Naik and Naik 2006; Tiwari et al. 2011; Post et al. 2019). Another study by Deregibus et. al. (2007), that the analysis by FACS analysis showed the expression by MVs of various adhesion molecules known to be present in the EPC plasma membrane, such as the intracellular adhesion molecule-1 (ICAM-1), integrin α4, CD44 and CD29 (integrin β1) (Deregibus et al. 2007). Therefore, the decrease in the adhesion capacity of cells exposed to PcsEMP and PiEMP may be related, at least in part, to the reduction of active integrins in the cell membrane.

We observed a more significant expression of VCAM-1 on endothelial cells treated with CtrlEMP and, even more, with PcsEMP and IsEMP compared to the control (without treatment), determined by immunocytochemistry. Several studies have demonstrated the proinflammatory property of EMPs (Curtis et al., 2009; Jansen et al., 2013). An increase in the VCAM-1 and ICAM-1 expression, as well as greater monocyte adhesion to the endothelium, is observed in endothelial cells treated with EMPs derived from cells exposed to high concentrations of glucose, a

condition similar to diabetes that is also commonly found in CKD patients, compared to untreated cells (Jansen et al. 2013). Similarly, EMPs from cells treated with TNF-α, which is also found at high levels in the CKD patients, induced the ICAM-1 expression in endothelial cells (Curtis et al., 2009; Lee et al., 2015). These findings indicate that EMPs can promote the pro-inflammatory phenotype in endothelial cells in CKD. The test to evaluate the monocyte migration capacity did not show any significant difference between treatments with EMPs. However, more studies should be performed to prove this finding. In addition, there was an increase in monocyte signaling by the expression of VCAM-1 in endothelial cells, showing that EMP, as well as MCP1, an attractive monocyte chemokine, are capable of promoting leukocyte transmigration to the inner layer, favoring atherosclerosis, activation leukocyte and thus promoting inflammation.

The migration capacity of endothelial cells can also be affected by EMPs. Our data were obtained from the wound healing assay and indicated that cells exposed to PcsEMP had increased cell migration capacity compared to cells treated with CtrlEMP. Recently, Guerrero et al. (2020) interestingly demonstrated that EMPs from cells exposed to p-cresol, a precursor to PCS, inhibited the repair of endothelial cells in wound healing assays (Guerrero et al., 2020).

We also observed that CtrlEMP, PcsEMP, IsEMP, and PiEMP did not alter ROS production in endothelial cells immediately exposed to EMPs. Despite this, Burger et al. (2016) demonstrated that EMPs carry NADPH oxidase subunits (i.e., Nox4 and p22phox) that are involved in ROS generation, in addition to higher levels of ROS in EMPs compared to untreated cells, determined by the lucigenin probe (Burger et al. 2016). Interestingly, EMPs from cells exposed to high glucose concentrations induce ROS production in endothelial cells after 30 min of treatment (Jansen et al., 2013).

We acknowledge that the present study has limitations, and further work is required. First, our experiments were performed with EA.hy926 cells, but it would also be interesting to use other cell models, such as human aortic endothelial cells (hAECs) and human microvascular endothelial cells (hMEC-1). It would also be relevant to analyze the in vitro effects of EMPs isolated from patients with CKD.

Besides that, further experiments on leukocyte activation in response to EMPs should be performed.

In conclusion, we demonstrated that uremic toxins such as PCS, IS, and Pi can induce the formation of EMPs, containing phosphatidylserine, CD31, CD144, and other biomolecules. These EMPs are bioactive and affect cell adhesion, the expression of inflammatory molecules such as VCAM-1, and the migration of recipient endothelial cells. Therefore, EMPs formed in uremic conditions play an important role in cell-cell communication and pathophysiological processes, making them potential biomarkers and therapeutic targets in CKD.

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8. CONCLUSÕES

- Foi possível caracterizar as MPEs em morfologia, concentração, tamanho da partícula e epítopos expressos na membrana.

- A concentração de uso das MPEs (10 µg/mL) foi determinado através do ensaio de Alamar Blue.

- A viabilidade celular foi alterada de forma significativa quando expostas a MPEs provenientes de PCS, IS e Pi em relação as MPEs provenientes de células de controle.

- A diminuição da capacidade de adesão das células expostas ao PcsEMP e PiEMP pode estar relacionada, pelo menos em parte, à redução das integrinas ativas na membrana celular.

- A reparação celular pode ter sido afetada pela falta de quimiocinas (SDF-1 e IL-8) que estimulam a migração de células progenitoras do endotélio até o local da lesão. Expressão de VCAM-1 nas células endoteliais indicam que EMPs podem promover o fenótipo pró-inflamatório nas células endoteliais na DRC. Em contrapartida a falta de migração leucocitária pode estar envolvida com a baixa expressão de células de adesão tais como E-selectina e P-selectina.

- Observamos que CtrlEMP, PcsEMP, IsEMP e PiEMP não alteraram a produção de ROS em células endoteliais imediatamente expostas a EMPs. Porém mais estudos devem ser realizados para comprovar a hipótese.

APÊNDICE

PRODUÇÃO CIENTÍFICA CONCOMITANTE AO DESENVOLVIMENTO DA TESE

ARTIGOS PUBLICADOS

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