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Virgínia Juliani Gomes

Envolvimento de urato monossódico e silibinina na modulação de monócitos de gestantes portadoras de pré-eclâmpsia

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Mestra em Tocoginegologia.

Orientadora: Profa. Dra. Maria Terezinha Serrão Peraçoli
Coorientadora: Dra. Mariana Romão Veiga

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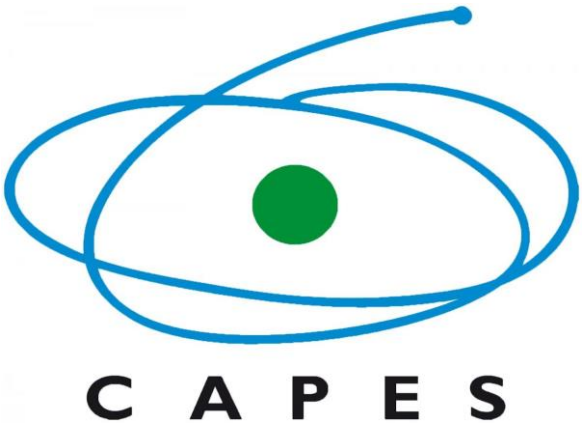

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Prefácio

A dissertação intitulada “Envolvimento de urato monossódico e silibinina na modulação de monócitos de gestantes portadoras de pré-eclâmpsia” encontra-se dividida em três capítulos.

Primeiramente, o capítulo I traz uma breve revisão de literatura a respeito da patogênese da pré-eclâmpsia e suas manifestações clínicas. Essas páginas abordam a classificação das subpopulações de células da linhagem monocítica-macrofágica e a importância de uma polarização macrofágica adequada para o desenvolvimento de uma gestação saudável. Também são rapidamente apresentados o papel de cristais de ácido úrico (urato monossódico) no desencadeamento da inflamação estéril presente nessa doença e as propriedades da silibinina, que explicam seu grande potencial como imunomodulador de monócitos.

O capítulo II apresenta o manuscrito *“Silibinin induces in vitro polarization of monocytes from preeclamptic women to M2 profile”* fruto do projeto de mestrado da aluna. Nesse trabalho, relatamos que o tratamento *in vitro* de monócitos de mulheres portadoras de pré-eclâmpsia com silibinina polariza as células para o perfil M2, sugerindo que esse flavonoide tem um efeito imunomodulador sobre a inflamação estéril característica da pré-eclâmpsia.

O capítulo III é composto por estudo desenvolvido durante o período de Bolsa de Ensino e Pesquisa no Exterior (BEPE-FAPESP) em parceria com o Instituto Karolinska (Suécia). O manuscrito intitulado “*Silibinin attenuates preeclampsia-derived monocytes-induced oxidative stress in human endotelial cells*” reafirma que a pré-eclâmpsia está associada com estresse oxidativo e disfunção endotelial, além de sugerir que o tratamento de monócitos com silibinina é capaz de reduzir a peroxidação lipídica, apesar de não influenciar na via de sinalização do óxido nítrico em células endoteliais de cordão umbilical humano (HUVEC).

Fluxograma dos Experimentos

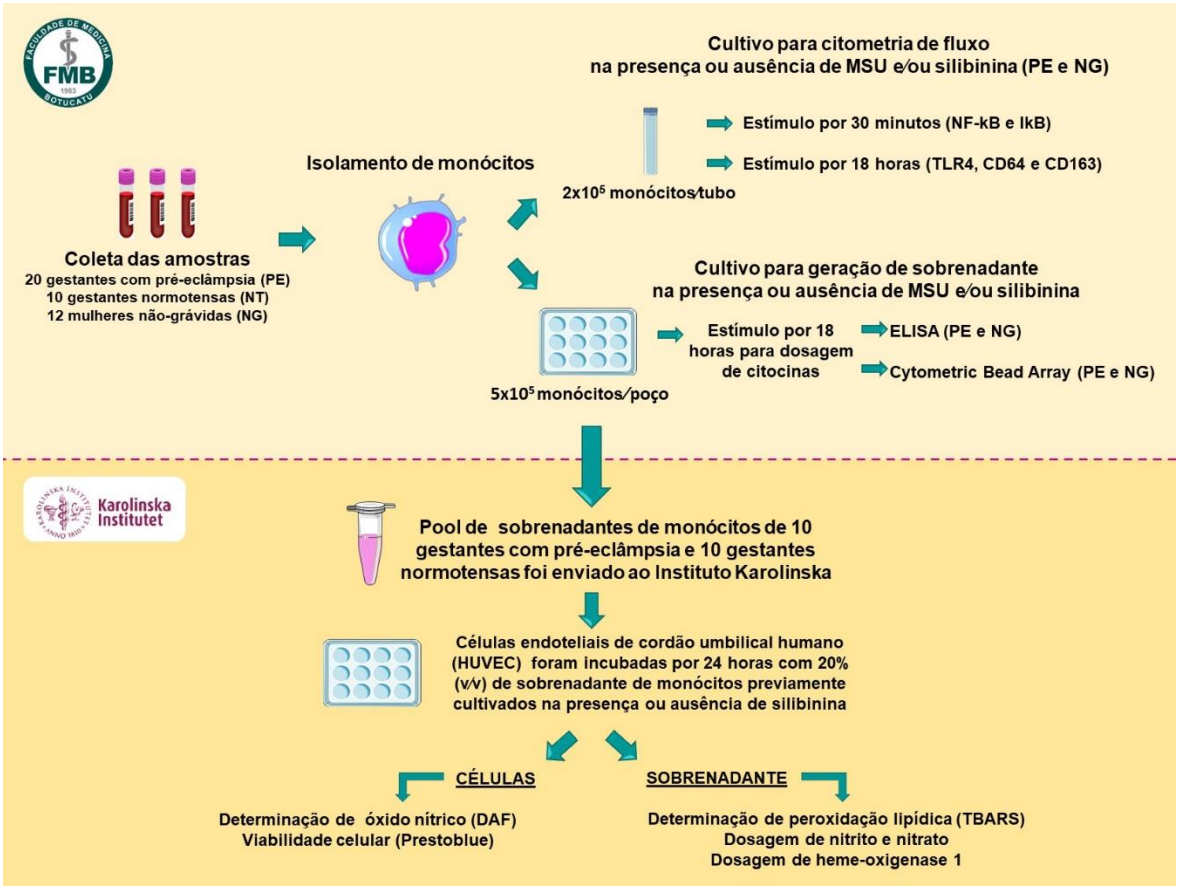

Figura 1. Fluxograma de experimentos sintetizando as etapas realizadas no Laboratório de Imunologia da Reprodução da UNESP (Botucatu, Brasil) e no período de Bolsa de Ensino e Pesquisa no Exterior (BEPE-FAPESP) no Instituto Karolinska (Estocolmo, Suécia).

Lista de abreviaturas

APC: fluorocromo aloficocianina
ARE: *antioxidant response element*
ANOVA: análise de variância
BSA: albumina do soro bovino
CD: *cluster of differentiation*
CD14: *cluster of differentiation 14*
CD16: *cluster of differentiation 16*
CD163: *cluster of differentiation 163*
CD206: *cluster of differentiation 206*
CD32: *cluster of differentiation 32*
CD64: *cluster of differentiation 64*
Co: grupo controle
DAMPs: padrões moleculares associados ao dano
DNA: ácido desoxirribonucleico
EDTA: ácido etilenodiamino tetra-acético
ELISA: ensaio de imunoabsorção enzimática
FcγR: receptores opsonínicos
FL: fluorescência
FSC: *forward scatter*, parâmetro de tamanho
°C: graus Celsius
GM-CSF: fator estimulador de colônia de granulócito macrófago
HMGB1: *High Mobility Group Box 1*
HO-1: hemeoxigenase-1
H_2O_2: peróxido de hidrogênio
HPLC: cromatografia líquida de alta eficiência
Hsp: proteína de choque térmico
HUVEC: células endoteliais de cordão umbilical humano

IFN-γ: interferon gama

IgG: imunoglobulina G

IKK: IκB quinase

IκB: proteína inibitória da via de sinalização de NF-κB

IL: interleucina

LPS: lipopolissacarídeo

M1: monócitos classicamente ativados

M2: monócitos alternativamente ativados

MDA: *malondialdehyde*

MFI: média de intensidade de fluorescência

min: minutos

MSU: urato monossódico

NLRP: *NOD-like receptor with a pyrin domain*

NF-κB: *nuclear factor kappa B*

Nfr2: *nuclear factor-erythroid-2-related factor 2*

NG: não-grávida

NP: *non-pregnant* (não-grávida)

NT: gestantes normotensas

O_2^-: ânion superóxido

$ONOO^-$: peróxido de nitrito

PAMPs: padrões moleculares associados a patógenos

PBMCs: Células mononucleares do sangue periférico

PBS: solução salina tamponada *(phosphate buffered saline)*

PE: pré-eclâmpsia

PE-Cy 7*: phycoeythrin*-Cy 7

PerCP-Cy5.5: fluorocromo proteína piridina clorofila conjugado com corante cianina

PRRs: receptores de reconhecimento de padrão

ROS: espécies reativas de oxigênio

rpm: rotações por minuto

RPMI: *Roswell Park Memorial Institute*, meio sintético complexo

Sb: silibinina

sCD163: receptor CD163 solúvel

SSC: *side scatter* - parâmetro de granularidade

TBA: ácido tiobarbitúrico

TBARS: substâncias reativas de ácido tiobarbitúrico

TCA: ácido tricloroacético

TGF-β1: fator transformador do crescimento beta 1

Th1: célula T helper 1

Th2: célula T helper 2

TLRs: receptores *Toll*

TNF-α: fator de necrose tumoral alfa

UA: ácido úrico

Lista de símbolos

g: gramas

mg: miligramas

mL: mililitro

mm: milímetro

mmHg: milímetro de mercúrio

p: nível de significância

pg: picograma

U/mL: unidade por mL

µg: micrograma

µL: microlitro

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Capítulo 1

Pré-eclâmpsia

A gestação normal é caracterizada por resposta inflamatória sistêmica leve, resultante da ativação da imunidade inata, que atua como mecanismo protetor do organismo materno contra infecções, uma vez que a reatividade da resposta imune específica se encontra ligeiramente diminuída (Wegmann et al., 1993; Borzychowski et al., 2006). A adaptação materna à gestação requer uma interação proeminente e rigidamente controlada entre as imunidades inata e adaptativa, para permitir o crescimento e desenvolvimento normais do semi-enxerto fetal (Van Rijn et al., 2008).

A pré-eclâmpsia (PE) é considerada uma síndrome específica da gravidez, sendo a principal causa de morbidade e mortalidade em 2 a 10% das gestações (ACOG, 2019). Os parâmetros clínicos que identificam o aparecimento dessa patologia são hipertensão arterial (PA ≥ 140 x 90 mmHg) associada ou não à proteinúria (≥ 300 mg/24h) a partir da vigésima semana de gestação ou após o parto, em mulheres previamente normotensas. Outras disfunções maternas também estão relacionadas com PE, como insuficiência renal, comprometimento hepático, complicações neurológicas ou hematológicas, disfunção útero-placentária ou restrição de crescimento fetal (Tranquilli et al., 2014; Mol et al., 2016; ACOG, 2019).

A PE caracteriza-se por intensa reação inflamatória sistêmica, lesão endotelial, agregação plaquetária, ativação do sistema de coagulação e aumento da resistência vascular generalizada (Roberts, 1998; Borzychowski et al., 2006), que parecem contribuir significativamente para a fisiopatologia da doença (Redman & Sargent, 2005). Assim, na PE observam-se ativação endógena de células inflamatórias como monócitos, granulócitos e células endoteliais (Redman et al., 1999; Borzychowski et al., 2006; Peraçoli et al., 2011), produção excessiva das citocinas pró-inflamatórias, interleucina-1 beta (IL-1β), IL-6, IL-8, interferon-gama (IFN-γ)

e fator de necrose tumoral alfa (TNF-α) (Redman & Sargent, 2003; Luppi & Deloia, 2006; Peraçoli et al., 2007; Pinheiro et al., 2013; Raghupathy, 2013), bem como por deficiência na produção de citocinas reguladoras, como IL-10 (Cristofalo et al., 2013; Peraçoli et al., 2013; Aggarwal et al., 2019).

Na PE, a ativação excessiva de monócitos e granulócitos intravasculares, bem como de macrófagos, sugere que a ativação do sistema imune inato pode causar problemas na progressão da gestação (Lok et al., 2009). Este distúrbio imunológico parece ter sua origem na placenta, resultante de lesão tecidual causada por isquemia/hipóxia, decorrente da falha de invasão das artérias uterinas pelo trofoblasto fetal (Huppertz, 2008). A diminuição da oferta local de oxigênio e nutrientes está associada à morte celular aumentada do trofoblasto (Wu et al., 2012), que pode resultar na liberação de mediadores de inflamação sistêmica materna como IL-6 e TNF-α, aumentando a expressão de moléculas de adesão e disfunção endotelial e contribuindo para a patogênese da PE (Kharfi et al., 2003; Harmon et al., 2016). Esses mediadores, liberados após necrose do trofoblasto (Huppertz et al., 2003) incluem DNA fetal, ácido úrico, *High Mobility Group Box 1* (HMGB1), produtos de matriz extracelular, IL-1α entre outros, caem na circulação materna e podem interagir com leucócitos circulantes, contribuindo para a intensa resposta inflamatória sistêmica, descrita na PE (Redman & Sargent, 2003). Monócitos de gestantes pré-eclâmpticas apresentam-se endogenamente ativados e liberam concentrações significativamente elevadas de TNF-α, IL-1β, IL-18, ânion superóxido e peróxido de hidrogênio (Peraçoli et al., 2011, Cristofalo et al., 2013; Matias et al., 2015). Esses resultados confirmam que a PE é caracterizada por estresse oxidativo e que monócitos circulantes podem representar importante fonte de radicais livres e citocinas inflamatórias nessa patologia. Assim, a resposta inflamatória sistêmica exacerbada, que na PE geralmente ocorre na ausência de infecção microbiana, tem sido descrita como inflamação estéril (Nadeau-Vallée et al., 2016; Brien et al.,

2018). Evidências da participação de monócitos nesse tipo de inflamação são representadas pela expressão gênica e proteica dos inflamassomas NLRP1 e NLRP3 associada à produção exacerbada de IL-1β, IL-18 e TNF-α por essas células, em gestantes portadoras de PE, que não apresentam sinais ou sintomas de infecção microbiana (Matias et al., 2015; 2019; Romão-Veiga et al., 2018).

Linhagem monocítica-macrofágica

Monócitos e macrófagos são importantes células da imunidade inata cujo processo de ativação está intimamente associado ao desenvolvimento da PE (Faas et al., 2014; Vishnyakova et al., 2019). Embora o mecanismo de ativação de monócitos na PE ainda não esteja totalmente esclarecido, uma possivel explicação parece estar relacionada ao fato de que monócitos maternos são ativados durante sua passagem pela placenta, uma vez que estabelecem contato com citocinas e fatores inflamatórios produzidos pelo sinciciotrofoblasto (Weel et al., 2016; Nonn et al., 2019). Citocinas, microvesículas e exossomos são liberados pelo sinciciotrofoblasto no sangue materno e podem manter o estado de ativação dessas células (Sokolov et al., 2016).

Uma vez infiltrados nos tecidos, monócitos se diferenciam em macrófagos, sendo seus precursores (Gordon & Taylor, 2005). Essas células estão envolvidas em vários processos como fagocitose, imunidade inata e adaptativa, atividade pró- e anti-inflamatória e, dependendo do microambiente tecidual onde se encontram, podem ser classificadas em duas principais subpopulações celulares que se adaptam e respondem à uma grande variedade de sinais: M1 ou classicamente ativados e M2 ou alternativamente ativados (Mantovani et al., 2004; 2013, Jena et al., 2019). O estado de ativação e função dessas células são marcadamente afetados por diferentes citocinas e produtos microbianos denominados padrões

moleculares associados a patógenos (PAMPs). Além disso, produtos endógenos liberados durante lesão tecidual, considerados padrões moleculares associados ao dano celular (DAMPs), ativam células da imunidade inata, promovendo a orientação da resposta imune adaptativa para perfil Th1 ou Th2, bem como expressão de funções efetoras especializadas e polarizadas (Mosser, 2003; Gordon & Martinez, 2010; Martin et al., 2011; Mantovani & Locati., 2013; Tian et al., 2015). Segundo Mantovani et al. (2004) a classificação M1 e M2 refere-se, portanto, a dois extremos de um espectro de ativação de macrófagos. Assim, macrófagos são células com plasticidade porque podem mudar de um estado ativado M1 para M2 ou regulador e vice-versa, sob efeitos de sinais específicos do ambiente onde se encontram (Porcheray et al., 2005; Bouhel et al., 2007; Tarique et al., 2015).

Essas células polarizadas diferem entre si pela expressão de receptores, produção de citocinas e funções efetoras. Células com perfil M1, decorrente da ativação por IFN-γ, TNF-α ou lipopolissacáride (LPS), expressam receptores opsonínicos do tipo FcγRI, II ou III (CD64, CD32, CD16), receptores TLR2 e TLR4 e produzem citocinas inflamatórias como TNF-α, IL-1β, IL-6, IL-12, IL-18 e IL-23, além de reativos intermediários do oxigênio e nitrogênio (Mantovani et al., 2004; Sica et al., 2009). Por outro lado, macrófagos ativados por IL-4 e IL-13 polarizam para perfil M2, que são caracterizado por maior expressão de receptores CD163 “scavenger” para complexos haptoglobina-hemoglobina (Kristiansen et al. 2001), possui propriedades anti-inflamatórias e imunorregulatórias (Zuwala-Jagiello, 2006; Akila et al., 2012) e receptor de manose (CD206), além de maior produção de IL-10, TGF-β1 e Arginase 1 (Mantovani et al., 2004; Malyshev & Malyshev, 2015).

Na interface materno-fetal, macrófagos estão presentes em todos os estágios da gestação e envolvidos em várias atividades, incluindo regulação da resposta imune, decidualização, invasão celular da placenta,

angiogênese, trabalho de parto e involução uterina pós-parto. O estado de ativação e função dessas células dependem do microambiente tecidual onde se encontram (Zhang et al., 2017). Numa gestação saudável, macrófagos da decídua se encontram inicialmente polarizados para o perfil M1, secretando citocinas e fatores de crescimento que auxiliam na implantação (Mor et al., 2011). Durante o segundo e terceiro trimestre de gestação, o perfil anti-inflamatório M2 prevalece, favorecendo a tolerância ao feto e permitindo seu crescimento no organismo materno (Svensson-Arvelund et al., 2014). Com a aproximação do momento do parto ocorre o desvio para o perfil M1, contribuindo para contração uterina e direcionamento do feto (Hamilton et al., 2012). A polarização macrofágica adequada e cronologicamente regulada é essencial para o desenvolvimento de uma gestação saudável (Figura 2) (Zhang et al., 2017; Vishnyakova et al., 2019). Sargent et al. (2006) sugerem que a transição para o perfil M2 durante o segundo trimestre de gestação estaria bloqueada na PE.

Estudos mostram que a classificação de macrófagos M1 e M2 pode ser estendida para monócitos do sangue periférico de pacientes com sepsis (Groselj-Grene et al., 2008), diabetes tipo 2 (Satoh et al., 2010), ou doenças autoimunes. O aumento da expressão de CD64 em monócitos de pacientes com lúpus eritematoso é considerado um marcador de atividade da doença (Kikuchi-Taura et al., 2015). Por outro lado, na leishmaniose cutânea, monócitos circulantes e macrófagos presentes nas lesões estão polarizados para o fenótipo M2. O tratamento quimioterápico nessa doença foi efetivo para restaurar o balanço M1/M2, importante para eliminar a infecção (Mukhopadhyay et al., 2015).

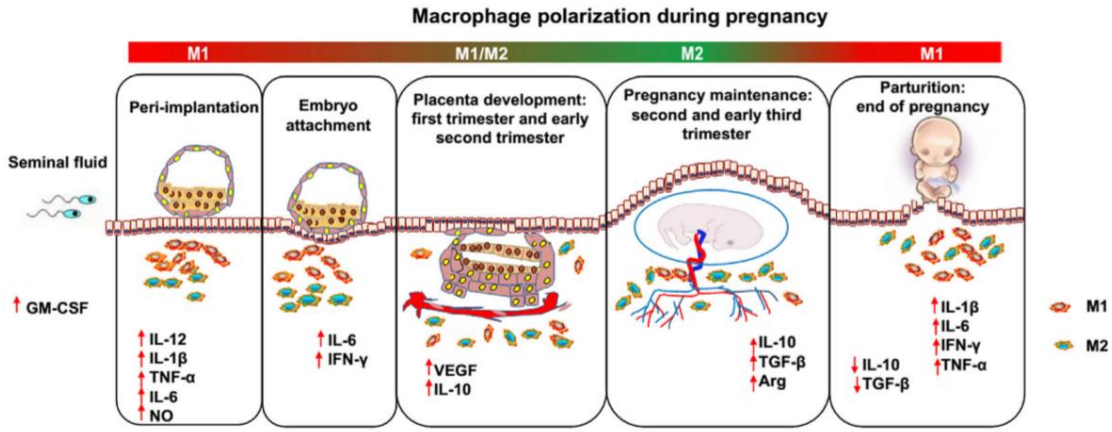

Figura 2. Dinâmica entre células M1 e M2 durante a gestação saudável. Logo após o coito, os níveis do fator estimulador de colônia de granulócitos e macrófagos (GM-CSF) são aumentados pela presença de TGF-β no fluído seminal, promovendo polarização para o perfil M1. Durante o período de implantação, macrófagos M1 produzem citocinas inflamatórias e mediadores químicos, como IL-6, IL-1β, TNF-α e óxido nítrico, auxiliando na implantação do embrião à decídua e no remodelamento vascular. Posteriormente, maiores níveis de progesterona são produzidos a fim de permitir o desenvolvimento fetal. Dessa forma, é estabelecido no útero um ambiente M2-dominante, apresentando menores concentrações de mediadores inflamatórios, maior produção de citocinas anti-inflamatórias e fagocitose de debris apoptóticos. Por fim, durante o período de parturição macrófagos M1 predominam sobre o perfil M2, promovendo a contração uterina, expulsão do bebê, ejeção da placenta e involução uterina (Figura adaptada de Zhang et al., 2017).

Em estudo prévio de nosso grupo, demonstramos que monócitos do sangue periférico de gestantes portadoras de PE estão endogenamente ativados e polarizados para perfil M1, com aumento na expressão de receptores TLR4 e CD64, diminuição de receptores CD163 e CD206 e produção aumentada de citocinas pró-inflamatórias (Medeiros et al., 2014). Além disso, em trabalho recente detectamos menor expressão do receptor CD163 em monócitos de gestantes com PE grave, associada à diminuição dos níveis plasmáticos da forma solúvel (sCD163) desse receptor e de IL-10. Paralelamente, a concentração plasmática das citocinas inflamatórias IL-1β e TNF-α foi significativamente mais elevada nessas gestantes e se correlacionou negativamente com os valores de sCD163. A associação

entre o perfil de citocinas pró-inflamatórias e a menor concentração de sCD163 e IL-10 no plasma das gestantes com PE grave sugere a ocorrência de um defeito na regulação da resposta inflamatória sistêmica na PE (Nunes et al., 2019).

Considerando que monócitos apresentam meia-vida de poucos dias na circulação sanguínea, suas concentrações e características fenotípicas podem ser consideradas um reflexo da gravidade do quadro da paciente com PE, sugerindo que o monitoramento desses fatores possui valor como teste de triagem e acompanhamento da doença (Vishnyakova et al., 2019). Devido a sua alta plasticidade, é possível que monócitos mudem de um estado ativado M1 para outro regulador M2 e vice-versa, dependendo dos sinais específicos presentes na circulação materna (Tarique et al., 2015; Zhang et al., 2017). Nesse sentido, a reprogramação dessas células pode se apresentar como interessante estratégia terapêutica para diversas doenças (Mantovani & Allavena, 2015). Dessa forma, a administração de moléculas moduladoras de monócitos pode vir a contribuir para a regulação do desbalanço da resposta inflamatória presente na PE.

Pré-eclâmpsia, hiperuricemia e silibinina

Em muitas pacientes com PE observam-se concentrações plasmáticas elevadas de ácido úrico, que se correlacionam com aumento da produção de TNF-α e de ânion superóxido por monócitos do sangue periférico (Peraçoli et al., 2011) e estão associadas com proteínas de choque térmico Hsp70 e citocinas inflamatórias como IL-1β, IL-12 e TNF-α (Peraçoli et al., 2013). A hiperuricemia está associada à gravidade da PE (Martin & Brown, 2010) e, portanto, considera-se que altos níveis de ácido úrico no plasma de gestantes pré-eclâmpticas podem representar uma contribuição direta à patogênese da doença pelo seu potencial de promover

inflamação (Bainbridge & Roberts, 2008).

O ácido úrico, sob a forma de cristais de urato monossódico (MSU) é considerado molécula pertencente ao grupo das DAMPs e pode se ligar aos receptores de reconhecimento de padrões (PRRs) expressos em células da imunidade inata, envolvidas na resposta inflamatória de monócitos (Kim et al., 2005; Mazouni et al., 2008). Dentre esses receptores destacam-se os receptores semelhantes a *Toll* (TLRs), que reconhecem e ligam-se a DAMPs (Kim et al., 2005) e PAMPs (Koga & Mor, 2008). Martin et al., (2011), estudando modelo murino para gota, observaram que cristais de MSU induzem a diferenciação de monócitos não-inflamatórios em macrófagos com perfil M1 de ativação. Além disso, MSU é ainda capaz de se ligar ao receptor TLR4, aumentando significativamente a expressão dessa molécula, NLRP3, IL-1β, CD40 e HLA-DR em células mesangiais renais (Xiao et al, 2015). Em trabalhos anteriores demonstramos que monócitos de gestantes portadoras de PE estimulados ou não com MSU apresentam ativação do inflamassoma NLRP3, da via TLR4/NF-kB e maior produção de IL-1β, IL-18 e TNF-α (Matias et al., 2015; 2019). O receptor NLRP3 é descrito como sensor de ácido úrico que induz a produção de IL-1β em linhagem celular de trofoblasto. Assim, a hiperuricemia associada à PE pode estar relacionada à ativação do inflamassoma NLRP3 no trofoblasto, aumentando assim os níveis de IL-1β na placenta e contribuindo para a patogênese da PE (Mulla et al., 2011).

A interação das DAMPs com os receptores TLRs induz resposta inflamatória pela via de sinalização do fator de transcrição nuclear NF-κB, uma proteína composta por duas subunidades a p50 e a p65, capaz de se ligar ao DNA e agir como um fator de transcrição em vários genes envolvidos nos processos inflamatórios, citoprotetores e carcinogênicos (Kang et al., 2002; Manna et al., 1999; Bannwart et al., 2010a; Raina et al., 2013). Em células não estimuladas, o NF-κB encontra-se no citoplasma ligado a proteínas inibitórias da família I-kappaB (IκB). Após o

reconhecimento das DAMPs pelos receptores, inicia-se um processo de fosforilação dependente da kinase – IKK, que pode ser do tipo: IKKα, IKKβ e IKKγ ou NEMO, seguido por uma degradação de IκB (Akira et al., 2001). Uma vez fosforilada, a IκB é poliubiquitinizada e rapidamente degradada pelo proteassoma, dissociando então o heterodimero p50/p65. Isso permite que o NF-κB migre para o núcleo e ligue-se à região promotora de genes que apresentam a sequência regulatória GGGACTTTCC, levando a um aumento da expressão do gene alvo (Lu & Wahl, 2005; Echeverri & Mockus, 2008) (Figura 3).

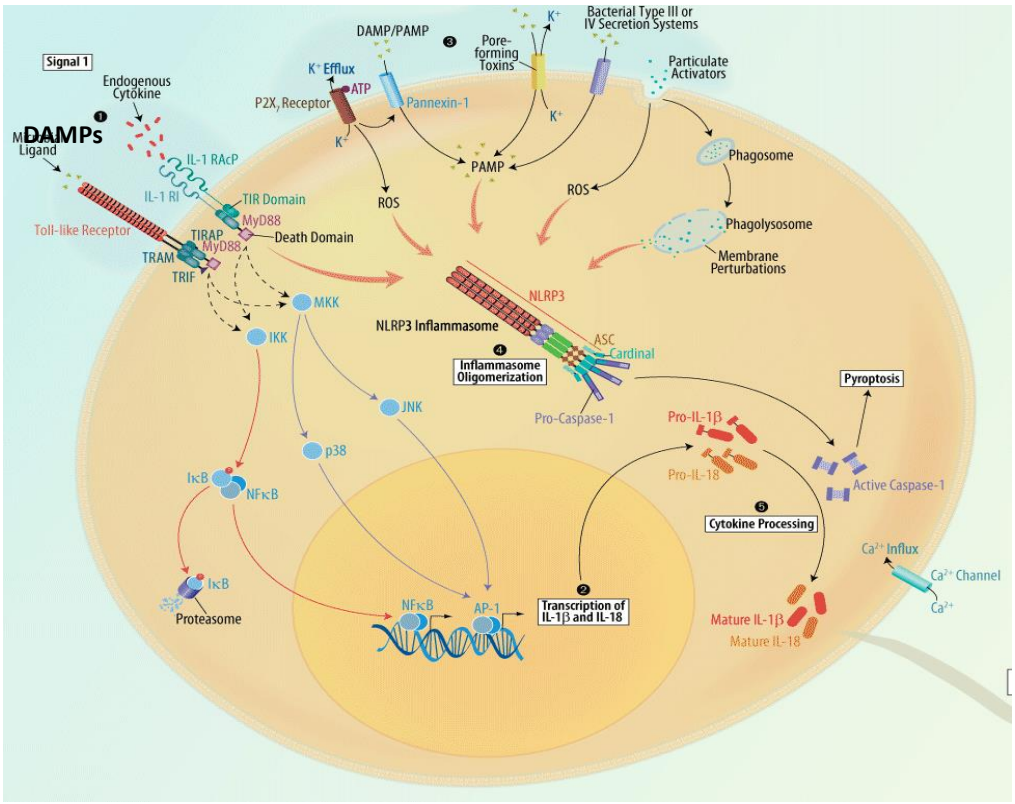

Figura 3. Cascata de ativação da via do NF-κB por DAMPs (Figura adaptada de R&D Systems; Minneapolis, MN, USA)**.**

Produtos naturais, extraídos de plantas, podem exercer efeito inibidor sobre processos inflamatórios, iniciados por ativação de NF-kB. A

silimarina é um flavonóide, extrato obtido de frutos e sementes de *Silybum marianum*, família Asteraceae, uma das mais antigas e tradicionais ervas medicinais, utilizada por sua ação hepatoprotetora há mais de dois mil anos (Kren & Walterová, 2005) (Figura 4). O componente mais ativo e abundante da silimarina é a silibinina, que assim como a silimarina, apresenta importantes atividades de citoproteção, anti-inflamatórias, anti-fibróticas, anti-oxidantes e anti-carcinogênicas (Kren & Walterová, 2005; Agarwal et al., 2006; Deep & Agarwal, 2010; Esmaeil et al., 2017). Entre os mecanismos moleculares envolvidos nesses efeitos destaca-se a ação inibidora da silibinina sobre o fator de transcrição nuclear kappa B (NF-κB). De maneira geral, a silibinina parece interferir na cascata de transdução de sinais controlada pelo NF-κB, suprimindo a fosforilação e degradação de IκB, diminuindo assim, a translocação de NF-κB para o núcleo. (Kren & Walterova, 2005; Comelli et al., 2007; Esmaeil et al., 2017; Matias et al., 2019).

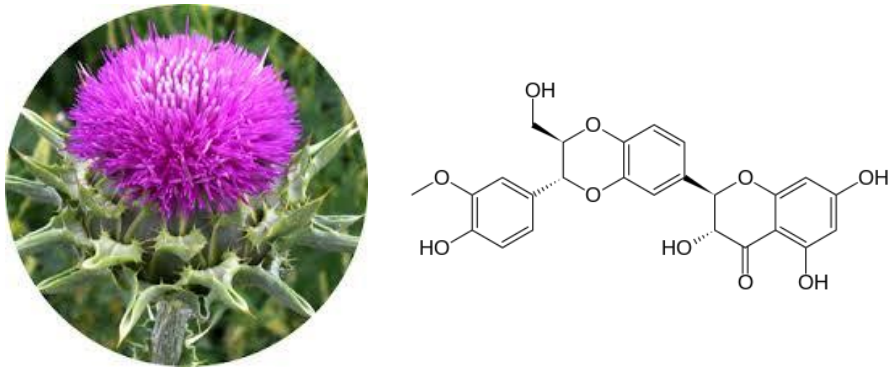

Figura 4. Imagem de *Silybum marianum* e estrutura química da silibinina. (Imagens de Bannwart et al., 2010b do Google em janeiro, 2020).

A silibinina tem sido empregada para tratamento de doenças humanas, devido sua toxicidade extremamente baixa, tornando o tratamento seguro e sem efeitos adversos (Hackett et al., 2013). Além disso, este flavonoide foi efetivo no tratamento de doenças hepáticas (Tamayo & Diamond, 2007) e como terapia adjuvante no câncer (Deep & Agarwal, 2007). Em trabalhos anteriores, realizados em nosso laboratório,

foi observado que a silibinina possui propriedades anti-oxidantes e anti-inflamatórias, demonstradas pela inibição dose-dependente sobre a liberação de H_2O_2, da produção de TNF-α, IL-1β, TGF-β e de prostaglandina E2 (PGE2), por monócitos de sangue periférico obtidos de indivíduos saudáveis estimulados com LPS e tratados com 50 μM de silibinina (Bannwart et al., 2010a,b). Além disso, inibiu a ativação de NF-κB e a síntese das citocinas pro-inflamatórias IL-1β e TNF-α, produzidas por células mononucleares de sangue periférico de gestantes portadoras de pré-eclâmpsia (Giorgi et al., 2012; Cristofalo et al., 2013). O tratamento com silibinina em modelo experimental de PE, induzido em ratas pela inibição da enzima óxido nítrico sintase por Nw-nitro-L-arginine methyl ester, melhorou o desempenho reprodutivo das ratas, normalizou os níveis da pressão arterial e o número de plaquetas, diminuiu os níveis de proteinúria e das citocinas inflamatórias IL-1β, TNF-α e interferon-gama (IFN-γ) detectados no soro dos animais (Souza et al., 2012). Em trabalho recente demonstramos que o tratamento in vitro de monócitos de gestantes pré-eclâmpticas com silibinina modula a inflamação estéril estabelecida nessas células, diminuindo a ativação dos inflamasomas NLRP1 e NLRP3 e da via TLR4/NF-kB (Matias et al., 2019)

Considerando que a PE é caracterizada por resposta inflamatória exacerbada, envolvendo a polarização de monócitos para perfil inflamatório, pela expressão de receptores extracelulares de ativação e aumento da produção de citocinas pró-inflamatórias, o emprego de moléculas moduladoras ou reguladoras de monócitos poderia contribuir para a melhor compreensão da imunidade inata e das vias de ativação dessas células envolvidas na fisiopatologia dessa síndrome da gestação.

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Capítulo 11

SILIBININ INDUCES IN VITRO POLARIZATION OF MONOCYTES FROM PREECLAMPTIC WOMEN TO M2 PROFILE.

Virgínia Juliani Gomes[1]*, Mariana Romao-Veiga[1], Mariana Letícia Matias[1], Priscila Rezeck Nunes[1], Vanessa Rocha Ribeiro[1], Amanda Carreira Devides[1], Camila Ferreira Bannwart-Castro[1], Graziela Gorete Romagnoli[1], José Carlos Peraçoli[1], Maria Terezinha Serrão Peraçoli[2].

[1] Botucatu Medical School, Sao Paulo State University (Unesp), Botucatu, Sao Paulo, Brazil
[2] Institute of Biosciences of Botucatu, Sao Paulo State University (Unesp), Botucatu, Sao Paulo, Brazil

*Corresponding author at: Department of Gynaecology and Obstetrics, Botucatu Medical School, São Paulo State University. Botucatu, São Paulo, Brazil, CEP 18618-687 Phone: 55 14 3880 0431
Email address: vi.juliani@hotmail.com

Abstract

Introduction: Preeclampsia (PE) is a specific syndrome of pregnancy characterized by an intense systemic inflammatory reaction. In the plasma of pregnant women with PE there are high levels of molecular structures associated with stress and cell death, called damage-associated molecular patterns (DAMPs) such as uric acid, which seem to contribute to the disease pathogenesis. DAMPs can activate monocytes shifting them to the M1 proinflammatory profile. The pro and anti-inflammatory imbalance in PE can be regulated through the administration of flavonoids with anti-inflammatory properties such as silibinin (Sb). **Objectives:** The present study evaluated the immunomodulatory effect of Sb on the expression of activation markers and the signaling pathway of the nuclear transcription factor NF-κB in monocytes of pregnant women with PE. **Methods:** Monocytes from pre-eclamptic women were cultured in the presence or absence of Sb, and the expression of surface molecules TLR4, CD64 and CD163, as well as the intracellular transcription factors IKB-α and NF-κBp65 was analyzed by flow cytometry. The concentration of cytokines IL-6 and IL-8 in the monocyte culture supernatant was determined by cytometric bead array and IL-1β, IL-10, TNF-α, IL-12p70, IL-23 and TGF- β by the ELISA immunoassay. The results were analyzed using parametric tests with a 5% significance level.
Results: The results of the present study showed that the in vitro treatment of monocytes from preeclamptic women with Sb downregulated the endogenous activation of NF-κB, expression of surface receptors TLR4 and CD64, and reduced the synthesis of the pro-inflammatory cytokines IL-1β, IL-8, IL-23 and TNF-α compared with cultures non-treated with Sb. The presence of this flavonoid in monocyte cultures increased the expression of CD163 and IκBα and the release of IL-10 and TGF-β in the culture supernatants, polarizing these cells from M1 to M2 profile. The anti-inflammatory activity of SB on the NF-kB activation pathway and induction of cell polarization to M2 profile was confirmed by an in vitro assay using monocytes from healthy, non-pregnant women. **Conclusion:** In vitro treatment of monocytes from preeclamptic women with Sb polarizes the cells to M2 profile, suggesting that Sb has an immunomodulatory effect on the sterile inflammation characteristic of preeclampsia.

Keywords: preeclampsia, monocyte subpopulations, silibinin, cytokines, NF-kB.

Introduction

Preeclampsia (PE) is a specific human gestational syndrome and the main cause of maternal-fetal mortality, morbidity and prematurity (Von Dadelzen et al., 2011; ACOG 2019). The more important clinical parameters for PE diagnosis are hypertension and proteinuria that occur from the 20th week of pregnancy. However, the onset of hypertension associated with other severe manifestations of PE such as neurologic or hematologic complications, kidney failure, liver impairment, utero-placental dysfunction or fetal growth restriction may occur in the absence of proteinuria and recently can be considered as PE (Tranquilli et al. 2014; Mol et al. 2016; ACOG 2019).

The exacerbated systemic inflammatory response characteristic of PE may be resultant from endogenous activation of innate immunity cells such as monocytes and granulocytes, and their excessive inflammatory cytokine production (Redman & Sargent, 2003; Borzychowski et al., 2006). Pro-inflammatory cytokines such as Interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6, IL-12 are associated with reduced levels of the regulatory cytokines IL-10 and transforming growth factor beta (TGF-β) in preeclamptic women (Cristofalo et al., 2013; Pinheiro et al., 2013; Raghupathy, 2013; Aggarwal et al., 2019). This disturbance seems to have its origin in the placenta, resulting from tissue damage caused by ischemia / hypoxia, due to the failure of uterine artery invasion by the fetal trophoblast (Huppertz, 2008). Then, decreased local oxygen and nutrient supplies are associated with increased trophoblast cell death (Wu et al., 2012), leading to release of inflammation mediators in maternal circulation, which interact and activate circulating leukocytes, contributing to the intense systemic inflammatory response described in PE (Redman & Sargent, 2003). Monocytes from preeclamptic pregnant women are endogenously activated and release significantly elevated concentrations of TNFα, Interleukin-1 (IL-1β), IL-18, superoxide anion and hydrogen peroxide (Peraçoli et al., 2011, Cristofalo et al., 2013; Matias et al., 2015). These results confirm that PE is

characterized by oxidative stress and that circulating monocytes may represent an important source of free radicals and inflammatory cytokines in this pathology. Thus, the exacerbated systemic inflammatory response, which in PE usually occurs in the absence of microbial infection, has been described as sterile inflammation (Nadeau-Vallée et al., 2016; Brien et al., 2018). Evidence of monocyte participation in this type of inflammation is represented by the gene and protein expression of nod-like receptor with a a pyrin domain 1 (NLRP1) and NLRP3 inflammasomes associated with the exacerbated production of IL-1β, IL-18 and TNF-α by these cells in preeclamptic women that do not show signs or symptoms of microbial infection (Matias et al., 2015; 2019; Romão-Veiga et al., 2018).

Once infiltrated into tissue, monocytes differentiate into macrophages (Gordon & Taylor, 2005). These cells are involved in various processes such as phagocytosis, innate and adaptive immunity, pro- and anti-inflammatory activity and, depending on the tissue microenvironment where they are can be classified into two main cell subpopulations: M1 or classically activated and M2 or alternatively activated (Mantovani et al., 2004; 2013, Atri et al., 2018; Jena et al., 2019). The activation state and the function of these cells are affected by cytokines, microbial and endogenous products released during tissue injury, inducing these cells to a M1 or M2 profile (Gordon & Martinez, 2010; Martin et al., 2011; Mantovani & Locati., 2013; Tian et al., 2015). According to Mantovani et al. (2004) classification M1 and M2 therefore refers to two extremes of a macrophage activation spectrum. Thus, macrophages are cells with plasticity because they can change from an activated state M1 to M2 or regulator state and vice versa, under the effects of environment-specific signals (Porcheray et al., 2005; Bouhel et al., 2007; Tarique et al., 2015; Zhang et al., 2017).

These polarized cells differ from each other by expression of receptors, cytokine production, and effector functions. M1 monocytes are activated by IFN-γ, TNF-α and bacterial lipopolysaccharide, express FcγRI, II or III opsonin receptors (CD64, CD32, CD16), TLR2 and TLR4 receptors

and produce the inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, IL-18 and IL-23, as well as oxygen and nitrogen intermediates (Mantovani et al., 2004; Martinez et al., 2008, Malyshev & Malyshev 2015). On the other hand, M2 monocytes are activated by IL-4, IL-10 or IL-13 and are directly involved with the immune response to parasites, tissue repair and allergic diseases (Martinez et al., 2008, Vandooren et al., 2009, Gordon & Martinez 2010, Mantovani & Locati 2013). This profile is characterized by higher expression of CD163 scavenger hemoglobin receptor, and mannose (CD206) receptor, has anti-inflammatory and immunoregulatory properties and secrets higher concentrations of IL-10 and TGF-β (Kristiansen et al., 2001; Mantovani et al., 2004; Zuwala-Jagiello et al., 2006; Malyshev & Malyshev 2015).

At the maternal-fetal interface, macrophages are present at all stages of pregnancy and have been involved in various activities, including regulation of the immune response, decidualization, placental cell invasion, angiogenesis, labor, and postpartum uterine involution. The state of activation and function of these cells depend on the tissue microenvironment where they are in (Zhang et al., 2017). In a healthy pregnancy, deciduous macrophages are initially polarized to the M1 profile, secreting cytokines and growth factors that aid implantation (Mor et al., 2011). During the second and third trimester of pregnancy, the anti-inflammatory profile M2 prevails, favoring tolerance to the fetus and allowing its growth in the maternal organism (Svensson-Arvelund et al., 2014). As labor approaches, deviation to the M1 profile occurs, contributing to uterine contraction and fetus delivery (Hamilton et al., 2012). Thus, a proper and chronologically regulated macrophage polarization is essential for the development of a healthy pregnancy (Zhang et al., 2017; Vishnyakova et al., 2019).

The M1 and M2 macrophage classification can be extended to human circulating monocytes in various diseases such as sepsis, type 2 diabetes, infectious and autoimmune diseases (Groselj-Grenc et al., 2008; Satoh et al., 2010; Mukhopadhyay et al., 2015; Kikuchi-Taura et al., 2015). In a previous study we demonstrated that monocytes from preeclamptic

women were polarized to M1 profile, showing higher expression of TLR4 and CD64 receptors, lower concentrations of CD163 and CD206 and higher production of pro-inflammatory cytokines when compared to normotensive (NT) pregnant women cells (Medeiros et al., 2014). Moreover, in a recently published article we detected lower expression of CD163 in monocyte surface, associated to decreased plasmatic levels of this receptor in its soluble form (sCD163) and IL-10 in women with PE. At the same time, the plasma concentration of inflammatory cytokines IL-1β and TNF-α was significantly higher in these preeclamptic women and was negatively correlated with sCD163 concentrations, suggesting that the regulation of the systemic inflammatory response is affected in PE (Nunes et al., 2019).

Hyperuricemia is commonly observed in preeclamptic women and correlates with higher production of TNF-α and superoxide anion by monocytes from peripheral blood (Peraçoli et al., 2011). Uric acid, in the form of monosodium urate (MSU) crystals, acts as a damage-associated molecular pattern (DAMP) and interacts with pattern recognition receptors (PRRs), such as toll like receptors (TLRs), which are involved in monocyte inflammatory response (Kim et al., 2005; Mazouni et al., 2008). In a previous study, we have reported that monocytes from preeclamptic women treated or not with MSU showed NLRP3 inflammasome activation and produced higher levels of IL-1β, IL-18 and TNF-α (Matias et al., 2015). The interaction of MSU with TLRs induces an inflammatory response by activating the transcriptional factor nuclear kappa B (NF-κB) pathway, responsible for regulating the transcription of several genes related to inflammatory, cytoprotective and carcinogenic processes (Manna et al., 1999; Kang et al., 2002; Raina et al., 2013). In unstimulated cells, NF-κB lies in the cellular cytoplasm bound to inhibitory protein IκB. After the recognition of a DAMP by TLR4, a cascade of kinases (IKKs) is activated, leading to IκB phosphorylation and further degradation by the proteasome (Akira et al., 2001). Thus, NF-κB can migrate to the nucleus and bind to promoter regions of inflammatory genes, resulting in greater expression of pro-inflammatory

genes (Lu & Wahl, 2005; Echeverri & Mockus 2008). Previous results obtained by our group demonstrated increased NF-κB activation in peripheral blood mononuclear cells from preeclamptic women, associated with higher production of IL-1β e TNF-α, when compared to normotensive pregnant women (Giorgi et al, 2012). We also demonstrated that monocytes from women with PE stimulated with MSU showed increased TLR4/NF-κB pathway activation (Matias et al, 2019). Then, the treatment of preeclamptic women monocytes with modulatory molecules might decrease the inflammatory processes detected in PE.

Silymarin is a polyphenolic flavonoid extracted from fruits and seeds of *Silybum marianum*, a plant from the Asteraceae family and considered one of the oldest medicinal herbs due to its hepatoprotective effect (Abenavoli et al., 2018). The most active and abundant component of Sylimarin is silibinin (Sb), which also presents important anti-inflammatory, antioxidative, anti-carcinogenic and cytoprotective activities (Kren & Walterová, 2005; Agarwal et al., 2006; Deep & Agarwal, 2010; Esmaeil et al., 2017). Silibinin has downregulatory effects on the NF-κB pathway, due to the inhibition of IκB degradation. Therefore, the translocation of NF-κB to the nucleus is reduced and the synthesis of proinflammatory cytokines by peripheral blood mononuclear cells is also inhibited (Kren & Walterová, 2005; Comelli et al, 2007; Esmaeil et al., 2017; Matias et al, 2019). Our research group have also reported that PBMCs from preeclamptic women cultivated with Sb showed decreased activation of NF-κB, NLRP1 and NLRP3 inflammasomes pathways coupled with decrease in TNF-α, IL-1β and IL-18 production (Giorgi et al., 2012; Cristofalo et al., 2013; Matias et al., 2019). Considering the anti-inflammatory properties of Sb, this study aimed to evaluate the immunomodulatory effect of this flavonoid on the expression of activation markers and on NF-κB signalling pathway in monocytes from preeclamptic women.

Materials and Methods

Subjects

Twenty preeclamptic women with gestational age ranging from 24 to 40 weeks were admitted to the Obstetric Unit of Botucatu Medical School, Sao Paulo State University, Brazil in the period between March 2018 and October 2019. PE was diagnosed in accordance with the American College of Obstetricians and Gynecologists, and it was defined as the onset of a persistently elevated blood pressure of 140/90 mmHg, associated or not with proteinuria (≥300 mg in urine collected during 24 h), and other severe clinical complications after 20 weeks of gestation (ACOG, 2019). The gestational age was calculated from the last menstrual period and confirmed by early (<12 weeks gestation) ultrasound examination. Exclusion criteria include prior PE, multiple gestation, illicit drug use, and pre-existing medical conditions such as diabetes, chronic hypertension, acute infections, autoimmune, renal and hepatic diseases. Proteinuria in 24-h urine was measured by a colorimetric method, the Technicon RA-XT automation system, and uric acid in plasma was assessed by uric acid enzymatic Trinder (Biotrol Diagnostic, Chennevières-lès-Louvres, France), in the Clinical Laboratory of Botucatu Medical School – UNESP. The study also included 12 healthy, non-pregnant (NP) women with no clinical pathology and blood donors of Botucatu Medical School that were matched by age with preeclamptic pregnant women. The Ethics Committee of the Botucatu Medical School approved the study (protocol number 3.754.036), and all women have provided the informed consent.

Blood collection

Blood for evaluation the expression of monocyte surface molecules (TLR4, CD14, CD64 and CD163), NF-κB transcription factor, IκBα molecule and cytokine production by these cells was collected from preeclamptic women in the moment of diagnosis, and in healthy NP women at the time of

blood donation. Blood samples (10 mL) were obtained by venipuncture of the antecubital vein and placed into a sterile plastic tube containing 10 U/mL EDTA (Becton Dickinson-BD Vacutainer; BD Biosciences, Franklin Lakes, NJ).

Monocyte culture and treatments

Peripheral blood mononuclear cells (PBMC) were obtained by gradient separation from Ficoll-Paque Premium (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the technique described in Peraçoli et al. (2011). Then, the cells were resuspended in RPMI 1640 culture medium (Sigma-Aldrich, SL, Missouri, USA) supplemented with 10% inactivated fetal bovine serum (complete medium), and counted with 5% Turk dye solution (1:1v/v). The cell concentration was adjusted to 1×10^7 PBMC/mL. For monocyte separation, the magnetic beads from Human Pan Monocyte Isolation Kit (Miltenyi Biotec, Germany) was employed according to the manufacturer's instructions. After isolation, the monocytes obtained were counted with 5% Turk's solution and the concentration was adjusted as needed for each of the following experiments.

Monocytes from preeclamptic women were cultured with or without 50 µM of Sb (Sigma-Aldrich) for 30 min and 18 h for NF-kB and cytokine determination, respectively. Silibinin concentration for monocyte culture was previously standardized (Bannwart et al., 2010). Monocytes fron non-pregnant women were cultured with or without 50µM of Sb and 50 µM of monosodium urate (MSU) as previously described (Matias et al., 2019).

Analysis of surface receptors and NF-kB signaling pathway in monocytes by flow cytometry

After acquisition, $2x10^5$ monocytes were distributed in cytometer tubes (BD Biosciences). Monocytes from preeclamptic women were treated with or without Sb and the cells from NP women were cultured with or without Sb and MSU as described above. Additionaly, monocytes from NP

women were incubated for 30 minutes with 10μM Bay 11-7082 NF-κB pathway inhibitor (Sigma-Aldrich), and in the presence or absence of MSU, SB or MSU+SB, for analysis of the intracytoplasmic factors NF-κB and IκBα. These cells were incubated with BD Biosciences antibodies with respective fluorochromes: anti-CD14 Alexa Fluor 488, anti-TLR4 phycoerythrin (PE), anti-CD64 phycoerythrin (PE) and anti-CD163 peridininchlorophyll protein-Cy 5.5 (PerCP-Cy 5.5) for 20 minutes in the dark at 4˚C. After centrifugation, cells were washed twice with phosphate buffered saline (PBS) containing 0,5% bovine serum albumin and centrifuged again for 10 min at 400g. The cells were then fixed and permeabilized using Fix Buffer (BD Biosciences) and Perm Buffer (BD Biosciences) for incubation with specific antibodies (BD Biosciences) and labelled with specific fluorochromes for the intracellular proteins NF-κB phycoeythrin-Cy 7(PE-Cy7) and IκBα Alexa FLuor 647 for 30 min in the dark and at room temperature. For each test, control tubes were incubated with specific isotypic antibodies for each fluorochrome (Alexa FLuor 488, PE, PerCP-Cy 5.5, PE-Cy7, and Alexa Fluor 647). The analyses were performed on a FACSCantoTM (BD Biosciences) flow cytometer using the FlowJo vX.10.6 (Tree Stars Inc.) to acquire and analyse cellular multiparameters. It was standardized the acquisition of 30,000 events per sample, and the population of interest was optimised, establishing a gating strategy. First, events were selected by size (FSC) and granularity (SSC) expected for monocytes. Then, duplets were discarded and fluorescence (FL) for CD14 was taken into account. From this gate, established on CD14 positive monocytes, the mean fluorescence intensities (MFIs) of TLR4, CD64, CD163, NF-κB, and IκBα were evaluated.

Cytokine determination

After monocyte isolation, $5x10^5$ cells/well from preeclamptic women were cultured with or without Sb and monocytes from healthy NP women were cultured with or without MSU and MSU+Sb for 18 h. Then, IL-1β, IL-10, IL-12p70, IL-23, TNF-α and TGF-β cytokines were determined in the

culture supernatants by enzyme-linked immunosorbent assay (ELISA), employing Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacture's instructions. Assay sensitivity limits of the kits were 1.0 pg/mL for IL-1β, 3.9 pg/mL for IL-10, 15 pg/mL for IL-12p70, 16.3 pg/mL for IL-23, 1.6 pg/mL for TNF-α and 15.4 pg/mL for TGF-β. For IL-6 and IL-8 determinations it was employed the cytometric bead array (CBA) technique using the Human Inflammatory Cytokine Kit (BD Biosciences) according to the manufacturer's protocol. The sensitivity limit was 2.5 and 3.6 for IL-6 and IL-8 respectively.

Statistical Analysis

The results were evaluated using parametric methods according to the statistical program GraphPad Prism, version 6.01 (GraphPad, CA, USA). Surface and intracelular molecules expression in monocytes from preeclamptic women were analysed by Paired Student's *t*-test, and results from healthy NP women were evaluated by analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The statistical significance was set at $p<0.05$.

Results

Clinical characteristics

Analysis of clinical characteristics of preeclamptic and non-pregnant women (Table 1) showed no statistical differences regarding age between the groups. Systolic and diastolic blood pressures were significantly higher in women with PE in comparison to NP women.

Table 1. Clinical and Laboratorial Characteristics

Characteristics	Preeclamptic Women (n=20)	Healthy Non-Pregnant Women (n=12)
Age (years)	30 (15 – 40)	29 (22-37)
Gestacional Age (weeks)	35 (26 – 39)	N/A
Systolic Blood Pressure (mmHg)	150 * (140 – 200)	120 (80-130)
Diastolic Blood Pressure (mmHg)	100 * (90 – 120)	80 (60-90)
Proteinuria (mg/24h)	500 (300 – 1880)	N/A
Uric Acid (mg/dL)	4.8 (3.9 – 8.9)	N/A

Values expressed in median, with maximun and minimun values between parentheses. *p<0,05 vs. NP women. N/A = not applicable.

Expression of TLR4, CD64 and CD163 surface receptors by monocytes from preeclamptic women

Figure 1 shows that the treatment of monocytes from preeclamptic women with Sb induces significantly lower expression of TLR4 and CD64 surface receptors (Figure 1 a and b) when compared to control cultures (Sb-). Moreover, monocyte inclubation in the presence of Sb (Sb+) increased the expression of CD163 (Figure 1 c and d) in these cells.

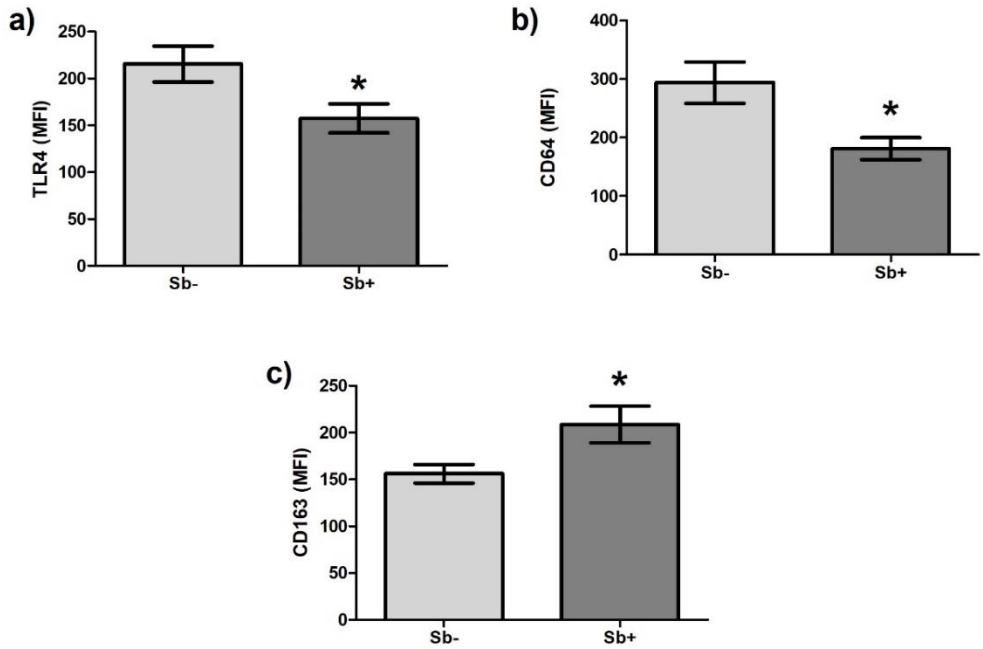

Figure 1. Expression of TLR4 (a), CD64 (b) and CD163 (c) by monocytes from 20 preeclamptic women. Cells were cultured in the absence (Sb-) or presence of silibinin (Sb+) for 18 hours. Results are expressed in mean ± SD of fluorescence intensity (MFI); ***** p≤0.05 vs Sb- (Paired Student's *t*-test).

Intracytoplasmic expression of NF-κB and IκB by monocytes from preeclamptic women

Monocytes from preeclamptic women incubated with Sb showed significantly lower expression of NF-κBp65 (Figure 2a) and increased intracytoplasmic levels of IκBα (Figure 2b) in comparison to cultures that were not treated with Sb.

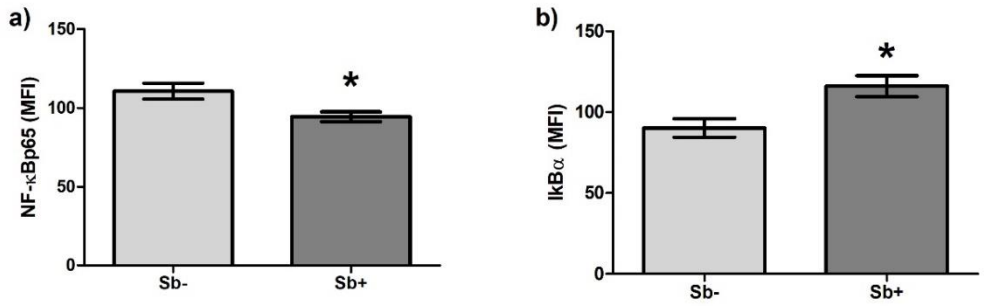

Figure 2. Expression of NF-κBp65 (a) and IκBα (b) by monocytes of 20 preeclamptic women. Cells were cultured in the absence (Sb-) or presence of silibinin (Sb+) for 30 minutes. Results are expressed in mean ± SD of fluorescence intensity (MFI); ***** p≤0.05 vs Sb- (Paired Student's *t*-test).

Cytokine determination in the supernatant of monocytes from preeclamptic women

Figure 3 shows that the treatment of monocytes with Sb decreased the production of IL-1β, IL-6, IL-8, IL-12p70, IL-23 and TNF-α when compared to control cultures (Figures 3 a-f). On the other hand, the presence of Sb in monocyte cultures increased the production of IL-10 and TGF-β (Figures 3g, 3h).

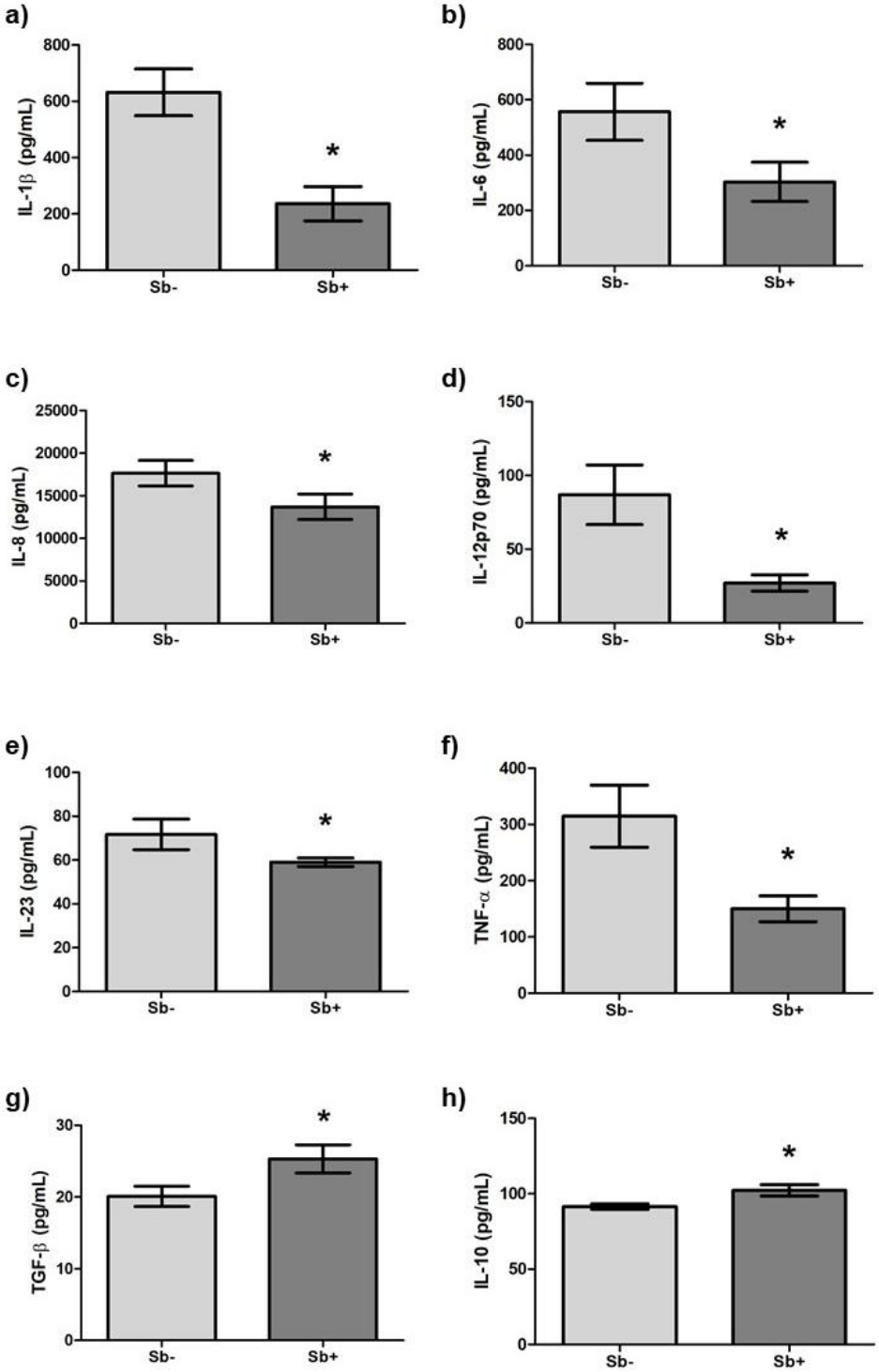

Figure 3. Concentrations of IL-1β (a), IL-6 (b), IL-8 (c), IL-12p70 (d), IL-23 (e), TNF-α (f), TGF-β (g) and IL-10 (h) detected in the supernatant of monocytes from 20 preeclamptic women. Cells were cultured in the absence (Sb-) or presence of silibinin (Sb+) for 18 hours. Results are expressed in mean ± SD. ***** p≤0.05 vs Sb- (Paired Student's *t*-test).

Expression of TLR4, CD64 and CD163 surface receptors by monocytes from healthy non-pregnant women

Flow cytometric analysis of monocytes from NP women showed that MSU treatment induced higher expression of TLR4 (figure 4a) and CD64 (figure 4b), in addition to lower expression of CD163 (figure 4d) by these cells compared to control (Co), Sb and MSU+SB treatments. Moreover, cell treatment with Sb or MSU+SB restored the control expression of TLR4 and CD64, and increased CD163 expression by monocytes.

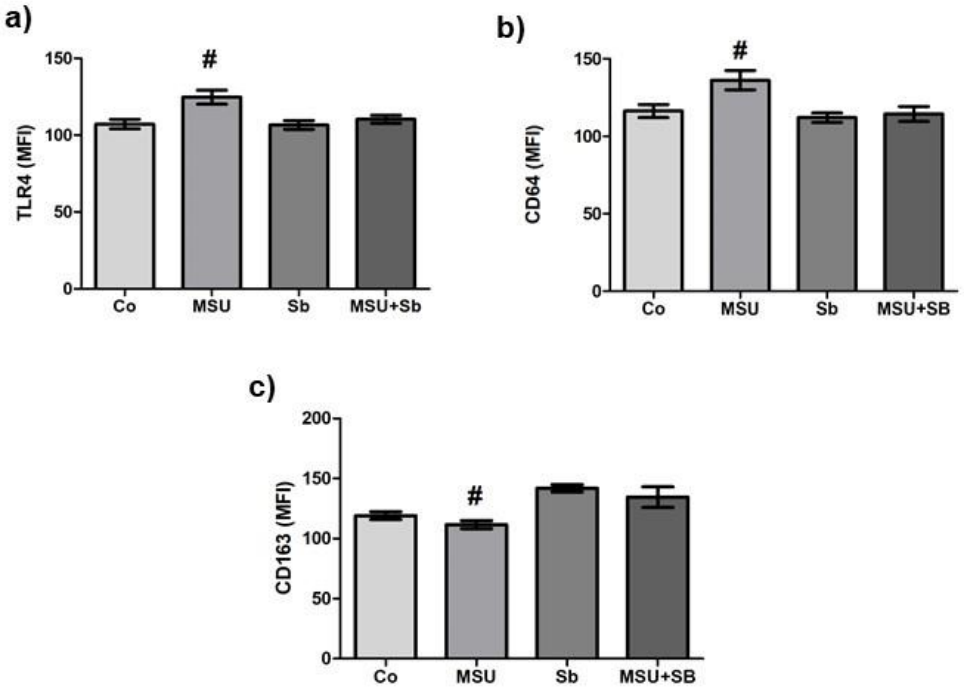

Figure 4. Expression of TLR4 (a), CD64 (b) and CD163 (c) by monocytes from 12 healthy non-pregnant women. Cells were cultured without treatment (Co) or in the presence of MSU, Sb or MSU+Sb for 18 hours. Results are expressed in mean $\pm$ SD of fluorescence intensity (MFI); #p≤0.05 vs all treatments (ANOVA).

Intracytoplasmic expression of NF-κB and IκB by monocytes from healthy non-pregnant women

Monocytes from NP women incubated with MSU expressed higher levels of NF-κBp65 (figure 5a) in comparison to control, unstimulated cultures (Co), Sb and MSU+Sb culture treatments. No statistical differences were observed in IκBα expression (figure 5b) after these treatments. Addition of the NF-κB pathway inhibitor, Bay 11-7082, to monocytes cultured

with MSU, Sb or MSU+SB decreased the activation induced by MSU, leading to no significant diferences between all treatments (Figures 5 c and d).

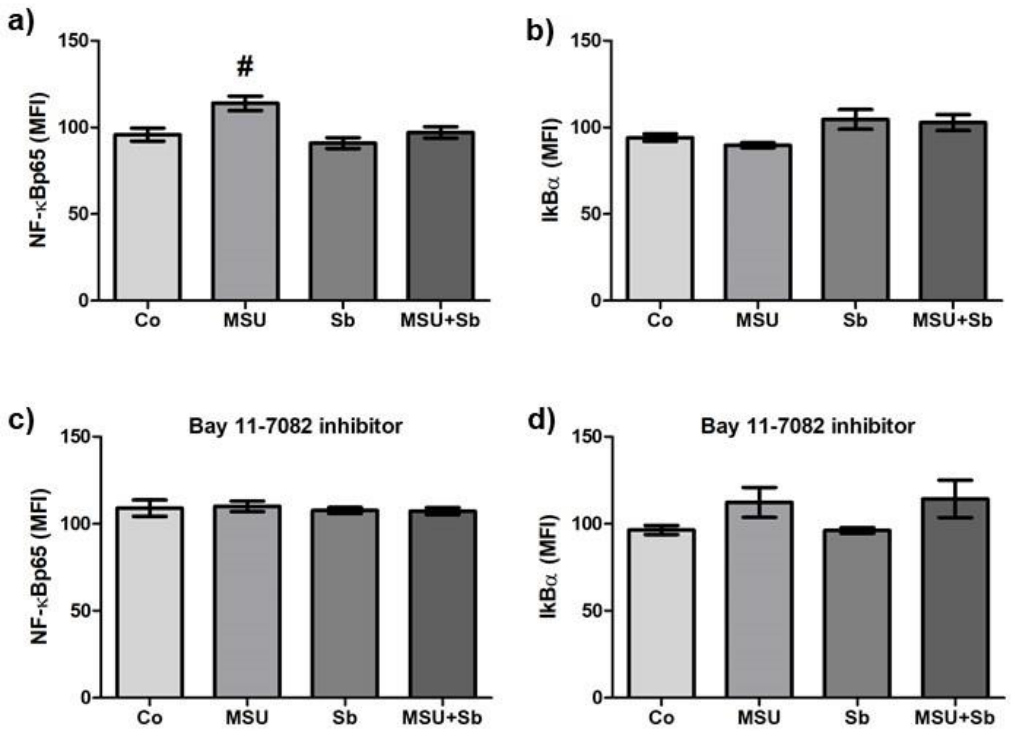

Figure 5. Expression of NF-κB (a) and IκBα (b) by monocytes from 12 healthy non-pregnant women. Cells were cultured without treatment (Co) or in the presence of MSU, Sb or MSU+Sb for 30 minutes. Expression of NF-κB (c) and IκBα (d) by monocytes from NP women treated with Bay 11-7082 NF-kB inhibitor and cultured with or without MSU, Sb or MSU+Sb for 30 minutes. Results are expressed in mean ± SD of fluorescence intensity (MFI). # p≤0.05 vs all treatments. (ANOVA).

Cytokine determination in the supernatant of monocytes from NP women

Figure 6 shows cytokine production by monocytes from NP women cultured in the absence (Co) or presence of MSU, Sb or MSU+Sb. Addition of MSU to the cultures induced higher concentrations of IL-1β (a), IL-23 (e) and TNF-α (f) and lower levels of IL-10 (h) when compared to the other treatments. Moreover, monocytes cultured with MSU showed higher levels of IL-8 (c) and lower concentrations of TGF-β (g) than the control and silibin-treated cultures. No statistical differences between treatments were

observed for IL-6 and IL-12p70.

Discussion

The present study investigated the immunomodulatory effect of silibinin on the expression of activation markers, and the NF-κB signaling pathway in monocytes from preeclamptic women. Our results demonstrated that Sb downregulates the endogenous activation of monocytes from PE women, since this flavonoid treatment decreased TLR4 and CD64 surface receptors expression by these cells. Additionally, Sb also reduced the synthesis of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12p70, IL-23 and TNF-α compared with cultures non-treated with Sb. These findings are in agreement with our previous study reporting reduction of TLR4/MyD88/NF-kB pathway gene expression as well as NF-κB concentration on nuclear extracts of monocytes from preeclamptic women were cultured with Sb (Matias et al., 2019). The immumodulatory effects of this flavonoid may be explained by silibinin's suppressive activity on the inhibitory protein IκB phosphorylation and further degradation, avoiding NF-κB's translocation to the nucleus, which consequently decreases the release of pro-inflammatory molecules (Kim et al., 2013; Sayrai, 2015; Esmaiel et al., 2017). In this sense, our data also show that the incubation with Sb induced higher expression of IκBα, and potentialized anti-inflammatory cytokines IL-10 and TGF-β production by monocytes from preeclamptic women. Endogenous levels of IL-10 and TGF-β have been reported as higher in the supernatant of monocytes from NT pregnant women than in cultures from women with preeclampsia (Cristofalo et al., 2013), suggesting that these cytokines play an important role in monocyte regulation in a healthy pregnancy.

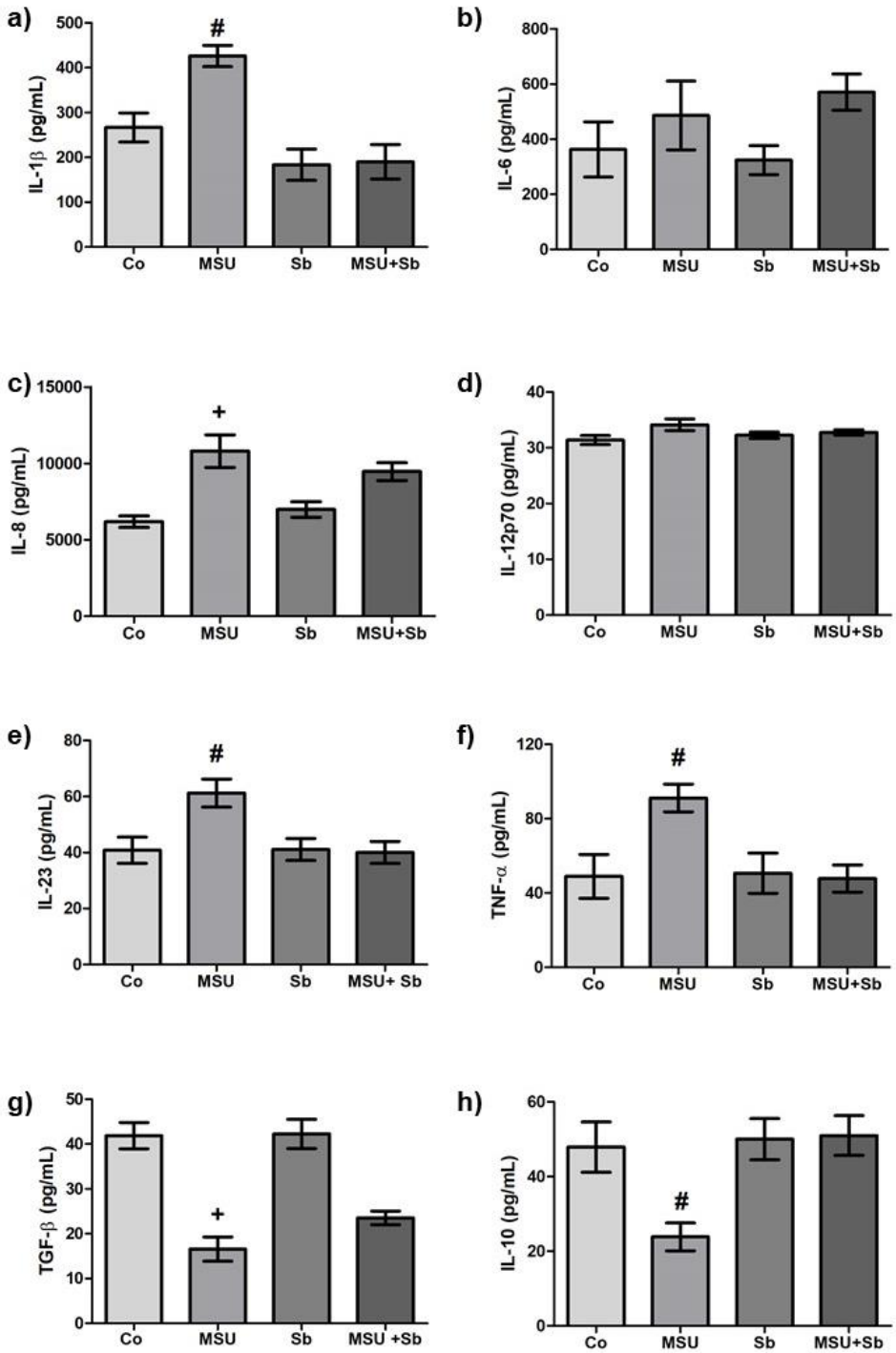

Figure 6. Concentrations of IL-1β (a), IL-6 (b), IL-8 (c), IL-12p70 (d), IL-23 (e), TNF-α (f), TGF-β (g) and IL-10 (h) detected in the supernatant of monocytes from 12 healthy non-pregnant women. Cells were cultured without treatment (Co) or in the presence of MSU, Sb or MSU+Sb for 18 hours. Results are expressed in mean ± SD. # p≤0.05 vs all treatments; + p≤0.05 vs Co and Sb (ANOVA).

Cytokines, chemokines and adhesion molecules are essential for the development of a normal pregnancy (Barrera et al., 2015; Cubro et al., 2018; Yockey & Iwasaki, 2018). Previous studies showed association between lower production of the anti-inflammatory cytokines IL-10 and TGF-β1 and increased release of IL-1β, IL-6, IL-12 and TNF-α by monocytes from preeclamptic women (Cristofalo et al., 2013; Medeiros et al., 2014; Chen et al., 2015; Matias et al., 2019). According to Cubro et al. (2018) IL-10 plays benefic role in normal pregnancy by promoting successful placentation, controlling inflammation, and regulating vascular function, and deficiency of IL-10 contributes to the development of PE. In the present study, the up-regulation of IL-10 production by monocytes from preeclamptic women after treatment with Sb confirms the potential and beneficial anti-inflammatory effect of this flavonoid for treatment of the systemic inflammatory response in PE. Thus, future studies on the mechanisms involved in IL-10 induction by Sb in monocytes are of utmost importance.

Previous results from our group demonstrated higher expression of TLR4 and CD64 coupled with diminished CD163 and CD206 concentrations in monocytes from PE women, in comparison to NT pregnant group, suggesting that monocytes from women with PE are endogenously activated and polarized to a M1 pro-inflammatory profile (Medeiros et al., 2014). This inflammatory profile of monocytes from pre-eclamptic women seems to be associated with their interaction with endogenous cellular products released during tissue injury and called DAMPs present in the maternal circulation. These DAMPs are represented by molecules such as uric acid (Matias et al., 2015), reactive oxygen intermediates, heat shock proteins Hsp70 (Asea et al, 2002; Peraçoli et al., 2013), proteins released from dead cells such as HMGB1 (Park et al., 2004; Zhu et al., 2015) and other injury products, which leak out from the extracellular matrix, such as fibronectin and hyaluronan (Said-Sadier & Ojcius, 2012; Romão-Veiga et al., 2018), that can create an inflammatory microenvironment propitious to

monocyte polarization towards the M1 profile.

Our results showed that preeclamptic monocytes treatment with Sb led to lower expression of the M1 markers TLR4 and CD64 receptors, while increases the expression of CD163, characteristic of M2 profile. To our knowledge, this is the first study evaluating the effects of Sb over monocyte polarization in PE.

The high expression of TLR4 in monocytes from preeclamptic women, detected in the present study is in accordance with the literature, reporting increased expression of the TLR4 receptor in peripheral blood monocytes from preeclamptic women (Al-ofi et al., 2012; Medeiros et al., 2014; Chen et al.,2015; Matias et al., 2019). This receptor increase in PE was also detected in neutrophils (Xie et al., 2005), umbilical cord mononuclear cells (Xia et al., 2010), and in the placenta (Bernardi et al., 2012), suggesting that this receptor and its pathway signaling contribute to the pathogenesis of PE (Abrahams et al., 2005). In addition, the elevated expression of CD64 receptor by monocyte from preclamptic women is in line with reports in pregnancy complications such as maternal infections (Naccasha et al., 2001), spontaneous premature delivery and in cases of premature rupture of the membranes (Gervasi et al., 2001; 2002). CD64 is constitutively present in monocytes and macrophages and has its expression increased in these cells under a pro-inflammatory condition such as rheumatoid arthritis (Luo et al., 2018), dengue (Naranjo-Gómez et al., 2019) and systemic lupus erythematosus (Kikuchi-Taura et al., 2015). In addition, it has been considered a biomarker for evaluation activity in systemic inflammatory diseases (Shimizu et al., 2018), and as an immunotherapeutic target for inflammatory diseases mediated by M1 macrophages (Akinrinmade et al., 2017). Thus, the downregulation of TLR4 and CD64 expression when monocytes from preeclamptic women were cultured with Sb reaffirms the flavonoids potential as an immunomodulator for the sterile inflammation established in PE.

Regarding to CD163 receptor expressed by monocytes from preeclamptic women, our results showed that treatment with Sb increased CD163 expression and IL-10 production by these cells. This haemoglobin scavenger receptor is considered an anti-inflammatory marker expressed exclusively by monocytes and macrophages (Etzederot & Moestrup, 2013). In PE, another recent study from our group have reported lower expression of CD163 in monocyte surface associated with decreased plasmatic levels of its soluble form (sCD163) in comparison to NT pregnant women (Nunes et al., 2019). Together, our results regarding surface receptors (TLR4, CD64 and CD163) expression and synthesis of pro and anti-inflammatory cytokines suggest that *in vitro* treatment of monocytes from PE women with Sb leads those cells to a M2 profile.

The effects of Sb on monocyte activation state, detected in preeclamptic women, was evaluated in monocytes from non-pregnant women (NP) cultured with MSU, as a model of cell activation induction by uric acid, a DAMP that plays an important role in PE (Matias et al., 2015). Higher concentrations of uric acid in plasma from preeclamptic women showed correlation with free radicals and inflammatory cytokines production by monocytes (Peraçoli et al., 2011) and may interact with TLRs or NLRP receptors, actions that lead to monocyte activation (Romão-Veiga et al., 2018). Our group has previously demonstrated that monocytes from preeclamptic women, incubated with MSU, presented higher activation of NLRP3 inflammasome and TLR4/NF-κB pathway, in addition to increase in IL-1β, IL-18 and TNF-α production (Matias et al., 2019).

In the present study, monocytes obtained from healthy NP women were cultured with or without MSU, Sb or MSU+Sb, and the surface receptors TLR4, CD64 and CD163 as well as pro- and anti-inflammatory cytokines were determined. The participation of the intracytoplasmic transcription factors NF-kB, IkB in these cultures was also evaluated. Results showed that similarly to what occurs in PE, MSU-stimulated

monocytes from NP showed significantly higher expression of TLR4 and CD64 and lower CD163 expression. Moreover, MSU induced higher production of IL-1β, IL-8, IL-23 and TNF-α and decreased IL-10 and TGF-β synthesis in comparison to control cultures. Regarding NF-κB pathway, MSU increased the expression of NF-κBp65 in monocytes from healthy NP women, while addition of MSU and Sb to the cultures restores not only the expression of TLR4, CD64 and NF-κBp65, but also the synthesis of IL-1β, IL-23, TNF-α and IL-10 back to basal levels found in control, non-stimulated cultures. The effect of MSU on NF-kB expression was abrogated after addition of the NF-κB pathway inhibitor, Bay 11-7082, to monocytes cultured with MSU, Sb or MSU+Sb, leading to non-significant differences between all treatments. These results, corroborate our previous studies demonstrating that Sb plays anti-inflammatory and antioxidant role by inhibing the activation of NF-κB, release of TNF-α and IL-1β and the production of superoxide and hydrogen peroxide by monocytes of preeclamptic women (Giorgi et al., 2012; Cristofalo et al., 2013). In addition to downregulation of TLR4/NF-kB pathway, Sb inhibits endogenous activation of NLRP1/NLRP3 inflammasomes in monocytes of preeclamptic women that may be involved in the intense systemic inflammation, characteristic of PE (Matias et al., 2019).

Conclusion

The results of the present study show that the *in vitro* treatment of monocytes from preeclamptic women with Sb downregulated the endogenous activation of NF-κB, expression of surface receptors TLR4 and CD64, and release of inflammatory cytokines by these cells. The presence of this flavonoid in monocyte cultures increased the expression of CD163 and IκBα and the release of IL-10 and TGF-β in culture supernatants, polarizing these cells from M1 to M2 profile. The anti-inflammatory activity of SB on the NF-kB activation pathway and induction of cell polarization to M2 profile was confirmed by an *in vitro* assay using monocytes from healthy,

non-pregnant women. Taken together, the results show that Sb has an immunomodulatory effect on sterile inflammation characteristic of preeclampsia.

Author contributions: VJG, MRV and MTSP conceived and designed the study, analyzed the results wrote the manuscript; JCP selected individuals for the study; VJG, MLM, PRN, VRR, ACC and CFBC performed the experiments. GGR assisted with her expertise in flow cytometry technique. All authors have read and approved the manuscript.

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Capítulo III

Monocytes from preeclamptic women previously treated with silibinin attenuates oxidative stress in human endothelial cells

Virgínia Juliani Gomes[1]*, Priscila Rezeck Nunes[1], Sarah McCann Haworth[2], Valéria Cristina Sandrim[3], José Carlos Peraçoli[1], Maria Terezinha Serrão Peraçoli[3], Mattias Carlström[2]

[1] Botucatu Medical School, Sao Paulo State University (Unesp), Botucatu, Sao Paulo, Brazil
[2] Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden
[3] Institute of Biosciences of Botucatu, Sao Paulo State University (Unesp), Botucatu, Sao Paulo, Brazil

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*Corresponding author at: Department of Gynaecology and Obstetrics, Botucatu Medical School, São Paulo State University. Botucatu, São Paulo, Brazil, CEP 18618-687 Phone: 55 14 3880 0431
Email address: vi.juliani@hotmail.com

Abstract

Preeclampsia is a gestational multiorgan syndrome characterized by hypertension, oxidative stress and endothelial dysfunction. Monocytes from peripheral blood of preeclamptic women are a source of free radicals and inflammatory cytokines. Imbalance between nitric oxide (NO) and reactive oxygen species is considered to play a role in the pathogenesis of preeclampsia, which may be improved by antioxidant therapy. This study aimed at evaluating if the supernatant of monocytes from preeclamptic women treated in vitro with the flavonoid antioxidant silibinin can modulate oxidative stress and improve NO bioavailability in human umbilical vein endothelial cells (HUVEC). The cells were incubated for 24h with supernatant of monocytes from healthy and preeclamptic pregnant women previously cultured with or without silibinin. Subsequently, cells and supernatants were used for various assays, i.e. DAF-FM™ and HPLC approaches to assess NO and its metabolites nitrate and nitrite. Determination of thiobarbituric acid reactive substances was used to evaluate lipid peroxidation. Heme oxygenase-1 (HO-1) was determined by ELISA. NO generation by HUVEC cultured with supernatant of monocytes treated with silibinin were not significantly different between preeclamptic and healthy groups. Nitrite levels were increased in HUVEC exposed to supernatant of preeclamptic monocytes treated with silibinin. Preeclamptic monocytes supernatants showed higher levels of nitrate regardless the treatment with silibinin. Treatment with silibinin reduced lipid peroxidation and increased HO-1 levels in HUVEC cultured with supernatant of monocytes from preeclamptic women. Taken together these results suggest that monocytes during preeclampsia can induce oxidative stress and endothelial dysfunction, which can be dampened by silibinin.

Key words: preeclampsia; hypertension; nitric oxide, antioxidant, flavonoid, monocytes, oxidative stress, human endothelial cells

1. Introduction

Preeclampsia (PE) is a multiorgan syndrome that affects 2-10% of pregnancies and is the leading cause of mortality, morbidity and premature labor (ACOG, 2019). This pathology is identified primarily by onset of clinical parameters such as hypertension and proteinuria from 20 weeks of gestation or by hypertension associated with maternal neurologic or hematologic complications, kidney failure, liver involvement or fetal growth restriction (Tranquilli et al., 2014; Mol et al., 2016). Moreover, PE is associated with endothelial cell dysfunction, elevated serum levels of inflammatory cytokines and oxidative stress (Borzychowski et al., 2006).

Poor placentation seems to induce a hypoxic-reperfusion injury causing enhanced oxidative stress in PE. In normal gestation, by 10-12 weeks of pregnancy, maternal blood flow in the placenta causes a local increased activity of antioxidant enzymes (Jauniaux et al., 2000). On the other hand, in pregnancies subsequently complicated by PE there is a decrease in enzymatic antioxidant capacity (Raijmakers et al., 2004).

Our previous studies have shown that monocytes from PE women are endogenously activated and therefore release significantly higher concentrations of tumour necrosis factor α (TNF-α) and reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) when compared to monocytes from normotensive (NT) healthy pregnant women (Peraçoli et al., 2011), suggesting that PE is coupled with oxidative stress and that the monocytes from maternal peripheral blood might be an important source of free radicals and inflammatory cytokines involved in the pathogenesis of this disease. These results are in agreement with previous findings demonstrating increased endothelial dysfunction and enhanced lipid peroxidation in pregnant women with PE (Cester et al., 1994; Brandão et al., 2014).

Nitric oxide (NO) importantly contributes to cardiovascular homeostasis by acting as a potent vasodilator (Osol et al., 2017). This signalling molecule causes vasodilation of the utero-placental arteries, which is vital for trophoblast invasion and remodelling of the endothelium (Kaufmann et al., 2003). Imbalance between NO and ROS seems to play an important role pathogenesis of PE (Matsubara et al., 2015). Novel strategies decreasing oxidative stress and increasing NO bioavailability may have therapeutic potential.

The transcription factor Nrf2 is a major redox-sensitive regulator of oxidative stress signaling (Ma et al., 2013; Van der Wijst et al., 2014). Nrf2 binds to the antioxidant response element (ARE) promoter sequence, regulating the expression of antioxidant proteins, including heme oxygenase-1 (HO-1), which is capable to inhibit oxidative stress and balance redox state in cells (Kweider et al., 2014). It has been demonstrated that the

inhibition of HO-1 activity increases blood pressure in pregnant rats (George et al., 2013), while induction of HO-1 attenuates placental ischemia-induced hypertension in a rat model of PE (George et al., 2011).

Those PE characteristics justify the interest in using antioxidants as therapeutic options. Silymarin is a polyphenolic flavonoid obtained from seeds and fruits of *Silybum marianum*, used as anti-inflammatory and hepatoprotective agent over two thousand years. Silibinin (Sb) is the main component (60-70%) and the most active one from sylimarin (Dixit et al., 2007), which presents anti-inflammatory, hepatoprotective, anti-fibrotic, anti-carcinogenic, antioxidative and cytoprotective effects (Kren & Walterová, 2005; Agarwal et al., 2006; Deep & Agarwal et al., 2010; Esmaiel et al., 2017) . Nowadays, Sb is still often prescribed as an alternative or a complementary hepatoprotective medicine. The protective action of Sb seems to be a result of its antioxidant properties (Post-White et al., 2007).

Considering that PE is characterized by oxidative stress and endothelial dysfunction, we investigated the hypothesis that administration of Sb may have therapeutic effects during PE by dampening immune cell activation and oxidative stress responses in human endothelial cells.

2. Research Design

2.1 Study Population

Peripheral blood from 10 pregnant normotensive women (NT) and 10 PE women were collected at the Obstetrics Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP – Brazil, between May 2018 and April 2019. Exclusion criteria included chronic hypertension or history of hypertension, multiple gestation, prior PE, illicit drug use, and pre-existing medical conditions such as diabetes, cancer, acute infectious disease, cardiovascular, autoimmune, renal and hepatic diseases. A pregnant woman was considered as having PE when she had hypertension (≥140/90 mmHg in systolic and diastolic pressure, respectively) associated with proteinuria (≥ 300 mg in 24-hour urine) after the 20th week of gestation. The Ethics Committee of the Botucatu Medical School approved all collections (protocol number 1.622.546 and 3.826.694) and all women gave written informed consent.

2.2 Collection of peripheral blood

Peripheral blood was collected at the time of disease diagnosis, and from the NT pregnant women at the time at which they matched to gestational age with PE women. Blood was collected into plastic tubes containing 5% EDTA. Ten mL of blood was collected by venepuncture from the antecubital vein of PE and NT women.

2.3 Monocyte cultures and collection of supernatant

Peripheral blood mononuclear cells (PBMC) were obtained by gradient separation from Ficoll-Paque Premium (GE Healthcare Bio-Sciences, Uppsala, Sweden), according to the technique described by Peraçoli et al. (2011). Then, the cells were resuspended in RPMI 1640 culture medium (Sigma-Aldrich, SL, Missouri, USA) supplemented with 10% inactivated fetal bovine serum (complete medium),and counted with 5% Turk dye solution (1:1v/v). The cell concentration was adjusted to 1×10^7 PBMC/mL. For monocyte separation, the magnetic beads from Human Pan Monocyte Isolation Kit (Miltenyi Biotec, Germany) was employed according to the manufacturer's instructions. After isolation, the monocytes obtained were counted with 5% Turk's solution and the concentration was adjusted as needed for each of the following experiments.

Monocytes from preeclamptic and normotensive pregnant women were cultured with or without 50 µM of Sb (Sigma-Aldrich) as previously standardized by Bannwart et al. (2010), after 18 hours of incubation monocyte supernatant was aspirated and storage at -80˚C.

2.4 Human umbilical vein endothelial cell and supernatant incubation

Human umbilical vein endothelial cell - HUVEC (Lonza, Switzerland) was cultured until reaching 80% confluence and then incubated for 24 hours in medium 199 (Gibco BRL Life Technologies, The Netherlands) with 20% (v/v) of supernatant obtained from monocytes previously cultured with or without Sb. The cells and supernatant obtained were used to perform the assays. The HUVEC cultured with the supernatant of monocytes from NT and PE pregnant women were incubated for 1 hour with PrestoBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, Califórnia, USA), in order to determine cell viability. The plate fluorescence was read using excitation wavelength of 560 nn in Spectramax iD3 multi-mode microplate reader (Hidex, Lablogic systems, Sheffield, UK).

2.5 Determination of TBARS to measure lipid peroxidation

Levels of lipid peroxidation in the supernatant of HUVEC cultures were measured by Thiobarbituric acid reactive substances (TBARS). An aliquot of 100 µL of supernatant was incubated on ice with 200 µL of 10% cold Trichloroacetic acid (TCA- Sigma-Aldrich) for 15 minutes. Meanwhile, standards were prepared in a serial dilution with 1,1,1,3-tetramethoxypropane (TMP- $C_7H_{16}O_4$, 133.75 mM, Sigma-Aldrich). The samples with TCA and the standards were centrifuged at 2.200g for 15 minutes at 4ºC. After that, 200 µL supernatant of the centrifuged samples and standards were placed in cryotubes with 200 µL of 0.67% thiobarbituric acid (TBA – Sigma – Aldrich) and incubated in boiling water bath at 95ºC for 50 min. Finally, the tubes were placed on ice for 3 min. Standards and samples were placed in a 96-well plate and the absorbance was measured

at 532 nm in Spectramax iD3 multi-mode microplate reader and the TBARS values were calculated using a malondialdehyde (MDA) standard curve. The values were expressed as nanomoles of MDA per mL.

2.6 Determination of nitric oxide species

HUVEC were plated ($5x10^4$ per well) and treated with 20% (v/v) of supernatant from monocytes in black 96-well plate. After 24h of treatment, the media was aspirated and 95 µL of saline solution (PBS) was added in each well. The plate was then incubated for 30 min at 37ºC. After the incubation, 5 µL of DAF-FM™ 200µM (Sigma-Aldrich, USA) was pipetted in each well and the plate was read for 2 hours at 37 ºC using Spectramax iD3. The fluorescence was measured at 495nm (excitation) and 535nm (emission), then expressed as arbitrary units to estimate NO species.

2.7 Measurement of nitrate and nitrite

Nitrate and nitrite levels were determined using a high performance liquid chromatography (HPLC) system (ENO-20; Eicom, USA). The ENO-20's high sensitivity and specificity were accomplished with the combination of a diazo coupling method and chromatography. The level of diazo compound is measured by absorbance at 540 nm (Spectramax iD3).

2.8 Measurement of HO-1

Heme-Oxygenase 1 concentrations in HUVEC supernatant after monocyte supernatant treatments were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit from R&D System (Minneapolis, MN, USA) following manufactory's instruction (Cat.No. DYC3776-5).

2.9 Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. A p value less than 0.05 was considered as statistically significant. PRISM software 6.0 (GraphPad, San Diego, CA, EUA) was utilized for the analysis.

3. Results

3.1 Cell Viability

Before performing any experiment, the cell viability of HUVECs incubated with supernatant of monocytes from PE and NT pregnant women was verified with PrestoBlue™ Cell Viability Reagent (Invitrogen, Carlsbad, Califórnia, USA). In all cases, viability was higher than 80% and no significant differences were found between groups, indicating that the treatments were not harmful to the cells (Fig. 1).

3.2 Measurement of lipid peroxidation

Lipid peroxidation were higher in the supernatant of HUVEC treated with the supernatant of monocytes from PE women when compared to cells treated with supernatant of monocytes from NT pregnant women (Figure 2). The supernatant of monocytes from PE women treated with Sb induced lower levels of lipid peroxidation when compared to cells that received supernatant of non-treated monocytes from women with PE.

3.3 Determination of NO-related species

Measurement of NO-related species, using DAF-FM fluorescence, did not reveal any significant difference between groups when HUVEC were exposed to supernatant of monocytes treated with Sb (Figure 3).

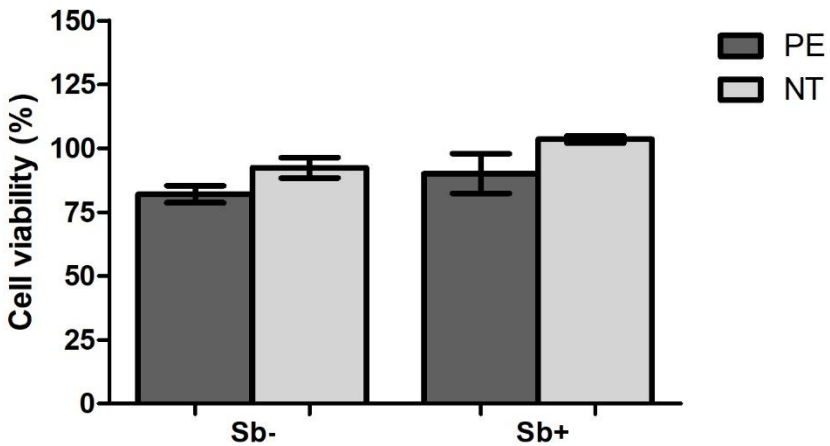

Figure 1. Effects of the treatments on human endotelial cells (HUVECs) viability, accessed by PrestoBlue™ assay. These cells were incubated with supernatant of monocytes (PE and NT) treated with (Sb+) or without (Sb-) silibinin. Data presented as means + SD for one independent experiement, performed in quintuplicates.

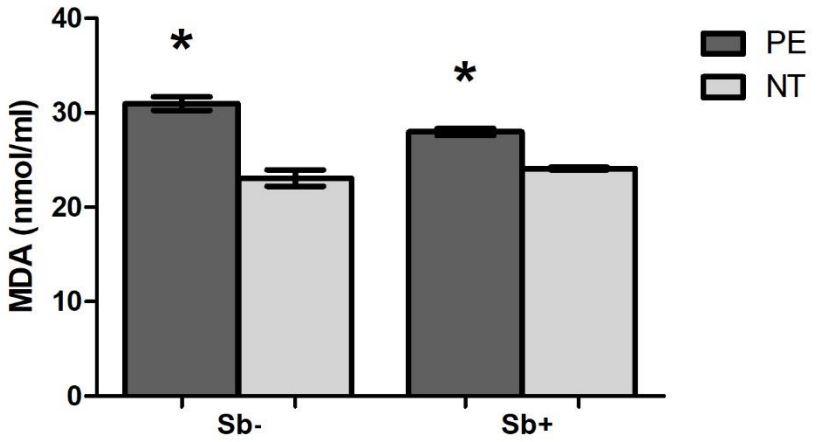

Figure 2. MDA levels in supernatant of HUVEC after 24h of incubation with supernatant of monocytes from PE and NT pregnant women treated with (Sb+) or without (Sb-) silibinin. Data presented as mean ± SD. One-way ANOVA followed by Bonferroni's multiple comparison test. * p<0,05 vs. all treatments.

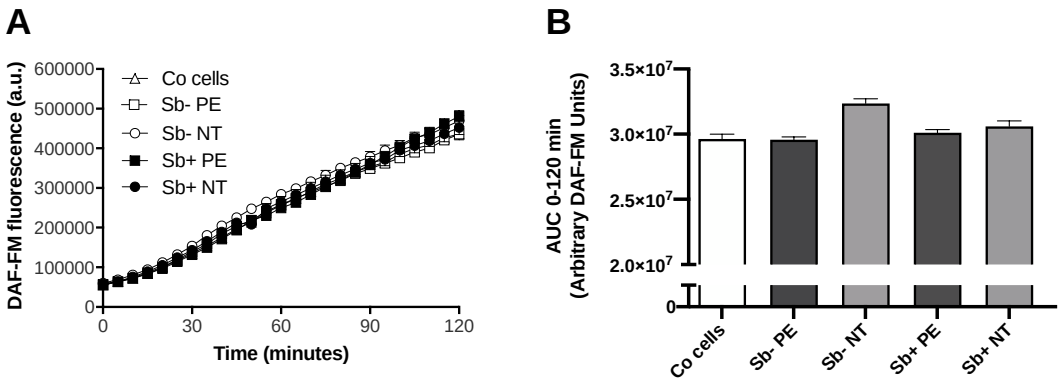

Figure 3. DAF-FM™ fluorescence continuous signal for 2 hours (A) and the area under the curve (B) in HUVEC exposed to supernatant of monocytes (PE-NT) treated with (Sb+) or without (Sb-) silibinin. Co cells = HUVEC without treatment.

3.4 Measurement of nitrite and nitrate

High performance liquid chromatography (HPLC) showed that levels of nitrite were significantly higher in HUVEC cultures that were incubated with the supernatant of monocytes from PE women treated with Sb (figure 4a) and the levels of nitrate were higher when HUVEC were treated with PE samples, regardless the Sb treatment (figure 4b).

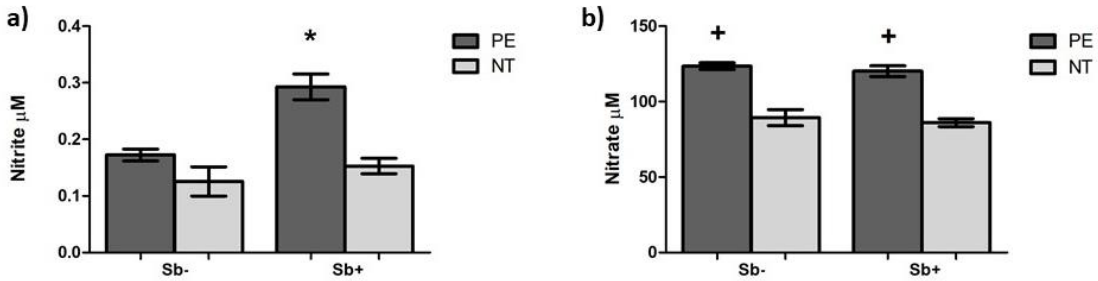

Figure 4. Levels of nitrite (a) and nitrate (b) in supernatant of HUVEC exposed to supernatant of monocytes (PE-NT) treated with (Sb+) or without (Sb-) silibinin. One-way ANOVA followed by Bonferroni's multiple comparison test. * $p<0,05$ vs. all treatments; + $p<0,05$ vs. Sb- NT and Sb+ NT.

3.5 Heme-Oxygenase-1

Figure 5 shows HO-1 levels in supernatant of HUVEC after monocyte supernatants treatments. HUVEC exposed to supernatant of monocytes from PE women treated with Sb showed higher levels of HO-1 compared to cells that received the supernatant of nontreated monocytes from women with PE.

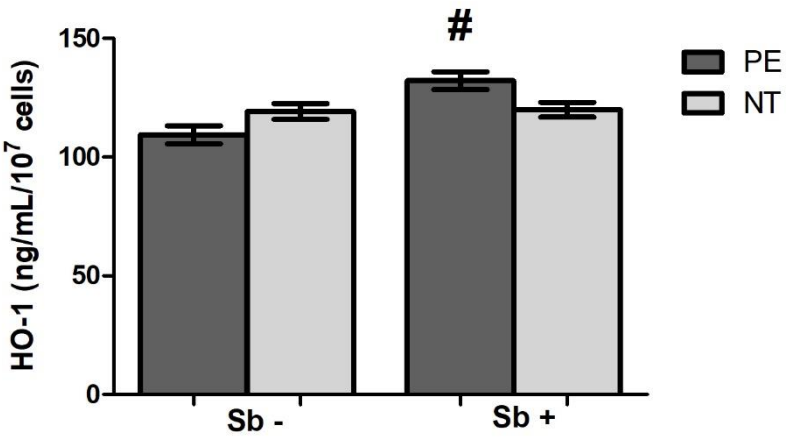

Figure 5. Levels of Heme oxygenase-1 (HO-1) in supernatant of HUVEC exposed to supernatant of monocytes (PE-NT) treated with or without silibinin. One-way ANOVA followed by Bonferroni's multiple comparison test. # $p<0,05$ vs. Sb- PE.

4. Discussion

Healthy pregnancy is characterized by adaptations in maternal cardiovascular system that allow a more effective delivery of oxygen and nutrients for the fetus. These changes include increased blood volume, decrease of vascular resistance and increased cardiac output (Wedel et al., 2009; Osol et al., 2017). NO is a major mediator of the cardiovascular system, acting as a potent vasodilator (Osol et al., 2017). Hodžić et al. (2017) showed that NO production increases with gestational age in healthy pregnancy. However, studies about NO production and activity in PE show conflicting results. Some studies report increased levels of NO in during PE in comparison with healthy pregnancies (Shaamash et al., 2000; Teran et al., 2009; Acauan et al., 2016), while others demonstrated decreased levels in women with PE (Mao et al., 2010; Ehsanipoor et al., 2013). The conflict between these findings indicates that NO production is not necessarily compromised in PE, but the bioactivity of NO may be altered in this disease (Acauan et al., 2016). NO presents a complex chemistry and its generation will inevitably culminate in the formation of reactive nitrogen species (Matsubara et al., 2010). In a context of oxidative stress, the exacerbated presence of superoxide anions (O_2^-) leads to the depletion of NO, forming peroxynitrite ($ONOO^-$), a reactive nitrogen specie that can damage lipid membranes, DNA and mitochondrial function. Thus, NO not only stops playing its vasodilator role, but also participate in endothelial disfunction (Haram et al.2019).

In the present study, we did not observe any statistical difference in NO generation between HUVEC exposed to supernatant from monocytes from

PE and NT pregnant women. The Sb treatment did not show any difference between groups, suggesting that this flavonoid does not act on the NO signaling pathway.

However, considering that the half-life of NO is extremely short, probably less than one second, the quantification of this molecule is considered not entirely reliable. NO is continuously converted to nitrite by autoxidation and to nitrate by oxyhaemoglobin in red blood cells (Lundberg et al., 2008). Nevertheless, both nitrite and nitrate anions are more stable metabolites found in blood and urine, and their levels in in plasma or urine has often been used to indirectly quantify NOS activity in vivo (Tsikas, 2005). Moreover, Kleinbongard et al. (2003) demonstrated that plasmatic nitrite levels retract endothelial-dependent NO production in humans and mammals. In addition, nitrate and nitrite are endogenously converted to NO, thereby acting as a storage of this molecule and as a complementation to the NO synthase-dependent pathway to produce NO (Lundberg et al., 1994; Benjamin et al., 1994)

Our results demonstrated significantly higher levels of nitrite in cultures exposed to supernatant of monocytes treated with Sb and levels of nitrate were increased when HUVEC were incubated with PE samples, despite the Sb treatment. Literature shows opposing results when it comes to levels of nitrite and nitrate in preeclampsia. Some reports demonstrated that those levels are increased in the PE plasma (Shaamash., 2000; Noorbakhsh et al., 2013), whereas others showed them decreased (Ehsanipoor et al., 2013; Eleuterio et al., 2013; Pimentel, 2013). These conflicting data may be a consequence of different diets (Keimer et al., 2003) and methodological approaches (Tsikas et al., 2005). Regarding nitrate levels, another possible explanation for these discrepant results is related to its urinary excretion (Hodžić et al., 2017). Renal impairment is considered rare in preeclampsia (1% of the cases) and yet proteinuria is a criterion for determining the disease severity (Pagani et al., 2019). In this sense, kidney injury may interfere in the levels of circulating nitrate in plasma and urine.

The insufficiency of antioxidants in PE women seem to be related to the increase of lipid peroxides in these pregnancies (Davidge et al., 1992). Lipid peroxidation is a commonly used indicator of oxidative stress, because it occurs when free radicals interact with membrane polyunsaturated fatty acids, which are transformed into secondary metabolites such as malondialdehyde (MDA) (Belo et al., 2004). The present study showed that HUVEC exposed to supernatant of monocytes from PE women demonstrated increased MDA levels when compared to cells incubated with NT supernatant. Moreover, HUVEC treated with PE samples showed lower levels of lipid peroxidation when monocytes were previously treated with Sb. These results are in agreement with a previous study from our research group that demonstrated that Sb decreases spontaneous O_2^- and H_2O_2 release and TNF- α production by monocytes from women with PE (Cristofalo et al., 2013).

The products of lipid peroxidation are carried by lipoproteins into the

circulation and may interact with and compromise other tissues, such as the endothelium (Burton et al., 2011). The increase of endothelial function is essential for the development of a healthy pregnancy. Brandão et al. (2014) reported that this endothelial adaptation was compromised in women who presented both early and late PE. Hypertension, proteinuria, edema, impaired platelet aggregation and other symptoms of PE can be intensified by endothelial dysfunction (Hellmo et al., 2018). Glomerular barrier integrity loss is a common example of an endothelial dysfunction consequence in PE, which results in the exacerbation of protein excretion in the urine (Wang et al., 2015). In severe cases of PE, the breakdown of the blood-brain barrier can lead to brain edema and seizures (Hammer et al., 2015).

Preeclampsia is marked by a deficient remodelling of the spiral arteries and compromised trophoblastic invasion of uterine vessels (Redman & Sargent, 2005). Our results demonstrated that HUVEC cultivated with supernatant of monocytes from PE women previously treated with Sb showed higher levels of HO-1 than cells that were exposed to supernatant of non-treated monocytes from women with PE. Recently, several studies have demonstrated the importance of HO-1 in the maintenance of a healthy pregnancy. Zhao et al. reported that low maternal levels of HO-1 in an experimental model in mice lead to modifications in uterine natural killer cell proliferation and maturation, poor formation of the maternal-fetal interface and failure in the vascular remodelling (Zhao et al., 2011). HO-1 serum concentration in PE patients have been described as lower than in healthy pregnancy (Anderson et al., 2018), but there have been also published conflicting data showing that HO-1 serum levels are increased in PE (Ozen et al., 2015).

In summary, the present study confirmed the fact that oxidative stress and endothelial dysfunction are increased in PE. To our knowledge, this is the first study that measured oxidative stress and explored the NO signaling pathway in human endothelial cells after the incubation with supernatant of monocytes from PE women. The treatment of monocytes with Sb was able to inhibit lipid peroxidation but did not seem to interfere with the bioavailability of NO in HUVEC.

Author contributions: VJG, MTSP, VCS and MC conceived and designed the study, analyzed the results and wrote the manuscript; JCP selected individuals for the study; VJG, PRN and SMH performed the experiments. All authors read and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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