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Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Tocoginecologia.

Orientadora: Profª. Titular Maria Terezinha Serrão Peraçoli
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Botucatu
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"Pois onde estiver o seu tesouro, aí também estará o seu coração."

Mateus 6:19-21

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"A vida é um instante entre duas eternidades."

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"Na minha experiência, você quase sempre pode desfrutar das coisas se decidir, com firmeza, fazê-lo."

Anne de Green Gables

Lucy Maud Montgomery

PREFÁCIO

O documento da Tese para obtenção do título de Doutora em Tocoginecologia, intitulado: "Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia" encontra-se dividido em 6 capítulos.

Inicialmente, a Introdução aborda uma revisão da literatura a respeito da patogênese da pré-eclâmpsia (PE) e suas manifestações clínicas. Também é apresentada a literatura relacionada aos eventos que desencadeiam a inflamação e ativação do inflamassoma, assim como o papel da vitamina D (VD) na PE.

O capítulo um apresenta o manuscrito intitulado "*Vitamin D decreases expression of NLRP1 and NLRP3 inflammasomes in placental explants from women with preeclampsia*", que foi submetido à publicação ao periódico *Cytokine.* Os resultados desse manuscrito mostram que o tratamento *in vitro* com VD sugere um papel imunomodulador na inflamação placentária de gestantes com PE e regulação do estresse oxidativo induzido pelo H_2O_2 em explantes placentários.

O capítulo dois contempla o manuscrito intitulado: "*Effects of vitamin D-induced supernatant of placental explants from preeclamptic women on oxidative stress and nitric oxide bioavailability in human umbilical vein endothelial cells*". Os resultados desse capítulo foram publicados no periódico *Brazilian Journal of Medical and Biological Research* (fator de impacto 2,046). Esse trabalho foi desenvolvido durante o estágio de pesquisa no exterior (BEPE-FAPESP), no Karolinska Institutet, Estocolmo (Suécia), com supervisão do Professor Mattias Carlström.

O capítulo três está apresentado em forma de manuscrito que será enviado para publicação, entitulado: *"Vitamin D modulates inflammasomes in placental explants of preeclamptic women and decreased apoptosis in huvec cultured with*

monosodium urate" e apresenta os resultados de expressão gênica e proteica de componentes do inflamassoma e citocinas inflamatórias em explantes placentários e também apoptose em células endoteliais (HUVEC) frente ao estímulo com urato monossódico (MSU). Esses dados mostram a ação da VD na modulação da ativação dos inflamassomas NLRP1/NLRP3 e na diminuição da produção de citocinas inflamatórias e apoptose.

No capítulo quatro, entitulado *"Vitamin D mantains viability and decreases apoptosis in HUVEC and modulates inflammation in placental explants from preeclamptic pregnant women cultured with TNF-α",* os resultados demonstraram que o tratamento *in vitro* com VD tem um papel imunomodulador na produção de citocinas em explantes placentários e apoptose em células endoteliais cultivadas com TNF-α. Esse capítulo também está apresentado em forma de manuscrito que será enviado para publicação.

Por fim, no capítulo cinco, encontram-se apresentadas as conclusões gerais do trabalho.

O capítulo seis contém os anexos referentes aos Termos de Consentimento Livre e Esclarecido e ao Protocolo do Comitê de Ética em Pesquisa.

Sumário

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ANOVA: análise de variância
cDNA: DNA complementar
cm: centímetros
DAMPs: padrões moleculares associados ao dano
DCFH: *dichlorodihydrofluorescein diacetate*
DMSO: *dimethyl sulfoxide*
DNA: ácido desoxirribonucleico
DR5: *death receptor 5*
EDTA: ácido etilenodiamino tetra-acético
ELISA: ensaio de imunoabsorção enzimática
EOPE: *early-onset preeclampsia*
EROs: espécies reativas de oxigênio
FITC: Isotiocianato de fluoresceína
GAPDH: gliceraldeído 3-fosfato- desidrogenase
h: horas
H_2O_2: peróxido de hidrogênio
HCl: ácido clorídrico
Hg: mercúrio
HMGB1: *high-mobility group box one*
HPLC: *high-performance liquid chromatography*
HUVEC: *Human Umbilical Vein Endothelial Cells*
IL: interleucina
IFN-γ: interferon gama
L: litro
LDH: lactato desidrogenase
LOPE: *late-onset preeclampsia*
MDA: malondialdeído
mg: miligramas
min: minuto
mL: mililitros
mM: milimolar
mm: milímetros
MSU: urato monossódico
NaCl: cloreto de sódio
ng: nanograma

NLRP: receptor *nod-like*
NT: normotensa
NO: óxido nítrico
NO_2^-: nitrato
NO_3^-: nitrito
ºC: graus Celsius
O_2^-: ânion superóxido
***p*:** nível de significância
PAMPs: padrões moleculares associados à patógenos
PBS: solução salina tamponada (*phosphate buffered saline*)
PE: pré-eclâmpsia
PI: *propidium iodide*
PRR: receptores de reconhecimento padrão
RNA: ácido ribonucleico
RNAm: RNA mensageiro
RPM: rotação por minuto
RPMI: meio sintético complexo (*Roswell Park Memorial Institute*)
ROS: *reactive oxygen species*
qPCR: reação em cadeia da polimerase quantitativa em tempo real
s: segundo
SD: desvio padrão (*standard deviation*)
SBF: soro bovino fetal
SNP: *single nucleotide polymorphisms*
TBARS: *thiobarbituric acid reactive substances*
Tbhp: *Tert-butyl hydroperoxide*
TCA: *trichloroacetic acid*
TMP: t*etramethoxypropane*
TNF-α: fator de necrose tumoral alfa
TRAIL: *tumor necrosis factor related apoptosis-inducing ligand*
VD: vitamina D
VDR: receptor de vitamina D (*vitamin D receptor*)
µg: micrograma
µL: microlitro
µM: micromolar
25(OH)D: 25-hidroxivitamina D
1,25(OH)D: 1,25- dihidroxivitamina D

Resumo

Gestações complicadas por pré-eclâmpsia (PE) estão associadas com isquemia/hipóxia placentária, estresse oxidativo e níveis elevados de citocinas pró-inflamatórias. A ativação do inflamassoma NLRP3, representada por maior expressão gênica e proteica dos componentes desse complexo relacionado à inflamação, como caspase-1, interleucina-1 beta (IL-1β) e *high-mobility group box-1* (HMGB1), foi descrita em placenta de gestantes pré-eclâmpticas, quando comparadas às gestantes normotensas (NT). A hiperativação do inflamassoma NLRP3 nos tecidos placentários pode estar envolvida no estado inflamatório sistêmico exacerbado da PE. O presente trabalho teve como objetivo avaliar a ação imunomoduladora da vitamina D (VD) sobre os inflamassomas NLRP1 e NLRP3 em explantes placentários de gestantes portadoras de PE e de NT e *Human Umbilical Vein Endothelial Cells* (HUVEC), bem como a apoptose nessas células. Foram avaliadas 30 gestantes assim distribuídas: 10 portadoras de PE precoce (idade gestacional < 34 semanas), 10 portadoras de PE tardia (idade gestacional ≥ 34 semanas) e 10 NT. Após a realização do parto cesáreo, fragmentos de aproximadamente 10 mg foram imediatamente retirados da região central da placenta, de modo que constituíssem amostras do citotrofoblasto viloso e da região do sincociotrofoblasto em contato com a face materna. Os explantes e as HUVEC foram cultivados na presença ou ausência de peróxido de hidrogênio (H_2O_2), urato monossódico (MSU), fator de necrose tumoral (TNF-α) e tratados com VD. Após 4 e 18 horas de cultivo os explantes e as células endoteliais foram submetidos à avaliação da expressão gênica e proteica de NLRP1, NLRP3, caspase-1, IL-1β, IL-18, TNF-α, HMGB1, *tumor necrosis factor related apoptosis inducing ligand* (TRAIL) e *vitamin D receptor* (VDR) por qPCR, ELISA e Western Blot. A apoptose nas células também foi avaliada por citometria de fluxo. De maneira geral, os resultados mostraram que o tratamento *in vitro* com VD é capaz de modular a inflamação placentária em gestantes com PE e diminuir o estresse oxidativo, induzido pelos tratamentos com MSU e H_2O_2 em explantes placentários e HUVEC, bem como aumentar a viabilidade e diminuir a apoptose nessas células estimuladas com TNF-α. A utilização de agentes com propriedades anti-inflamatórias e antioxidants, como a VD é um campo de pesquisa que deve ser mais explorado em futuras investigações para entender o mecanismo pelo qual a VD atua na imunomodulação da resposta inflamatória na PE.

Palavras-chave: Pré-eclâmpsia, explantes placentários, HUVEC, vitamina D, citocinas, inflamassomas, apoptose.

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota".

Madre Teresa de Calcutá

INTRODUÇÃO

A pré-eclâmpsia (PE) é uma síndrome específica da gestação humana, considerada a principal causa de morbidade e mortalidade entre 2 a 8% das gestações no mundo todo (ACOG, 2020) e uma das principais causas de morte materna no Brasil, responsável por 37% das causas de morte obstétricas diretas (Laurenti et al., 2009). Os parâmetros clínicos que caracterizam essa patologia são: hipertensão arterial e proteinúria a partir da vigésima semana de gestação ou nos primeiros dias após o parto. Entretanto, outras disfunções maternas também podem estar relacionadas com a PE, como insuficiência renal, envolvimento hepático, complicações neurológicas ou hematológicas, disfunção útero-placentária ou restrição de crescimento fetal (Tranquilli et al., 2014; Mol et al., 2016).

A gestação normal é caracterizada por resposta inflamatória sistêmica leve, representando uma resposta de adaptação do organismo materno para permitir o crescimento e desenvolvimento fetal (van Rijn et al., 2008). Essa adaptação é resultado da ativação da imunidade inata, levando à reação inflamatória leve, que não indica doença, mas atua como mecanismo protetor do organismo materno contra infecções, visto que a resposta imune adaptativa se encontra ligeiramente diminuída (Wegmann et al., 1993).

Durante o segundo trimestre de gravidez, ocorre o processo de remodelação das artérias espiraladas maternas que, com a invasão do trofoblasto, se diferenciam fenotipicamente em células endoteliais (Collins et al., 2012). A vasculogênese garante o suprimento sangüíneo adequado para a placenta e o suporte nutricional e respiratório do feto. Entretanto, observa-se que em placentas de gestantes com PE a invasão trofoblástica é inadequada, acontecendo em apenas 30-50% das artérias (von Dadelszen et al., 2003). Essa falência na remodelação vascular pode levar à má perfusão e isquemia placentárias (George & Granger, 2011). A isquemia acontece devido ao fato de que as artérias não são suficientemente remodeladas, ocorrendo uma perfusão desordenada do fluxo sanguíneo para o espaço interviloso. Somado a um suprimento inadequado de nutrientes e oxigênio, ocorre a redução da área de superfície disponível para troca entre mãe e feto, o que pode contribuir para os desfechos desfavoráveis relacionados à gestação (Turco & Moffet, 2019).

A fisiopatologia da PE ainda não está totalmente esclarecida, mas

atualmente sabe-se que a isquemia placentária tem fundamental importância nesse processo, já que a liberação de produtos resultantes de má perfusão na circulação materna pode levar à disfunção endotelial sistêmica (Burton et al., 2019).

Além da isquemia placentária, o estresse oxidativo também é uma característica marcante em gestações complicadas pela PE (Burton & Jauniaux, 2004; Myatt & Cui, 2004) e pode ser resultante de lesão causada por hipóxia e reperfusão (Burton & Hung, 2003) e/ou deficiência das defesas antioxidantes (Perkins, 2006). Peraçoli et al. (2011) relataram a ativação endógena de monócitos de gestantes portadoras de PE, associada à produção elevada de ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e fator de necrose tumoral-α (TNF-α) por essas células, demonstrando que a PE é caracterizada por estresse oxidativo.

Os produtos resultantes da disfunção endotelial e estresse oxidativo podem agir como mediadores inflamatórios, ativando o sistema imune inato, o primeiro mecanismo pelo qual o organismo responde imediatamente a infecções e injúrias. Receptores de reconhecimento padrão (*pattern recognition receptors - PRRs*) reconhecem moléculas liberadas por patógenos ou células danificadas. Esses sinais moleculares são chamados de padrões moleculares associados ao dano (*damage-associated molecular pattern - DAMPs*) ou à patógenos (*pathogen associated molecular pattern - PAMPs*) (Gong et al., 2020).

As DAMPs podem estar presentes no plasma das gestantes e são consideradas importantes indutoras da resposta inflamatória. Alguns exemplos de DAMPs são moléculas como reativos intermediários do oxigênio, proteínas de choque térmico, proteínas liberadas de células mortas como o *high-mobility group Box-1* – HMGB1, e ácido úrico (Asea et al., 2002; Park et al., 2004).

Receptores de reconhecimento padrão, importantes na resposta inflamatória, fazem parte da família *Nod-like* ou NLR (proteína com domínio de oligomerização nucleotídica), são proteínas citosólicas capazes de reconhecer PAMPs e DAMPs citoplasmáticos e recrutar outras proteínas, formando complexos de sinalização que promovem a inflamação, denominados inflamassomas (Lamkanfi et al., 2007).

Entre todos os inflamassomas descritos até o momento, o *NLR family pyrin domain containing 3* (NLRP3) é o melhor caracterizado (Kelley et al., 2019). A ativação de NLRP3 pode ocorrer devido a vários estímulos, entre eles a produção

de espécies reativas de oxigênio (EROs) (He et al., 2016), que são os primeiros reativos intermediários produzidos durante esse processo, estando envolvidos na formação e ativação de NLRP3 e sendo responsáveis pela liberação de agentes inflamatórios durante a resposta imune (Abais et al., 2015).

Ainda não se sabe detalhadamente como as EROs atuam na ativação do inflamassoma. Estudos recentes indicam que elas são induzidas por ativadores de NLRP3 e são essenciais como mensageiros secundários para a sinalização de ativação de NLRP3 (Martinon, 2010). O_2^- e H_2O_2 podem agir como segundo sinal para ativar inflamassomas em podócitos, células importantes em lesões renais. Abais et al. (2014) mostraram que a decomposição da H_2O_2 pela catalase previne a agregação de inflamassomas NLRP3 em podócitos de camundongos, o que pode diminuir a injúria glomerular. Bauernfeind et al. (2009) investigaram o papel das substâncias capazes de inibir a produção de EROs, e encontraram evidências de que essas substâncias não necessariamente ativam o inflamassoma, mas fazem parte de sua indução. Não existem na literatura estudos que relacionam as EROs à ativação do inflamassoma na PE e melhores investigações são necessárias para adquirir mais conhecimento sobre essa via e desenvolver possíveis alvos terapêuticos.

Nunes et al. (2018) demonstraram que H_2O_2 leva à ativação de NLRP3 em explantes placentários de gestantes normotensas (NT), como consequência da indução de estresse celular, com aumento da expressão de caspase-1, interleucina 1β (IL-1β) e TNF-α. Esse processo inflamatório também está associado com baixa produção de interleucina 10 (IL-10), uma citocina essencial na gestação normal, auxiliando na placentação adequada, controlando a inflamação excessiva e regulando a expressão gênica de IL-1β e TNF-α (Moore et al., 2001; Cubro et al., 2018).

A ativação do inflamassoma gera formas ativas da interleucina IL-1β e IL-18 (Abbas et al., 2012). A IL-1β é uma citocina inflamatória importante, liberada durante a resposta inflamatória endotelial e, de maneira interessante, a ativação de NLRP3 produz grandes quantidades de IL-1β (Godo & Shimokawa, 2017). Além disso, esse processo contribui para liberação de HMGB1 e a morte celular induzida por patógenos bacterianos (Schroder & Tschopp, 2010; Gross et al., 2011). A ligação de DAMPs, como o ácido úrico, na forma de urato monossódico (MSU),

pode ativar o inflamassoma NLRP3 (Lamkanfi et al., 2007). Em trabalho recente, foi demonstrado que monócitos de gestantes com PE estimulados com MSU tem a expressão gênica do inflamassoma NLRP3 aumentada (Matias et al., 2015).

NLRP1 e NLRP3 também são expressos em células imunes, células endoteliais e em outros tipos celulares como células placentárias (Abrahams, 2011; Pontillo et al., 2013). Weel et al. (2017) avaliaram a ocorrência do inflamassoma NLRP3 em placenta de gestantes portadoras de PE, com o objetivo de desvendar o papel do inflamassoma na interface materno-fetal. Foram detectados níveis elevados de RNA mensageiro (RNAm) de NLRP3, caspase-1, IL-1β, TNF-α e HMGB1 em tecido placentário, bem como aumento significativo dos níveis de caspase-1, IL-1β, TNF-α e HMGB1 em homogenato de placenta de gestantes portadoras de PE, quando comparadas às NT. Esses resultados sugerem que a hiperativação do inflamassoma NLRP3 nos tecidos placentários pode estar envolvida no estado inflamatório sistêmico exacerbado da PE (Weel et al., 2017).

A inflamação persistente no endotélio estimula a secreção de vários mediadores inflamatórios, e por isso é um processo importante em alguma doenças inflamatórias, pois podem ativar diversos padrões de sinalização. A IL-1β é liberada durante a resposta inflamatória endotelial e a ativação de NLRP3 produz grandes quantidades de IL-1β (Godo & Shimokawa, 2017). Células endoteliais são alvo para IL-1β e também a produzem durante a inflamação (Wang et al., 2011). Além disso, o excesso de produção de EROs pode aumentar o catabolismo do óxido nítrico (NO), levando à diminuição da sua disponibilidade. Esse desbalanço entre EROs-NO leva à expressão de genes relacionados a inflamação, o que por sua vez pode prejudicar a função endotelial (Shihata et al., 2016).Observando a importância das células endoteliais na PE, a utilização de HUVECS se torna uma boa alternativa de modelo experimental *in vitro* (Caldeira-Dias et al., 2018; Gonçalves-Rizzi et al., 2018; Caldeira-Dias et al. 2019).

A apoptose é um mecanismo celular intrínseco que leva a auto-destruição celular. É um fenômeno fisiológico necessário na embriogênese, estresse celular e remoção de células lesadas, bem como para controlar a proliferação celular anormal em condições patológicas (Hockenbery et al., 1994). Esse processo celular é induzido por estímulos específicos e ocorre em células que participam de vias

relevantes para o organismo (Thompson, 1995). A apoptose é essencial para a homeostase dos tecidos humanos, incluindo a placenta. O aumento da apoptose do trofoblasto durante a PE pode contribuir para supressão da angiogênese e o crescimento placentário levando ao desenvolvimento da doença (Barakonyi et al., 2014).

Recentemente, alguns autores têm discutido a apoptose em trofoblastos humanos e sua possível implicação no desenvolvimento normal, remodelação, e envelhecimento da placenta. Esse processo é importante para a placentação adequada, mas pode estar envolvido na fisiopatologia de doenças relacionadas à gestação. A apoptose no trofoblasto aumenta em placentas normais de acordo com a progressão da gestação, e uma elevada incidência de apoptose é observada em gestações com PE e restrição de crescimento fetal (Shawn et al., 2005; Sinha et al., 2016).

O TNF-α foi a primeira citocina identificada como sendo capaz de induzir a apoptose em trofoblastos humanos. Em combinação com o interferon gama (IFN-γ), o TNF-α estimulou a apoptose em sincício e citotrofoblastos (Garcia-Lloret et al., 1996; Pijnenborg et al., 2000). O TRAIL (*tumor necrosis factor related apoptosis-inducing ligand*) é o terceiro receptor de morte no sistema de regulação da apoptose no sistema imune (Barakonyi et al., 2014). Durante a PE, o aumento na expressão de TRAIL por células trofoblásticas é provavelmente um mecanismo contra sinais apoptóticos de linfócitos e células *natural killer* maternas (Sokolov et al., 2009). Chaemsaithong et al. (2014) mostraram que os níveis plasmáticos de TRAIL solúvel são menores em mulheres com PE em comparação com as normotensas. Zauli et al. (2012) sugeriram um papel preditivo de TRAIL solúvel (sTRAIL) como um risco para desenvolvimento de distúrbios hipertensivos na gestação, como a PE.

Um dos ligantes de TRAIL, *death receptor 5* (DR5), é detectado em células trofoblásticas. O estímulo com citocinas como o TNF-α pode levar ao redirecionamento de DR5 para a superfície celular e consequentemente aumentar a suscetibilidade das células trofoblásticas ao TRAIL. É sugerido que o estímulo excessivo com TNF-α pode causar dano celular, como ocorre na PE (Barakonyi et al., 2014).

A vitamina D exerce inúmeros efeitos no organismo. Em relação ao sistema imune inato e adaptativo, ela é capaz de estabelecer um estado imune mais tolerogênico, principalmente por apresentar atividades reguladoras sobre a resposta inflamatória. Durante a gestação normal, a vitamina D é produzida por células trofoblásticas placentárias e da decídua humana, sendo responsável por exercer um papel anti-inflamatório em vários órgãos, incluindo a placenta (Xu et al., 2014). Esse hormônio possui atividades estruturais e fisiológicas semelhantes à progesterona nos estágios iniciais da gestação, desempenhando um papel importante na implantação, placentação e manutenção da gestação saudável (Monastra et al., 2018). Durante a gestação humana, a conversão de 25(OH)D em 1,25(OH)2D está aumentada na placenta, demonstrando que ela e a decídua são importantes no metabolismo da vitamina D (Murthi et al., 2016).

Estudos recentes relatam deficiência de vitamina D em gestantes portadoras de PE (Robinson et al., 2013; Xu et al., 2014; Mohaghegh et al., 2015) além da ocorrência de parto prematuro (Yuan et al.. 2019; Pashapour et al., 2019), enquanto outros mostram associação entre essa deficiência e o risco de desenvolvimento de PE (Aghajafari et al., 2013; Bodnar et al., 2014; Akbari et al., 2018). Esses resultados da literatura sugerem que a suplementação de vitamina D pode proteger o organismo materno por sua influência sobre a função vascular, modulação da resposta imune, homeostase e capacidade de manutenção do trofoblasto (Hyppönen et al., 2013; Hutabarat et al, 2018). Apesar dessas evidências, mais estudos clínicos, utilizando a VD para tratamento de síndromes da gestação, devem ser conduzidos baseados no histórico clínico e pessoal das pacientes, bem como questões étnicas e raciais (Cyprian et al., 2019; Dovnik et al., 2018).

Em conclusão, os estudos mostram que o estresse oxidativo, a ativação do inflamassoma associada à produção aumentada de citocinas inflamatórias, assim como apoptose aumentada são descritos na placenta de mulheres com gestações complicadas por PE. Portanto, o tratamento *in vitro* de explantes placentários de gestantes pré-eclâmpticas e HUVECs com a VD poderia contribuir para um melhor conhecimento dos processos envolvidos na inflamação sistêmica da PE e das células endoteliais e sugerir o emprego da VD como tratamento adjuvante dessa importante síndrome da gestação.

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Capítulo 1

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VITAMIN D DECREASES EXPRESSION OF NLRP1 AND NLRP3

INFLAMMASOMES IN PLACENTAL EXPLANTS FROM WOMEN WITH PREECLAMPSIA

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ABSTRACT

This study aimed to evaluate the immunomodulatory effect of vitamin D (VD) on the NLRP1 and NLRP3 inflammasomes in placental explants from preeclamptic (PE) and normotensive (NT) pregnant women. Placental explants from eight PE and eight NT pregnant women were obtained after caesarean section and were cultured with or without hydrogen peroxide (H_2O_2), VD or H_2O_2+VD. Gene and protein expression of NLRP1, NLRP3, HMGB1, caspase-1, interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and IL-18 were determined by quantitative PCR and Western blotting/ELISA. Compared to NT pregnant women, the endogenous gene expression of NLRP1, NLRP3, HMGB1, IL-1β, TNF-α and IL-18 was significantly higher in explants from PE women and became decreased after treatment of the explants with VD. Similarly, VD decreased the protein expression of NLRP3, caspase-1, HMGB1, IL-1β, TNF-α and IL-18 in PE explants. Placental explants from NT women cultured with H_2O_2 showed increased gene and protein expression of NLRP1, NLRP3, caspase-1, IL-1β, TNF-α and HMGB1, while H_2O_2 was also able to increase TNF-α and caspase-1 gene expression in preeclamptic explants. Treatment with H_2O_2+VD decreased gene and protein expression of NLRP1, NLRP3, caspase-1, HMGB1, IL-1β, TNF-α and IL-18 in both PE and NT explants stimulated with H_2O_2. Together, the results demonstrated that NLRP1 and NLRP3 inflammasomes are upregulated in the placental tissue of PE women. The *in vitro* treatment suggests that VD may play an immunomodulatory role in the placental inflammation of preeclamptic women and downregulates oxidative stress induced *in vitro* by H_2O_2 in placental explants.

Keywords: Preeclampsia, placental explants, inflammasome, cytokines, vitamin D.

1. INTRODUCTION

A normal pregnancy is characterized by an inflammatory status, as a response to the maternal organism to allow foetal growth and development [1]. Maternal adaptations to pregnancy require strictly controlled regulation of innate and adaptive immunities to ensure a successful pregnancy [2].

In parallel, in preeclampsia (PE), an exclusive pregnancy syndrome, a generalized systemic inflammatory process and vascular endothelial damage associated with placental dysfunction occurs [3]. This evidence suggests that the pathophysiology of PE has direct interference from the placenta [4].

Preeclampsia is responsible for most cases of morbidity, mortality and preterm birth, affecting approximately 2–8% of all pregnancies. Diagnosis of this disease is performed from the 20th week of pregnancy or in the first days postpartum and is based on the development of hypertension with or without proteinuria [5]. More recently, other maternal commitments have been associated with diagnoses, such as thrombocytopenia, impaired liver function, renal insufficiency, pulmonary oedema and new-onset cerebral or visual disturbances [6].

Oxidative stress is a remarkable event that contributes to complications in PE [7,8]. The increase in oxidative stress in the placenta is represented by release of a variety of mediators of endothelial dysfunction, such as lipid peroxides [9], proinflammatory cytokines [10] and microparticles derived from the syncytiotrophoblast [11]. Thus, PE is also characterized by the production of inflammatory substances, in addition to endogenous products released during tissue damage, most commonly known as "danger signals" or damage-associated molecular patterns (DAMPs), such as reactive oxygen species (ROS), heat shock proteins, uric acid, and proteins released from dead cells (such as *high mobility group-box 1* (HMGB1)) [12-14].

These DAMPs bind to pattern recognition receptors (PRRs) expressed in innate immunity cells [15]. One of the most important PRRs is part of the family of NLR receptors (protein with nucleotide oligomerization domain), which are cytosolic proteins capable of recognizing cytoplasmic DAMPs and pathogen-associated molecular patterns (PAMPs) and recruiting other proteins, forming signalling complexes that promote inflammation [16]. These signalling complexes

are called inflammasomes and generate active forms of interleukins, such as IL-1β, IL-18 [17], and release HMGB1 to the extracellular environment, acting as a proinflammatory mediator [18], contributing to the onset of the inflammatory response in PE [19].

ROS may be considered as a danger signal driving inflammation [20]. It is not yet known in detail how ROS act in the activation of the inflammasome, but studies indicate that they are induced by NLRP3 activators and are essential as secondary messengers for signalling NLRP3 activation [21]. The decomposition of hydrogen peroxide (H_2O_2) by catalase prevents the aggregation of NLRP3 inflammasomes in mouse podocytes, which can decrease glomerular injury [22]. In a recent study, we demonstrated that the treatment of placental explants obtained from normotensive pregnant women with H_2O_2 led to activation of the NLRP3 inflammasome as a consequence of induced cell stress and increased caspase-1 gene and protein expression of the proinflammatory cytokines IL-1β and TNF-α, which is associated with lower IL-10 production [23].

Vitamin D exerts numerous effects on the organism. This hormone can establish a more tolerogenic immune status, mainly because of its regulatory activities on the inflammatory response [24]. During normal pregnancy, vitamin D is produced by placental and human deciduous trophoblastic cells and is responsible for an anti-inflammatory role in several organs, including the placenta [25]. This hormone plays an important role in the implantation, placentation, and maintenance of a healthy pregnancy. During human pregnancy the conversion of 25(OH)D to 1,25(OH)2D is increased in the placenta, demonstrating the importance of this organ in the metabolism of vitamin D [26].

Vitamin D deficiency is reported in preeclamptic women [25,27], while other studies show an association between this deficiency and the risk of developing PE [28,29], suggesting that vitamin D supplementation may protect the maternal organism by its influence in the modulation of the immune response [24].

Considering the oxidative stress described in the placenta of pregnancies complicated by PE, as well as activation of the NLRP3 inflammasome receptor with increased production of proinflammatory cytokines induced by H_2O_2, this study aimed to evaluate the immunomodulatory effect of vitamin D on the NLRP1 and

NLRP3 inflammasomes in placental explants from preeclamptic and normotensive pregnant women.

2. METHODS

1.1. Study population

Placentas from eight preeclamptic (PE) and eight normotensive (NT) pregnant women were collected in the Obstetrics Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP-Brazil, between July 2017 and February 2018. Gestational age was calculated from the last menstrual period and confirmed by ultrasound dating. The diagnosis of PE was made considering when, without a history of hypertension, a pregnant woman developed hypertension (blood pressure ≥ 140/90 mmHg) with or without proteinuria (≥ 300 mg in a 24-hour urine) after the 20th week of gestation [5]. The NT group did not present with a history of hypertensive disorders in pregnancy.

1.2. Exclusion criteria and ethics statement

Exclusion criteria included chronic hypertension, multiple gestations, prior preeclampsia, illicit drug use, and pre-existing medical conditions, such as diabetes, cancer, acute infectious disease, cardiovascular, autoimmune, renal and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all women involved in the study (Protocol number: 3.383.792). Also, all experiments were performed following relevant guidelines and regulations.

1.3. Collection of placental tissue

Placentas were collected from delivery by elective caesarean section. Immediately after delivery, all placentas considered for the study were examined macroscopically and processed within 10 min, as previously described [23], to constitute placental explants.

1.4. Culture of placental explants with hydrogen peroxide and vitamin D

A total of 11 mg of human villous tissue was cultured according to Nunes et al. [23] in 24-well plates (SPL Life Sciences, Korea) over 24 h for stabilization. After this time, placental explants from PE and NT women were cultured for 24 h with or without hydrogen peroxide (100 μM), vitamin D (100 nM) and with or without hydrogen peroxide plus vitamin D (Sigma-Aldrich, SL, Missouri, USA). Explant viability was verified by the release of the intracellular enzyme lactate dehydrogenase (LDH) into the incubation medium after 24, 48, 72 and 96 h of incubation, as described previously [23]. The same investigation was performed to verify the viability of the explant under treatment with H_2O_2 and VD over 24 h. LDH concentrations were determined with a LDH assay kit (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's protocol.

1.5. Expression of transcripts related to inflammation

Placental explants from eight PE and eight NT pregnant women were employed to determine the expression of the genes encoding NLRP1, NLRP3, HMGB1, caspase-1, IL-1β, TNF-α and IL-18 proteins at the transcriptional level. Total RNA was extracted from the placental explants after culture using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's protocol, and the quantitative polymerase chain reaction (qPCR) was performed according to Weel et al. [30]. Briefly, isolated RNA was treated with DNAse I Amp Grade (Invitrogen). Subsequently, the synthesis of complementary DNA (cDNA) was conducted using the ImProm-II TM Reverse Transcription System, according to the manufacturer's protocol. The qPCR was performed using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA), and the variants of the studied targets were aligned in the MEGA 5.1 program. Each primer was subsequently selected by the software Primer-BLAST. Primers located in exon-exon junctions guarantee the purity of the reaction, namely the absence of any genomic DNA that may contaminate it. The primer sequences used in this study are shown in Table 1.

Table 1. Primers for NLRP1, NLRP3, HMGB1, caspase-1, IL-1β, TNF-α, IL-18

and GAPDH. Numbers indicate nucleotide positions in the corresponding transcripts.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank
NLRP1	(1728)TCCGGCTCCCATTAGACAGA(1747)	(1810)AGACCCATCCTGGCTCATCT(1791)	NM_033004.3
NLRP3	(2826)GAGGAAAAGGAAGGCCGACA(2845)	(2917)TGGCTGTTCACCAATCCATGA(2897)	NM_004895.4
HMGB1	(1404)TACGAAAAGGATATTGCTGC(1423)	(1505)CTCCTCTTCCTTCTTTTTCTTG(1484)	NM_001313893.1
CASP1	(1065)AGACATCCCACAATGGGCTC(1084)	(1172)TGAAAATCGAACCTTGCGGAAA(1151)	NM_033292.3
IL1B	(544)GAGCAACAAGTGGTGTTCTCC(564)	(653)AACACGCAGGACAGGTACAG(634)	NM_000576.2
TNF	*(325)GCTGCACTTTGGAGTGATCG(344)*	*(462)GGGTTTGCTACAACATGGGC(443)*	*NM_000594.3*
IL18	(438)ACTGTAGAGATAATGCACCCCG(459)	(517)AGTTACAGCCATACCTCTAGGC(496)	NM_001562.3
GAPDH	(684)CGTGGAAGGACTCATGACCA(703)	(801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Each reaction was set in duplicate, and the conditions for the qPCR were as follows: initial denaturation at 96 °C for 2 min and then 40 cycles at 95 °C for 15 s and 60 °C for 60 s, followed by a melting curve. Expression values of the analysed transcripts were normalized to that of the enzyme encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve [31]. To analyse the relative expression, after the analysis of gene expression, we chose an RNA sample obtained from each group, which received a relative value of 100. All other samples received values for that sample.

1.6. Cytokine determinations

The concentrations of IL-1β, TNF-α and IL-18 in supernatants of placental explants from NT and PE women, obtained after treatment with or without H_2O_2 and VD, were determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Assay sensitivity limits were 1.0 pg/mL for IL-1β, 1.6 pg/mL for TNF-α, and 5.15 pg/mL for IL-18.

1.7. Obtaining homogenate from placental explants

Placental explants from three PE and three NT pregnant women were cultured for 24 h in the presence or absence of H_2O_2, VD or H_2O_2 plus vitamin D and previously stored at -80 °C were removed for thawing, and the placental fragment sample was weighed and documented in grams of tissue. These fragments were then transferred to the tissue homogenizer container containing 2 mL of protease inhibitor buffer: 150 mM/L NaCl and 20 mM/L Tris pH 7.5, 5 mM/L EDTA and 1 mM/L L phenylmethylsulphonyl fluoride (pH 7.3). After homogenization in an ice bath, the material was placed in test tubes and centrifuged at 3500 rpm for 20 min at 4 ºC. The supernatant was then aliquoted into 0.5 mL microtubes and stored at -80 °C. The supernatant obtained from the culture of placental explants was used to determine the total protein concentration by Lowry's method [32] for NLRP3, NLRP1, caspase-1 and HMGB-1 determinations.

1.8. Electrophoresis and blotting

After determining the homogenate protein concentration of placental explants, 30 μg of proteins were treated with Laemli sample buffer (Bio-Rad, Hercules, CA, USA) and β-mercaptoethanol at 95 °C for 5 min, then the proteins were separated on SDS-PAGE and after electrophoresis, transferred to a nitrocellulose membrane. Nonspecific protein binding was blocked by incubating the membranes in 5% skim milk in Tris-HCl buffer containing 0.1% Tween 20 (TBST) for 45 min at room temperature. Membranes were subsequently incubated for 18 h with rabbit polyclonal antigen-specific primary antibodies: anti-NLRP1, anti-NLRP3, anti-caspase-1 and anti-HMGB1 (Novus Biologicals, Littleton, CO, USA) at dilution on manufacturer recommended concentrations containing TBST. After washing, the membranes were incubated with rabbit specific secondary antibody diluted in TBST for 1 h. Immunoreactive components were revealed by the Amersham™ ECL Select™ Western Blotting Detection Reagent luminescent kit (GE Healthcare®, Little Chalfont, United Kingdom).

1.9. Statistical analysis

The results were evaluated using parametric and non-parametric methods according to the statistical program GraphPad Prism, version 6.01 (GraphPad, CA,

USA). The clinical characteristics of preeclamptic and normotensive pregnant women were analysed by Mann-Whitney *U* test. Gene and protein expression of NLRP1, NLRP3, HMGB1, caspase-1, IL-1β, TNF-α and IL-18 by placental explants were evaluated by analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

2. RESULTS

2.1. Clinical and laboratory characteristics of preeclamptic and normotensive women

The clinical and laboratory characteristics of the groups are presented in Table 2. Regarding age, there was no statistical difference between the groups. However, gestational age was lower in women with PE compared to that in NT women. In addition, PE was associated with hypertension, hyperuricemia and proteinuria.

Table 2. Characteristics of pregnant women.

Variable/Groups	*Normotensive (n=8)*	*Preeclampsia (n=8)*
Age (years)	29 (18-41)	28 (16-39)
Gestational age (weeks)	40 (36-41)	*34 (30-39)
Systolic blood pressure (mmHg)	120 (110-120)	*155 (140-70)
Diastolic blood pressure (mmHg)	70 (60-80)	*100 (90-110)
Uric acid (mg/dL)	3.2 (2.3-4.7)	*5.7 (3.7-6.9)
Proteinuria (mg/24 h)	< 300	*730 (300-7540)

Results are expressed as median (range: minimum-maximum.) *($p<0.05$) vs. NT; (Mann-Whitney *U* test).

3.2. Placental explants viability was preserved in 24 h culture and among different concentrations of H_2O_2 and VD

Results of LDH enzyme determination in the supernatants from explant cultures are shown in Figure 1. Placental explants cultured for 24 h presented satisfactory viability with the lowest level of LDH (20.85 ± 3.28 nmol/min/mL) (Figure 1A); therefore, this time of culture was used for the entire experiment. Analysis of LDH release at 24 h culture with H_2O_2 and VD did not show a significant difference (Figure 1B).

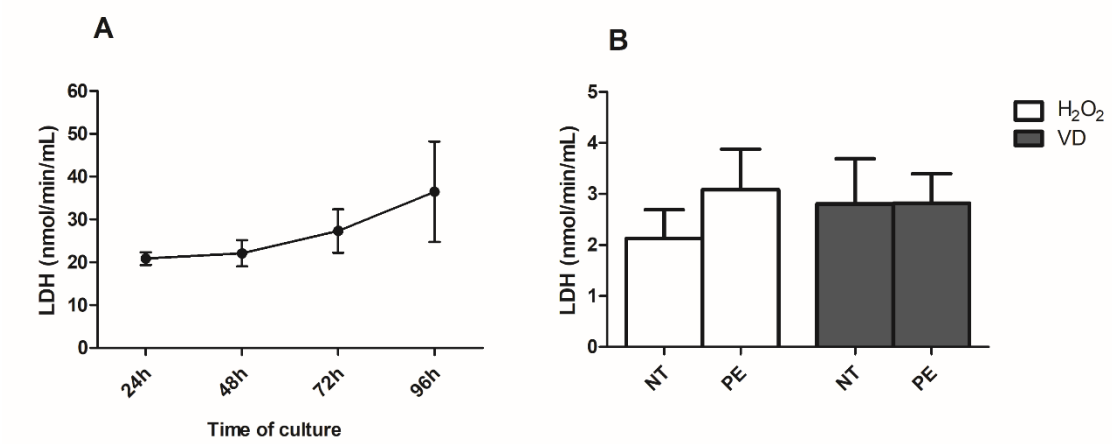

Figure 1. Cell viability of placental explants. Concentration of lactate dehydrogenase (LDH) detected in supernatant of placental explants cultured during 24, 48, 72 and 96 h (A). Concentrations of LDH in 24 h cultures with H_2O_2 and VD (B). Data are presented as mean ± SD (ANOVA).

3.3. Gene and protein expression of cytokines are higher in placental explants from the PE group and lower with VD treatment

Figure 2 shows the gene and protein expression of IL-1β (A/D), TNF-α (B/E) and IL-18 (C/F), respectively, in placental explants and in supernatants from culture of these placental explants obtained from preeclamptic and NT pregnant women. The endogenous gene and protein expression of IL-1β, TNF-α and IL-18 were significantly higher in the PE group than in the NT group. On the other hand, VD treatment of placental explants decreased protein and mRNA levels of these cytokines in the PE group. Explant culture with H_2O_2 increased gene and protein

expression of IL-1β and TNF-α, as well as gene expression of IL-18, in the NT group compared to those in control cultures. This treatment also led to higher levels of TNF-α mRNA compared to those in control cultures in the PE group. Cultures treated with H_2O_2+VD compared to those only treated with H_2O_2 showed a decrease in protein and gene expression of TNF-α, as well as in mRNA levels of IL-1β and IL-18, in the PE and NT groups and in protein expression of these cytokines in supernatants of preeclamptic placental explants.

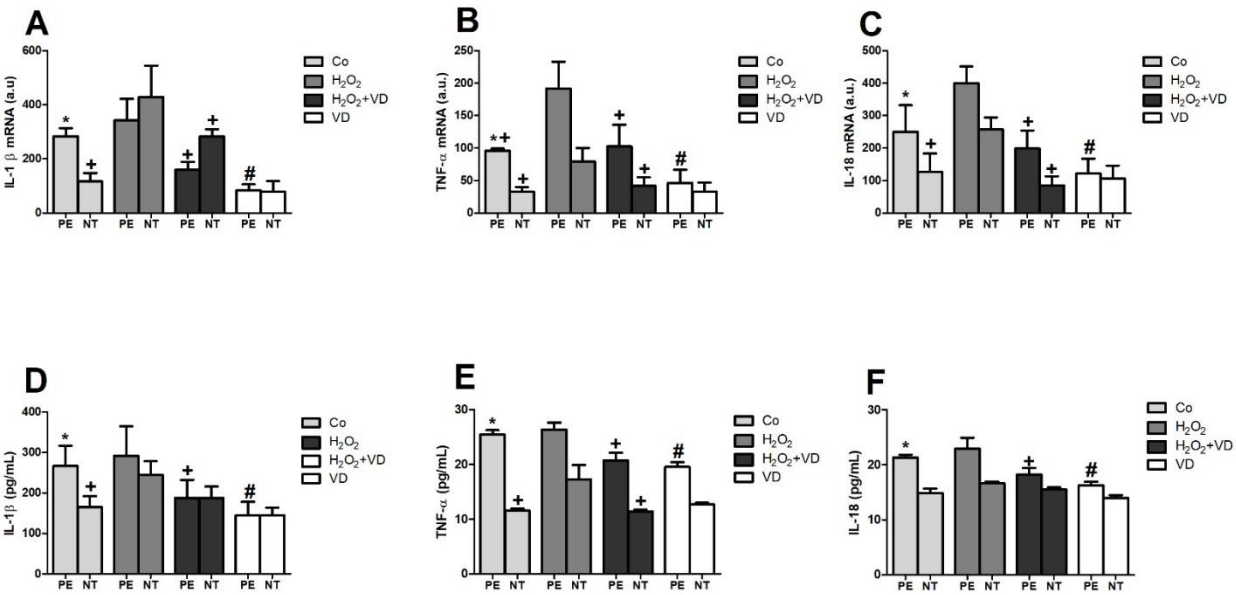

Figure 2. Gene and protein expression of IL-1β (A/D), TNF-α (B/E) and IL-18 (C/F) in placental explants and supernatants of preeclamptic (PE) and normotensive (NT) pregnant women after treatment with or without H_2O_2, VD, and H_2O_2+VD. Results are expressed in mean ± SD. *($p<0.005$) vs. Co NT, +($p<0.005$) vs. H_2O_2, #($p<0.005$) vs. Co PE (ANOVA followed by Tukey's multiple comparison test).

3.4. Gene expression shows that vitamin D was able to decrease inflammasome activation in placental explants

The endogenous gene expression of NLRP1, NLRP3 and HMGB1 was significantly higher in the PE explants compared to those in the NT explants and also higher in cultures treated with H_2O_2 compared to control cultures in the NT group (Figure 3). Caspase-1 was also higher in both the PE and NT groups when

cultured with H_2O_2. The addition of vitamin D to the explant cultures of preeclamptic women decreased the gene expression of NLRP1, NLRP3, caspase-1, and HMBG1. Treatment with H_2O_2+VD decreased gene expression of NLRP1, NLRP3, caspase-1, and HMGB1 in both PE and NT placental explants as compared to those only treated with H_2O_2.

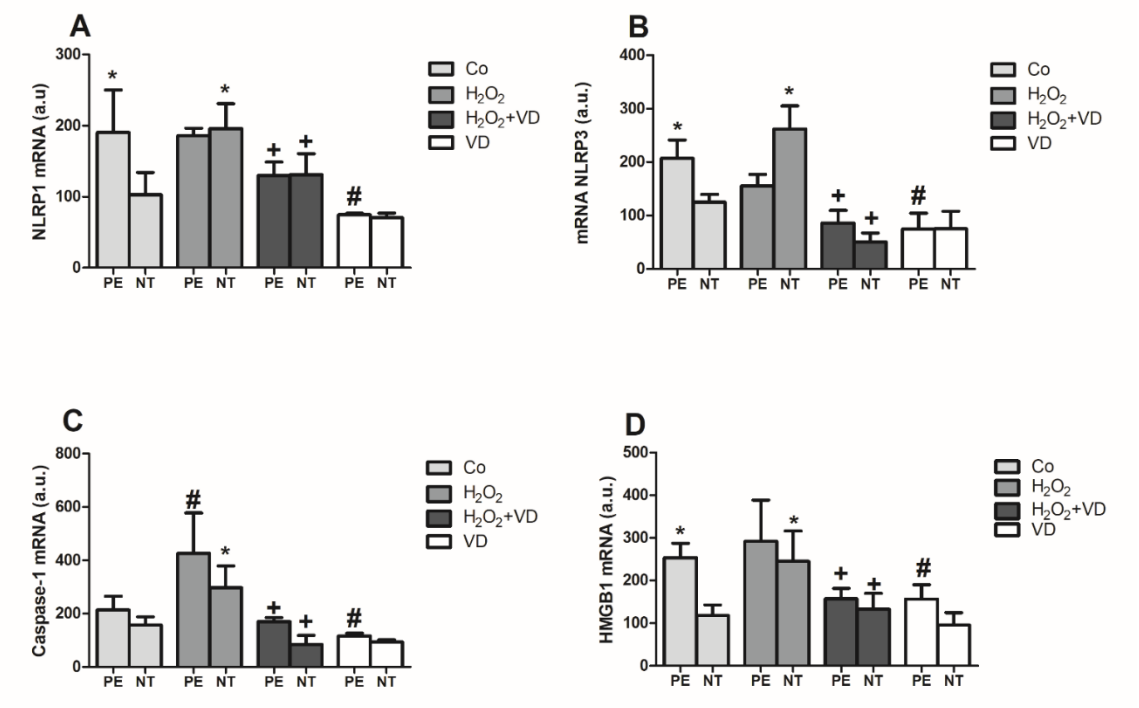

Figure 3. Gene expression of NLRP1 (A), NLRP3 (B), caspase-1 (C) and HMGB1 (D) in placental explants of preeclamptic (PE) and normotensive (NT) pregnant women after treatment with or without H_2O_2, VD, and H_2O_2+VD. Results are expressed in mean ± SD. *($p<0.005$) vs. Co NT, +($p<0.005$) vs. H_2O_2, #($p<0.005$) vs. Co PE (ANOVA followed by Tukey's multiple comparison test).

3.5. Culture with vitamin D also decreased expression of inflammatory proteins in placental explants

High protein expression of NLRP3 (Figure 4A and 5A) and caspase-1 (Figure 4C and 5C) was detected in explants cultured with H_2O_2 compared to control cultures in both PE and NT explants, as well as HMGB1 in the NT group (Figure

5D). On the other hand, compared with H_2O_2 stimuli, treatment with H_2O_2+VD or VD decreased protein expression of NLRP3, caspase-1 and HMGB1 in the PE group (Figure 4A, 4C and 4D). In the NT group, the protein expression of NLRP1, NLRP3, caspase-1 and HMGB1 was decreased after explant treatment with H_2O_2+VD or VD (Figure 5A-5D).

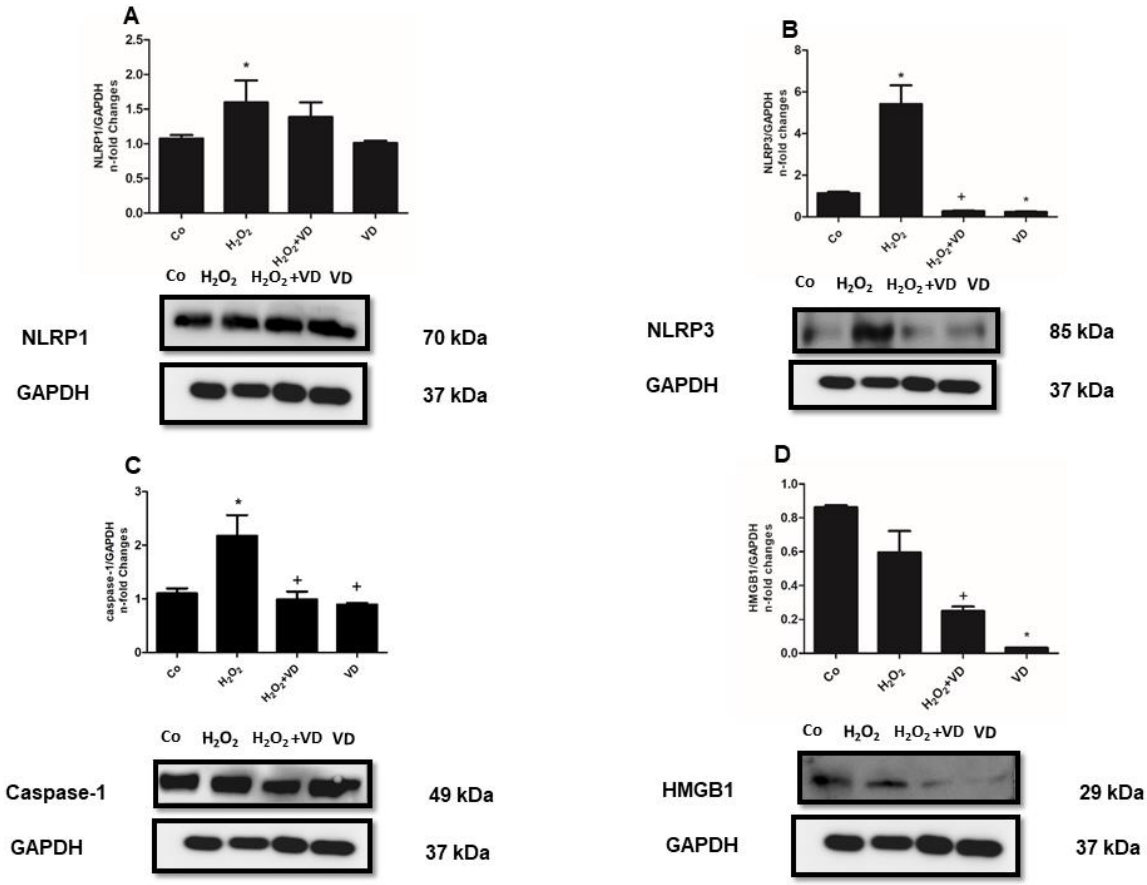

Figure 4. Protein expression of NLRP1 (A), NLRP3 (B), caspase-1 (C) and HMGB1 (D) after treatment with or without H_2O_2, VD, and H_2O_2+VD in placental explants of preeclamptic (PE) women. Bands are presented by densitometry analysis normalized by respective values of GAPDH. Results are expressed in mean ±SD. *($p<0.005$) vs. control (Co), +($p<0.005$) vs. H_2O_2 (ANOVA followed by Tukey's multiple comparison test).

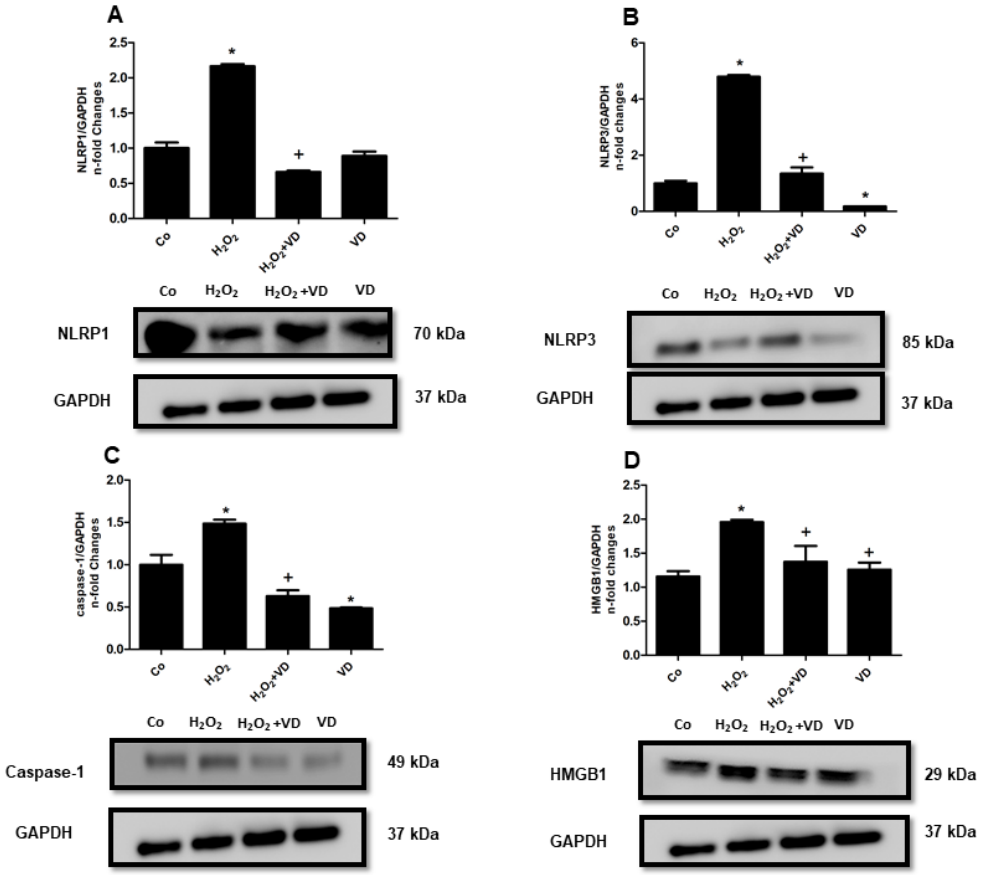

Figure 5. Protein expression of NLRP1 (A), NLRP3 (B), caspase-1 (C) and HMGB1 (D) after treatment with or without H_2O_2, VD, and H_2O_2+VD in placental explants of normotensive (NT) pregnant women. Bands are presented by densitometry analysis normalized by respective values of GAPDH. Results ate expressed in mean ± SD. *($p<0.005$) vs. control (Co), +($p<0.005$) vs. H_2O_2 (ANOVA followed by Tukey's multiple comparison test).

4. DISCUSSION

The present study shows significant increases in both gene and protein expression of the inflammatory cytokines IL-1β, IL-18, TNF-α and HMGB1, as well as NLRP1 and NLRP3 inflammasomes, in placental explants of preeclamptic women, confirming the contribution of NLRP3 hyperactivation to the adverse placental inflammation in PE [30,33]. These results are in line with our previous

studies showing increase expression of NLRP3, caspase-1, IL-1β and TNF-α in placental villi and placental homogenate from pregnant women with severe PE [30]. It is known that NLRP3 is the most studied of all inflammasomes, and the results of our group corroborate with literature showing the activation of the NLRP3 inflammasome, as well as its role in placental inflammation and in the pathogenesis of preeclampsia [33-35]. Besides endogenous activation of the NLRP3 inflammasome, our results showed, for the first time, the activation of the NLRP1 inflammasome in placental explants of preeclamptic women, confirming the involvement of both inflammasomes in placental inflammation. To the best of our best knowledge, studies with the NLRP1 inflammasome in humans show its dysfunction in large number of diseases, acting in a dichotomous role in either augmenting or attenuating disease, and contributing to its pathogenesis [36]. Recent revisions report that single nucleotide polymorphisms (SNPs) in the vicinity of the NLRP1 gene have been associated with autoimmune, autoinflammatory, infectious and neurologic diseases [36,37]. In previous studies, we reported activation of the NLRP1 and NLRP3 inflammasomes in peripheral blood monocytes from preeclamptic women [13,38]. The activation of the NLRP1 inflammasome in monocytes and placental tissue might be explained by association of the NLRP1 variant rs12150220 (L155H) with development of preeclampsia, suggesting a role of this inflammasome receptor in the pathogenesis of this disorder [39].

HMGB1 gene expression by placental explants was also elevated in women with PE compared to NT pregnant women and agrees with previous studies showing higher gene expression of HMGB1 in placentas from PE women, as well as its higher protein levels in preeclamptic maternal circulation [14,17]. Compared with NT pregnancies, placentas from severe and early-onset preeclamptic women showed significantly higher protein expression of HMGB1 in the syncytiotrophoblast [18].

In the present study, placental explants from preeclamptic and NT pregnant women were treated with VD. The results showed that this hormone decreased production of IL-1β, TNF-α and IL-18, as well as NLRP1, NLRP3, caspase-1 and HMGB1 gene expression, in preeclamptic placental explants, in accordance with other *in vitro* studies showing the influence of VD in the down-regulation of

proinflammatory immune responses [24,40]. To our knowledge, this is the first study showing that vitamin D was able to decrease the endogenous activation of the NLRP1 and NLRP3 inflammasomes, as well as the production of inflammatory cytokines, in placental explants from preeclamptic women.

Previous studies have demonstrated that oxidative stress is capable of activating an inflammatory response, highlighting the importance of ROS in the regulation of NLRP3 [41,42]. In our study, increased oxidative stress was induced by stimulation of placental explants with H_2O_2, and showed more prominent NLRP1, NLRP3, capase-1 and HMGB1, as well as expression of proinflammatory cytokines, in the NT group. Caspase-1 participates in the activation of inflammasomes during the oxidative stress state, and the production of IL-1β is associated with tissue damage and inflammation [43]. Although explants of placenta from preeclamptic women were also stimulated by H_2O_2, no significant difference were detected between endogenous and H_2O_2-induced activation of the NLRP1 and NLRP3 inflammasomes, as well as IL-1β, IL-18 and HMGB1 gene and protein expression. These results may be due to the basal pre-activation state of placenta from the preeclamptic group and may reflect the inflammatory profile found in PE [25].

The treatment of explants with VD, employed alone or in combination with H_2O_2, led to a decrease in gene and protein expression of NLRP1, NLRP3, caspase-1, and HMGB1 in both the PE and NT groups.

Several studies in the literature suggest that low VD levels during pregnancy are associated with the risk of developing PE and preterm birth, [28,44,45] and alternatives aiming to decrease or prevent the activation of the inflammasome using natural products can be used in an attempt to contain the inflammatory state of PE [38,46]. The placenta acts to adapt and defend the maternal organism in an attempt to compensate for inflammation and oxidative stress. Defects in the placentation process can culminate in pregnancy-related disorders, such as preeclampsia, which may be negatively supported by vitamin D deficiency. Thus, this hormone may play a role in homeostasis and trophoblast survival capacity [47]. Based on this evidence, vitamin D supplementation seems to be performed at the beginning of gestation or during preconception due to its potential related to inflammation. Indeed, the effects

of vitamin D supplementation in the prevention and treatment of pregnancy disorders are not completely understood. More studies are necessary to start supplementation based on the personal history of pregnant women, and more clinical trials should be done to improve information about doses considering racial and ethnic features [40,48]. Besides that, vitamin D supplementation during pregnancy has been clearly shown to be safe both in the mother and foetus and may be recommended given its benefits.

In conclusion, the present study demonstrated that the *in vitro* treatment of placental explants from preeclamptic women with VD downregulates the endogenous activation of the NLRP1 and NLRP3 inflammasomes and proinflammatory cytokines. These results suggest that VD may play an immunomodulatory role in the placental inflammation of preeclamptic women and downregulates oxidative stress induced *in vitro* by H_2O_2 in placental explants. Future research is needed to understand the mechanism of VD in the immunomodulation of placental inflammation and the characteristics of preeclampsia.

Author contributions: PRN, MRV and MTSP conceived and designed the experiments, analysed data and wrote the paper; JCP, LGO, JRR and VTMB selected pregnant women for the study; PRN, MRV, MLM, VRR, and CJCF performed the experiments. All authors read and approved the manuscript.

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Capítulo 2

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Temos a satisfacao de informar que seu manuscrito "11073.R1 - Effects of vitamin D-induced supernatant of placental explants from preeclamptic women on oxidative stress and nitric oxide bioactivity in HUVEC" foi aceito quanto ao merito científico para publicacao no Brazilian Journal of Medical and Biological Research.

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EFFECTS OF VITAMIN D-INDUCED SUPERNATANT OF PLACENTAL EXPLANTS FROM PREECLAMPTIC WOMEN ON OXIDATIVE STRESS AND NITRIC OXIDE BIOAVAILABILITY IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Abstract

The study evaluated the effect of the supernatant of placental explants from preeclamptic (PE) and normotensive (NT) pregnant women after tissue treatment with or without vitamin D (VD) on oxidative stress and nitric oxide (NO) bioavailability in human umbilical vein endothelial cells (HUVEC). Placental explants were prepared from eight NT and eight PE women, and supernatants were obtained after incubation with or without hydrogen peroxide (H_2O_2) and/or VD. HUVEC were cultured for 24 h with supernatants, and the following parameters were analyzed in HUVEC cultures: NO, nitrate (NO_3^-), and nitrite (NO_2^-) levels, lipid peroxidation, and intracellular reactive oxygen species (ROS). Results showed that the production of NO_3^-, NO_2^-, malondialdehyde (MDA), and ROS were significantly higher in HUVEC treated with explant supernatant from PE compared to NT pregnant women, while the supernatant of PE explants treated with VD led to a decrease in these parameters. A significantly high production of NO was detected in HUVEC cultured with control supernatant of NT group, and in cultures treated with supernatant of PE explants treated with VD. Taken together, these results demonstrated that cultures of placental explants from PE women with VD treatment generated a supernatant that decreased oxidative stress and increased the bioavailability of NO in endothelial cells.

Key words: Oxidative stress; Placental explants; Preeclampsia; Vitamin D

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Introduction

Preeclampsia (PE) is a specific human syndrome of pregnancy characterized as the main cause of morbidity, mortality, and preterm birth, affecting as many as 10% of all pregnancies. Clinical diagnosis is performed from the twentieth week of pregnancy or in the first days after delivery, based on the development of hypertension with or without proteinuria (1) in addition to maternal manifestations such as thrombocytopenia, impaired liver function, renal insufficiency, pulmonary edema, and new-onset cerebral or visual disturbances (2).

Nitric oxide (NO) is a key signaling molecule in the cardiovascular system, controlling vascular tone, neurotransmission, redox signaling, cellular respiration, and host defense (3). This molecule participates actively in the pregnancy processes such as trophoblast invasion and placental development, representing the main vasodilator in the placenta (4). Disturbances in the NO system, coupled with oxidative stress, contribute to vascular dysfunction in preeclamptic women (5). Oxidative stress, characterized by excessive formation of reactive oxygen species (ROS), can impair endothelial nitric oxide synthase (eNOS) function (6) and consequently decrease bioavailability of NO. Together with the excessive production of ROS, the placenta from preeclamptic women shows an intense inflammatory process. ROS are involved in the injuries signaling to the immune system (7) and can orchestrate the inflammatory response by the release of hydrogen peroxide (H_2O_2) from damaged tissues leading to the recruitment of leukocytes to the lesion site (8).

Vitamin D (VD) has several effects on the organism, modulating cardiovascular and immune cell functions. Concerning the innate and adaptive immune system, it can establish a more tolerogenic immune status, by its regulatory activities on the inflammatory response. During normal pregnancy, VD is produced by placental trophoblast cells and human decidua and is responsible for anti-inflammatory effects in various organs, including the placenta (9).

This hormone plays an important role in the implantation, placentation, and maintenance of healthy gestations. During human pregnancy, the conversion of inactive 25(OH)-D to the active form 1.25(OH)2-D is increased in the placenta

demonstrating that this tissue and decidua are important in the bioactivation of VD (10). Recent studies have reported VD deficiency in preeclamptic women PE (11,12) and others have shown an association between deficiency and the risk of developing PE, suggesting that supplementation may modulate the immune response in this pathology (13).

Recently, we demonstrated the *in vitro* activation of NLRP3 inflammasome in placental explants from normotensive (NT) pregnant women as a consequence of the H_2O_2-induced cellular stress, which is initiated by ROS release, as well as increased gene expression of inflammatory cytokines (14). Furthermore, the increase of ROS formation could decrease the bioavailability of NO, since some researchers have suggested that PE should be characterized by a disruption of vascular dilation mediated by NO and disturbed by ROS (15). Therefore, the use of immunomodulatory substances like VD for the *in vitro* treatment of explants may lead to a better understanding of the systemic inflammation in PE and possibly propose alternative ways to treat this syndrome.

Considering that the placenta of women with PE shows oxidative stress, exacerbated inflammation, and NO system imbalance, this study aimed to evaluate the effect of the supernatant of placental explants from PE and NT pregnant women treated with or without VD on oxidative stress and NO bioavailability in human umbilical vein endothelial cells (HUVEC). We intended to observe whether the employment of substances with an immunomodulatory effect such as VD on placental tissue can be used to reduce oxidative stress in endothelial cells.

Material and Methods

The methodology employed in this study is demonstrated in a schematic figure Supplementary Figure S1).

Study population and ethics statement

Placentas and blood from 8 NT and 8 PE pregnant women were collected. These pregnant women were admitted to the Obstetrics Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP, Brazil, between November 2017 and August 2018. Gestational age was calculated from the last menstrual period and

confirmed by ultrasound dating. A pregnant woman was considered preeclamptic when, without a history of hypertension, she developed hypertension (≥140/90 mmHg) associated or not with proteinuria (≥300 mg in 24-h urine) after the 20th week of gestation (1). NT pregnant women did not present a personal history of hypertensive disorders before or during pregnancy. Proteinuria in 24-h urine was analyzed by a colorimetric method, the Technicon RA-XT automation system (Asinteg, Argentina), and uric acid was assessed by uric acid enzymatic Trinder (Biotrol Diagnostic, India) in the Clinical Laboratory of Botucatu Medical School.

Exclusion criteria included chronic hypertension, multiple gestations, prior preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, cancer, acute infectious disease, and cardiovascular, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all women involved in the study (Protocol number: 3.383.792). This work was carried out under the Code of Ethics of the World Medical Association (Declaration of Helsinki). Also, all mandatory laboratory health and safety procedures were complied with in the course of conducting any experimental work reported in this paper and all experiments were performed following relevant guidelines and regulations.

Collection of placental tissue

Placentas were collected at delivery by elective cesarean section. Immediately after delivery, all placentas considered for the study were examined macroscopically and processed within 10 min. Fragments of approximately 5×5 cm were immediately removed from the central region of the placenta, constituting samples in contact with the maternal side (basal plate). After this initial collection, smaller fragments were washed in phosphate buffered saline (PBS) and separated from the decidual layer that is normally adhered to the basal plate. The terminal portions of the villi were observed in PBS (the villi were seen floating freely in the liquid) and were dissected into small sections to constitute explants.

Culture of placental explants with hydrogen peroxide and vitamin D

Placental tissue was obtained according to a previous study (14). Briefly, a total of 11 mg of human villous tissue was cultured in RPMI 1640 culture medium (Thermo Fisher, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), 40 mg/mL antibiotic/antimycotic (Sigma-Aldrich), and 10% fetal bovine serum (Gibco BRL Life Technologies, The Netherlands) in 24-well plates (SPL Life Sciences, Korea) during 24 h for stabilization. After stabilization, placental explants from PE and NT pregnant women were cultured for 24 h with or without H_2O_2 (100 μM) (Sigma-Aldrich) as a stimulus for oxidative stress induction, and with or without VD (100 nM) (Sigma-Aldrich), considered an immunomodulator. Placental explant supernatants obtained were stored at –80°C for later experiments and analyses.

Cytokine determinations

The concentrations of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-18 in supernatants of placental explants from NT and PE women, obtained after treatment with or without H_2O_2 and VD, were determined by Quantikine ELISA kits (R&D Systems, USA) according to the manufacturer's instructions. Assay sensitivity limits were 1.0 pg/mL for IL-1β, 1.6 pg/mL for TNF-α, and 5.15 pg/mL for IL-18.

Vitamin D and vitamin D receptor (VDR) determination

Blood sampling and vitamin D determination. Peripheral blood (10 mL) was obtained by venipuncture from the antecubital vein of 8 PE women at the time of PE diagnosis and of 8 NT pregnant women at the time they were matched to gestational age with PE women. Blood was collected into plastic tubes containing 5% EDTA. After blood centrifugation at 4°C for 10 min at 1,200 g, the plasma fraction was removed and aliquots were stored at –80°C until vitamin D determination.

Vitamin D, 25(OH)D, was determined by the automated chemiluminescence microparticle immunoassay (CMIA) with an Architect 25-OH Vitamin D assay kit, by the Architect® i2000 analyzer (Abbott®, USA). The analytical sensitivity was 1.9 ng/mL and the coefficient of variation within and between assays was <10%, as

described in the kit. The reference range was 0.0–160.0 ng/mL, according to the method. Values ≥30 ng/mL were considered sufficient, from 21 to 29 ng/mL insufficient, and <20 ng/mL deficient (16).

VDR gene expression

Placental explants from 8 PE and 8 NT pregnant women were employed to determine the expression of VDR at the transcriptional level. Total RNA was extracted from the placental explants after culture using the Total RNA Purification Kit (Norgen Biotek Corp., Canada) following the manufacturer's protocol, and the reverse transcription-coupled polymerase chain reaction (qPCR) was performed as described previously (14). Briefly, isolated RNA was treated with DNAse I Amp Grade (Invitrogen, USA). Subsequently, the synthesis of complementary DNA (cDNA) was conducted using ImProm-II TM Reverse Transcription System, according to the manufacturer's protocol (Promega, USA).

The qPCR was performed using RT GoTaq-qPCR Master Mix (Promega, USA) and the variants of the studied targets were aligned in the MEGA 5.1 program (www.megasoftware.net) and each primer was subsequently selected by the software Primer-BLAST (NIH, USA). Primers located in exon-exon junctions guarantee the purity of the reaction, namely the absence of any genomic DNA that may contaminate it. The VDR primer sequence used in this study was (590)TGGAGACTTTGACCGGAACG(609)
(704)GCTTCGCCTGAAGAAGCCT(686). Each reaction was set in duplicate and the conditions for the qPCR were as follows: initial denaturation at 96°C for 2 min and then 40 cycles at 95°C for 15 s and 60°C for 60 s, followed by a melting curve. Expression values of the analyzed transcripts were normalized to that of the enzyme-encoding glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) as follows:(684)CGTGGAAGGACTCATGACCA(703)
(801)GGCAGGGATGATGTTCTGGA(782). The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve (17). To analyze the relative expression, after the analysis of gene expression, we chose an RNA sample obtained from each group, which received a relative value of 100. All other samples received values for that

sample.

HUVEC culture and incubation with supernatant of placental explants

HUVEC (Lonza, Switzerland) were acquired with a certification proving that the cells were from the designated type. All experiments were performed using cells in the sixth passage and in quintuplicates. HUVEC were cultured until reaching 80% confluence and then incubated for 24 h in medium 199 (Gibco BRL Life Technologies) with 20% (v/v) supernatant pool from placental explants of PE women, and from NT explants previously cultured with or without H_2O_2 and/or VD as described above. The cells and HUVEC media were used to perform the assays. PrestoBlue® Cell Viability Reagent (Invitrogen) was employed to determine whether the supernatants pre-treated with H_2O_2 and/or VD were harmful to HUVEC showing cell viability.

Measurement of nitrate, nitrite, and NO species

Nitrate and nitrite levels produced by HUVEC after placental explant supernatant incubation for 24 h were assessed in HUVEC supernatant using a high-performance liquid chromatography (HPLC) system (ENO-20; Eicom, USA) as previously described (18). The ENO-20's high sensitivity and specificity were accomplished with the combination of a diazo coupling method and chromatography. The level of the diazo compound was measured by absorbance at 540 nm using a Spectramax iD3 multi-mode microplate reader (Hidex, Lablogic Systems, UK).

A total of $5x10^4$ HUVEC per well were plated onto a black 96-well plate (Sigma-Aldrich). After 24 h of incubation with placental explant supernatants, the cells were washed with 95 µL of PBS and incubated for 30 min at 37°C. After this period, cells were loaded with 5 µL of DAF-FM™ (10 µM) (Sigma-Aldrich) and read for 60 min in 37°C using Spectramax iD3. The fluorescence signal was measured in a microplate reader (excitation 495 nm, emission 535 nm) and is reported as arbitrary units.

Measurement of lipid peroxidation – TBARS

Levels of lipid peroxidation in the supernatant of HUVEC cultured with pre-treated placental explants supernatant for 24 h were measured by thiobarbituric acid reactive substances (TBARS). An aliquot of 100 µL of supernatant was mixed with 200 µL of 10% cold trichloroacetic acid (TCA, Sigma-Aldrich), before being placed on ice for 15 min to precipitate protein.

During this time, standards were prepared in a serial dilution with 1,1,1,3-tetramethoxypropane (TMP, C7H16O4, 133.75 mM, Sigma-Aldrich). Then, the samples with TCA were centrifuged at 2,200 g for 15 min at 4°C. After centrifugation, 200 µL of the supernatants and standards were placed in cryotubes with an equal volume of 0.67% thiobarbituric acid (TBA, Sigma-Aldrich) and boiled at 95°C for 50 min. Finally, the tubes were placed on ice for 3 min. Standards and samples were placed on a 96-well plate and the absorbance was measured at 532 nm (Spectramax iD3) and the TBARS values were calculated using the malondialdehyde (MDA) standard curve. The values are reported as nanomoles of MDA per mL.

Levels of intracellular ROS

Intracellular ROS was quantified in the supernatant of HUVEC cultured with pre-treated placental explants supernatant using 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) by fluorescence with 2V,7V-dichlorofluorescein diacetate. Tert-butyl hydroperoxide (tBHP) at 1000 µM was added 2 h before reading as a positive control. After 24 h of treatment with placental explant supernatants, HUVEC were incubated with 100 µM DCFH-DA diluted in dimethyl sulfoxide (DMSO) for 30 min at 37°C. Then, cells were washed with PBS (pH 7.4) and the relative levels of fluorescence were quantified in a spectrophotofluorimeter (Hitachi F-4500, Japan, 485 nm excitation and 520 nm emission). The measured fluorescence values are reported as fluorescence intensity.

Statistical Analysis

Comparisons between groups were assessed by nonparametric tests (Mann-Whitney U test) and parametric analysis of variance (ANOVA) followed by

Bonferroni's multiple comparison test. Results were evaluated using the statistical program PRISM, (Graph Prism, version 6.01, GraphPad, USA) and statistical significance was accepted at $P<0.05$.

Results

Clinical and laboratory characteristics showed worse clinical outcomes in PE women

The differences between clinical and laboratory data of PE and NT pregnant women are reported in Table 1. There was no statistical difference in age between the groups. However, gestational age was lower in women with PE compared to NT. As expected, PE had worse clinical outcomes compared to NT pregnant women, and were associated with hypertension, hyperuricemia, and proteinuria.

VD and VDR were lower in plasma and placental explants from PE women

Figure 1A shows vitamin D 25 (OH)D levels in the plasma of PE and NT pregnant women. Figure 1B presents VDR gene expression in PE and NT placental explants. Both the VD levels and the receptor's gene expression were significantly lower in preeclamptic women.

Inflammatory cytokines were lower in supernatant from explants treated with VD

Figure 2 shows the concentrations of cytokines in supernatant of placental explants from PE and NT pregnant women cultured in the absence or presence of H_2O_2 and VD. Protein expression of IL-1β, TNF-α, and IL-18 was significantly higher in supernatants from control cultures of PE compared to NT pregnant women. In the PE group, treatment with VD showed decreased expression of IL-1β, TNF-α, and IL-18 compared to control explants supernatant. The H_2O_2+VD treatment showed lower protein expression of IL-1β, TNF-α, and IL-18 compared to H_2O_2 treatment in cultures of NT and PE explants.

Cell viability of HUVEC when treated with supernatants

After performing the dosages of VD and cytokines, the supernatant of PE

and NT explants was used to treat the cultures of HUVEC. Before carrying out the culture, a viability test was performed to observe if the supernatants were harmful to the cells. HUVEC viability after 24-h culture with placental explant supernatant from PE and NT women pre-treated with H_2O_2, H_2O_2+VD, and VD is presented in Supplementary Figure S2. No significant differences were found between treatments regarding cell viability.

Levels of nitrite and nitrate were significantly lower in HUVEC treated with supernatant of PE explants cultured with VD

Figure 3 shows nitrite and nitrate concentrations in supernatant of HUVEC after culture with supernatants of PE and NT placental explants pre-treated with H_2O_2, H_2O_2+VD, and VD. Levels of nitrite and nitrate were significantly higher in HUVEC cultures treated with PE control supernatant compared to NT control. Similarly, we observed higher levels of nitrite and nitrate in HUVEC cultures submitted to treatment with supernatant of placental explants from NT women treated with H_2O_2 compared to control supernatant. On the other hand, treatment of HUVEC with supernatant of PE explants cultured with VD led to a decrease in nitrite and nitrate levels compared to control supernatant, and also in the treatment with supernatant of placental explant of NT women cultured with H_2O_2+VD compared to only H_2O_2.

HUVEC treated with NT supernatant produced higher levels of NO

Figure 4A shows the NO fluorescence signal at 120 min after HUVEC were exposed to placental explant supernatants (Control NT and Control PE). There was a significant difference between HUVEC treated with Control NT supernatant compared to cells exposed to Control PE supernatant, with higher NO production in Control NT supernatant-treated cells. Figure 4B–D shows NO generation by HUVEC after 60 min of incubation with DAF-FM™ after treatment with supernatant of placental explants.During this time of incubation (60 min), a significantly higher NO production by HUVEC treated with Control NT supernatant compared to Control PE supernatant-treated cells as well as a high production of NO in VD PE supernatant treated cells compared to Control PE were observed

(Figure 4B). When cultured with pre-treated H_2O_2 NT supernatant, the cells showed lower NO production (Figure 4C). There was no significant difference between the production of NO in H_2O_2 supernatant treated cells compared to H_2O_2 +VD (Figure 4D).

Measurement of lipid peroxidation – TBARS

The levels of MDA are shown in Figure 5A. Significantly higher MDA production was detected in HUVEC treated with Control PE explant supernatants compared to Control NT as well as compared to the VD PE group. MDA levels were significantly higher in the generated supernatant of HUVEC treated with H_2O_2 NT supernatant compared to Control NT and H_2O_2+VD NT, as well as in the H_2O_2 PE-treated group compared to H_2O_2+VD PE.

Supernatants from PE explants cultured with vitamin D decreased levels of intracellular ROS in HUVEC

Figure 5B shows the ROS levels in the groups. Oxidative stress was higher in HUVEC cultured with PE control supernatant compared to the NT one. Supernatants from NT placental explants cultured with H_2O_2 increased oxidative stress in HUVEC, while supernatants from PE explants cultured with vitamin D decreased oxidative stress in these cells.

Discussion

In the present study, we demonstrated that placental explants from PE women cultured without stimulus produced endogenous levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-18. These results are in accordance with our previous studies showing a significant increase in NLRP3 inflammasome, caspase-1, IL-1β, and TNF-α in placental villi and in placental homogenate from PE women compared to NT pregnant women (19) and suggested the involvement of the placenta in the exaggerated inflammatory state that characterizes PE. In addition, this inflammatory process and the intense oxidative stress originated in the placenta may give rise to endothelial dysfunction in PE (7). Therefore, we evaluated the effect of the supernatant from placental explants of PE

and NT pregnant women treated with or without VD on oxidative stress and NO bioavailability in HUVEC. In our understanding, no studies have evaluated the effect of these supernatants on endothelial cells function. Thus, we studied placentas from NT pregnant women at term and placentas of PE women in the last trimester of pregnancy, at the time of delivery.

The present study demonstrated that HUVEC cultured with endogenous supernatants of non-treated placental explants from PE women produced increased levels of NO_2^-, NO_3^-, MDA, and ROS. These results suggest that the endogenous activation state of PE placental tissues triggered oxidative stress in these endothelial cells. In opposition, significantly higher production of NO was detected after HUVEC treatment with control supernatant of NT placental explants compared with the PE group, as well as after treatment with supernatant obtained from PE explants cultured with VD. Thus, these results demonstrated that HUVEC treated with supernatant from placental explants pre-cultured with VD showed a decrease in oxidative stress and an increase in the bioavailability of NO. This hypothesis may be raised since the concentration of IL-1β, TNF-α, and IL-18 in the supernatant of placental explants from preeclamptic women decreased after treatment with VD, showing its modulatory effect on inflammatory cytokines production, particularly for its regulatory activities on the inflammatory response (20).

It has been proposed that the decrease in NO production in the placenta could cause abnormal tissue perfusion, which is observed in PE states. Regarding systemic production of NO, it was shown that the vasodilation of the brachial artery mediated by NO-dependent flow was approximately three times lower in preeclamptic women compared to normal pregnant women (21). The NO system is also deranged in PE, as NO is a potent vasodilator, acting in order to induce relaxation in vascular smooth muscle cells (22). Decreased levels of NO have been reported in PE (23), and could be correlated with metabolic changes, such as hypertension, proteinuria, and platelet dysfunction (24).

Oxidative stress is an important phenomenon in PE (25) that could result from injury caused by hypoxia and reperfusion (26) and/or deficiency of antioxidant defenses (27). Peraçoli et al. (28) reported the endogenous activation of monocytes of women with PE, demonstrated by correlation among high production of

superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and TNF-α by these cells, and high serum levels of uric acid, contributing to the enhanced oxidative and inflammatory state characteristic of PE. Hyperuricemia was also detected in the preeclamptic women in the present study, associated with high production of inflammatory cytokines by placental explants. According to other authors, production of uric acid concomitant with O_2^- generation decreases NO bioavailability, leading to endothelial dysfunction (29). On the other hand, uric acid is also known as a powerful antioxidant through scavenging radical species and could also be regarded as a compensatory mechanism to counteract oxidative stress (29–31). Therefore, as hyperuricemia is a frequent finding in severe cases of PE, studies on oxidant and antioxidant properties of uric acid are of utmost importance to understand the pathophysiology of PE.

Several studies have been trying to show the effects of antioxidant therapy in women with PE. A systematic review performed by Rumbold et al. (32) concluded that supplementation with any antioxidant during pregnancy compared with control or placebo is associated with a reduced risk of developing PE. Similarly, Vadillo-Ortega et al. (33) showed that antioxidants in combination with L-arginine are effective in cases of PE risk. Evidence of association between vitamin D status and PE has been shown in various studies, but clinical trials did not show an independent effect of supplementation in cases of PE prevention. However, problems regarding dose, timing, and duration of supplementation with VD have not been completely explored (34).

Some authors have already shown that normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, but antioxidants also increase (35). In opposition, women with PE present an insufficient production of antioxidants to offset the increase in oxidative stress and lipid peroxidation (36).

An important oxidative stress biomarker, MDA is a product of fatty acid oxidation and an indicator of lipid peroxidation. Some authors showed increased MDA levels in the blood of preeclamptic women (37). MDA binds to TBARS, which are also elevated in the blood of women with PE (37), reflecting an oxidative stress status. In the present study, we observed a significant increase in MDA and intracellular ROS in HUVEC treated with supernatant control from placental

explants of PE compared to NT. On the other hand, the treatment with VD supernatant decreased these levels, demonstrating the antioxidant and anti-inflammatory effect. Wimalawansa (38) highlighted the effects of VD as one of the key controllers of systemic inflammation and oxidative stress, downregulating oxidative stress, cell and tissue damage, and the aging process. In the same way, the hypovitaminosis is enhances oxidative stress and systemic inflammation. VD is also known as a potent anti-oxidant that facilitates balanced mitochondrial activities, preventing oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage (38). Our findings are consistent with previous studies suggesting that the overproduction of free radicals and lipid peroxidation are important factors in the pathogenesis of preeclampsia (39). In summary, oxidative stress could contribute to the cytotoxic mechanisms in PE, inducing cellular damage, leading to endothelial cell injury, inflammation, and angiogenic imbalance (40).

In conclusion, our results suggest that placental explants of preeclamptic women treated with VD can decrease oxidative stress and lipid peroxidation, as well as increase the bioavailability of NO in HUVEC.

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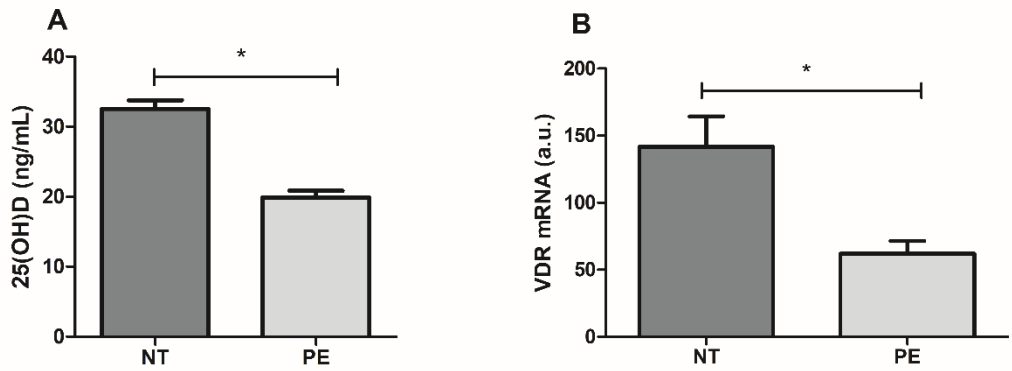

Figure 1. Levels of vitamin 25 (OH) D in plasma (A) and vitamin D receptor (VDR) gene expression (B) in the placental explants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8). Data are reported as means±SD. *P<0.05 (Mann-Whitney U test). a.u.: arbitrary units.

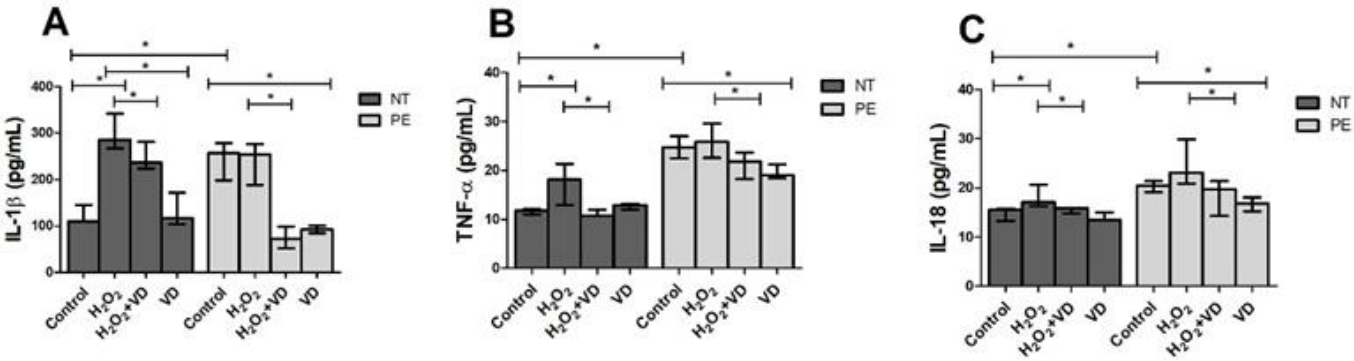

Figure 2. Concentration of cytokines in supernatant of placental explants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8) under different treatments. Data are reported as median with range. *P<0.05 (Mann-Whitney U test). IL-1β: interleukin-1 beta; TNF-α: tumor necrosis factor-alpha; IL-18: interleukin-18.

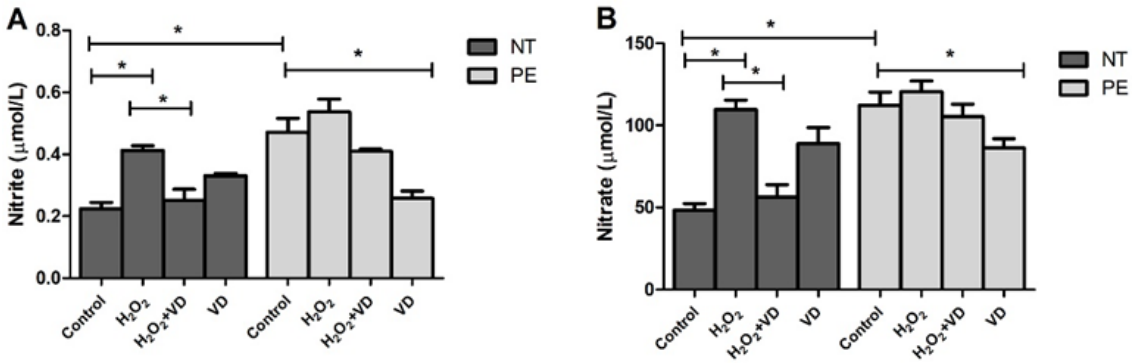

Figure 3. Levels of nitrite (A) and nitrate (B) in the supernatant of human umbilical vein endothelial cells treated with supernatant of placental explants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8), cultured in the absence (Control) or presence of H_2O_2, H_2O_2+VD, and VD. Data are reported as means±SD for one independent experiment, performed in quintuplicates. *P<0.05 (ANOVA followed by Bonferroni's multiple comparison test).

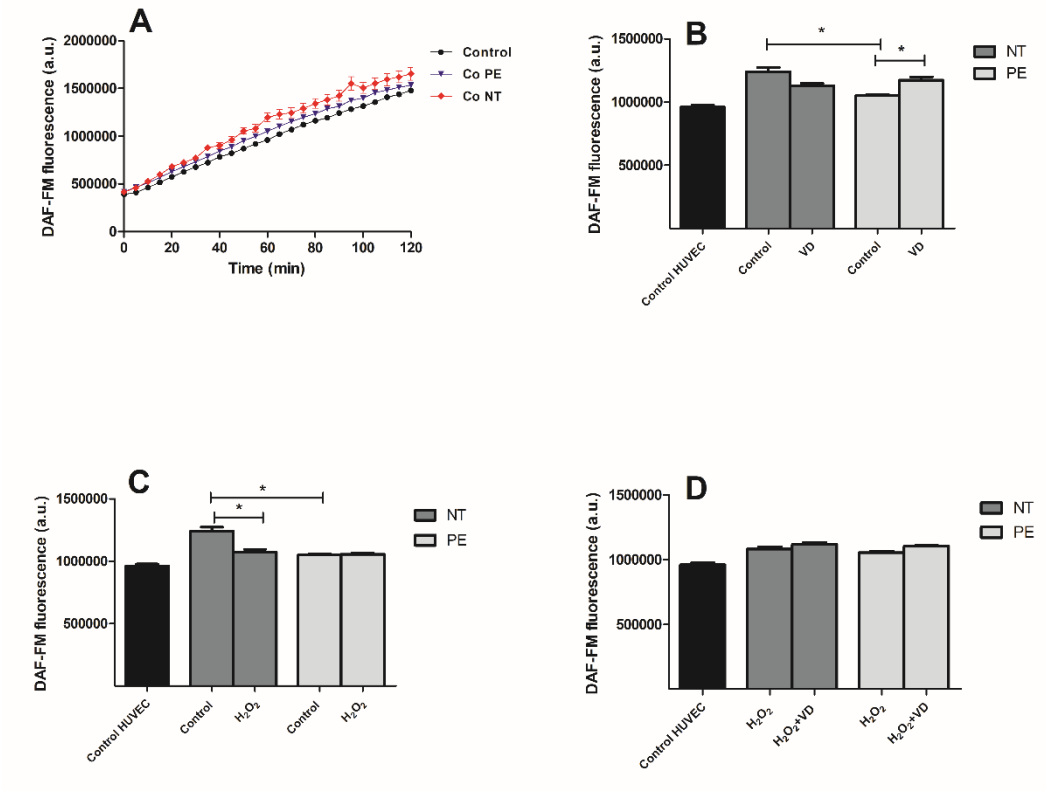

Figure 4. Nitric oxide fluorescence signal measured using DAF-FM™ after human umbilical vein endothelial cells (HUVEC) were exposed to placental explant supernatants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8). A, Control, Control NT, and Control PE; B, effect of treatment with vitamin D (VD) at 60 min; C, effect of treatment with H_2O_2 at 60 min; and D, effect of treatment with H_2O_2+VD at 60 min. Data are reported as means±SD for one independent experiment, performed in quintuplicates. *P<0.05 (ANOVA followed by Bonferroni's multiple comparison test). a.u.: arbitrary units.

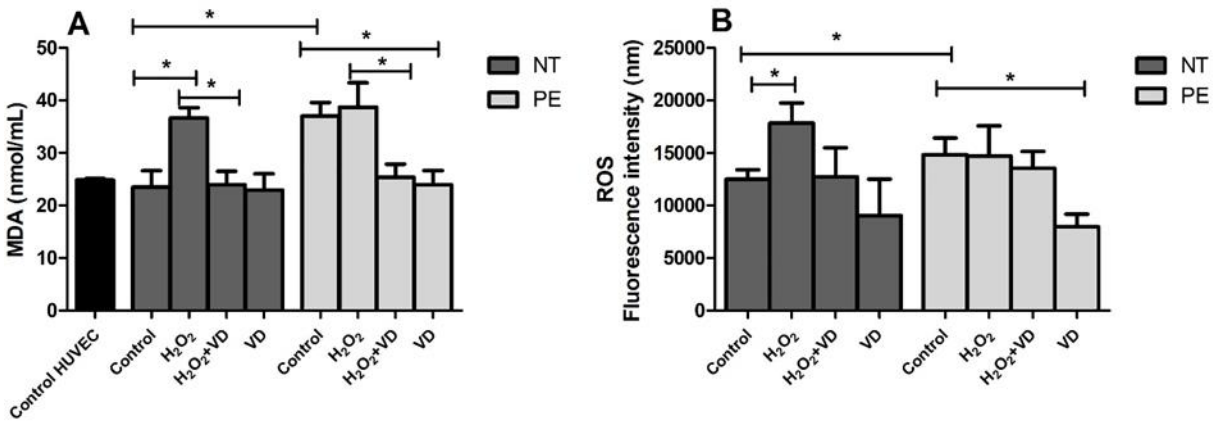

Figure 5. Malondialdehyde (MDA) levels (A) and levels of intracellular reactive oxygen species (ROS) (B) evaluated by fluorescence of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) in the supernatant of human umbilical vein endothelial cells (HUVEC) treated with supernatant of placental explants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8), cultured in the absence (Control) or presence of H_2O_2, H_2O_2+vitamin D (VD), and VD. Data are reported as means±SD. *P<0.05 (ANOVA followed by Bonferroni's multiple comparison test).

Table 1. Characteristics of the pregnant women.

Variable	Normotensive (n=8)	Preeclampsia (n=8)
Age (years)	29 (18–41)	28 (16–39)
Gestational age (weeks)	40 (36–41)	*34 (30–39)
Systolic blood pressure (mmHg)	120 (110–120)	*155 (140–70)
Diastolic blood pressure (mmHg)	70 (60–80)	*100 (90–110)
Uric acid (mg/dL)	3.2 (2.3–4.7)	*5.7 (3.7–6.9)
Proteinuria (mg/24 h)	<300	*730 (300–7540)

Data are reported as median (minimum-maximum.) *P<0.05) vs normotensive (Mann-Whitney U test).

Supplementary Material

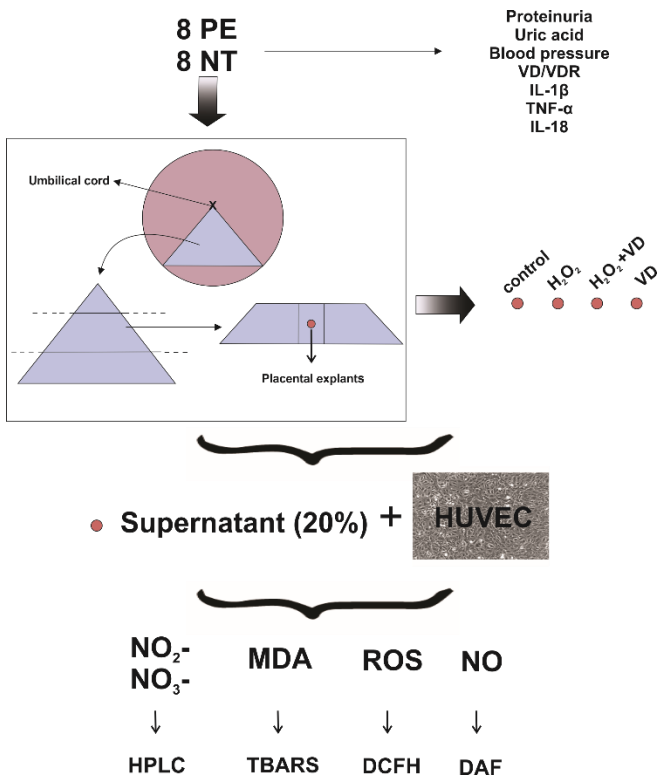

Figure S1. Methodology scheme of the study.

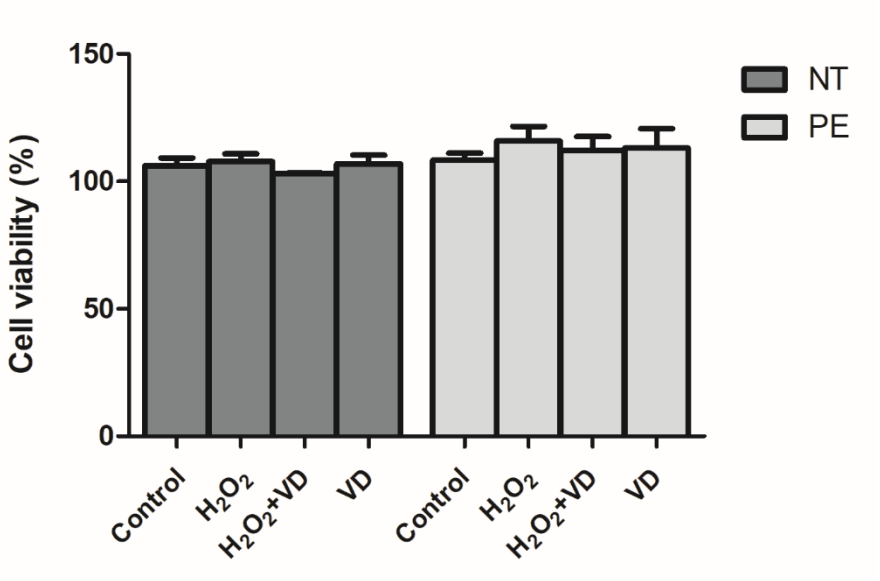

Figure S2. Viability of human umbilical vein endothelial cells (HUVEC) exposed to the supernatant of placental explants from preeclamptic (PE) (n=8) and normotensive (NT) pregnant women (n=8), cultured in the absence (Control) or presence of H_2O_2, H_2O_2+VD, and VD, using Prestoblue® assay. Data are reported as means±SD of two independent experiments performed in quintuplicates.

Capítulo 3

Os resultados referentes à esse capítulo serão enviados para publicação em periódico internacional

VITAMIN D MODULATES INFLAMMASOMES IN PLACENTAL EXPLANTS OF PREECLAMPTIC WOMEN AND DECREASED APOPTOSIS IN HUVEC CULTURED WITH MONOSODIUM URATE

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ABSTRACT

This study aimed to evaluate the vitamin D (VD) immunomodulatory effect on the NLRP1/NLRP3 inflammasomes in placental explants from preeclamptic (PE) and normotensive (NT) pregnant women, as well as on apoptosis in *Human Umbilical Vein Endothelial Cells* (HUVEC). Placental explants from 10 late-onset PE (LOPE), 10 early-onset PE (EOPE) and 10 NT pregnant women were cultured with or without monosodium urate (MSU) and VD. Gene and protein expression of NLRP1, NLRP3, HMGB1, caspase-1, *interleukin-1 beta* (IL-1β), and IL-18 were determined by quantitative PCR and Western blotting/ELISA. *Annexin V-fluorescein isothiocyanate* (FITC)/*Propidium iodide* (PI) staining was used to determine the apoptosis rate in HUVEC after treatments. Compared to NT pregnant women, the endogenous gene expression of NLRP1, NLRP3, HMGB1, IL-1β, and IL-18 was significantly higher in explants from EOPE and became decreased after treatment of the explants with VD. Similar decrease in protein expression of NLRP1, NLRP3, caspase-1 and HMGB1 was detected in explants from EOPE and NT after culture with MSU+VD. MSU was able to decrease HUVEC viability, induce NLRP1 and NLRP3 inflammasome activation, and increase the rate of apoptosis, while the addition of VD increased viability and decreased inflammation and apoptosis in these cells. The results demonstrated that NLRP1 and NLRP3 inflammasomes are upregulated in the placental tissue of EOPE women, associated with high production of inflammatory cytokines. The *in vitro* treatment with VD downregulated placental inflammation and decreased apoptosis in HUVEC stimulated with MSU, suggesting its immunomodulatory role in systemic inflammation of PE.

Key words: Preeclampsia, placental explants, HUVEC, MSU, inflammasome, cytokines, vitamin D.

1. INTRODUCTION

Normal pregnancy is characterized by a regulated inflammatory status, as a response to the maternal organism to allow foetal growth and development [1], whereas preeclampsia (PE) proves to be a disease that involves generalized systemic inflammatory process and vascular endothelial damage associated with placental dysfunction [2].

As a multifactorial disease, PE is responsible for most cases of morbidity, mortality and preterm birth, and affects approximately 2–8% of all pregnancies [3]. Clinical diagnosis is performed from the 20th week of pregnancy or in the first days postpartum, in a previously normotensive pregnant women. The main symptoms that define the diagnosis of PE are the onset of hypertension associated or not with proteinuria. More recently, thrombocytopenia, impaired liver function, renal insufficiency, pulmonary oedema and new-onset cerebral or visual disturbances have been associated with PE devopment [4,5].

Early-onset PE (EOPE), that manifests before 34 weeks of gestation, and late-onset PE (LOPE), that occurs from 34 weeks of pregnancy, should be treated as distinct pathologies from the etiological point of view and in relation to the prognosis. The two forms share some etiological characteristics in common, but differ in relation to several risk factors, leading to different outcomes [6].

The pathophysiology of PE is not yet fully understood, but it is currently known that placental ischemia is fundamental in this process, since the release of products resulting from poor perfusion in the maternal circulation can lead to systemic endothelial dysfunction [2]. These products can act as inflammatory mediators, activating the innate immune system, the first mechanism by which the organism responds immediately to infections and injuries. Pattern recognition receptors (PRRs) recognize molecules released by pathogens or damaged cells. These molecular signals are called damage-associated molecular pattern (DAMPs) or pathogen associated molecular pattern (PAMPs) [7].

DAMPs bind to PRRs expressed in innate immunity cells. One of the most important PRRs is part of the family of NLR receptors (protein with nucleotide

oligomerization domain), which are cytosolic proteins capable of recognizing cytoplasmic DAMPs and recruiting other proteins, forming signalling complexes that promote inflammation [8]. Inflammasome is a large protein complex that acts as a signalling receptor reacting to PAMPs and DAMPs and mediates a highly inflammatory state, generating active forms of interleukins, such as IL-1β, IL-18 [9], and releasing HMGB1 to the extracellular environment, acting as a proinflammatory mediator and contributing to the onset of the inflammatory response in PE [10,11].

Among all the described inflammasomes, the NLR family pyrin domain containing 3 (NLRP3) is the best characterized and when activated in endothelial cells, during pathological conditions, this complex can aggravate endothelial dysfunction, contributing to the development of various diseases [12].

In the literature, the role of inflammasome in the maternal-fetal interface has been little studied. The involvement of the NLRP3 inflammasome in placental immune defense was described by Pontillo et al. The authors showed that stimulation of placental cells, such as first trimester cytotrophoblast and human deciduous cells with lipopolysaccharide (LPS) led to an increase in the expression of caspase-1, IL-1β and NLRP3 [13]. The secretion of IL-1β by fetal membranes and mononuclear cells isolated from umbilical cord, placenta and maternal blood, obtained from normal term pregnancy and stimulated with LPS is dependent on caspase-1, suggesting that the NLRP3 inflammasome appears to be necessary for the processing of IL-1β in placental tissue [14]. Mulla et al. (2011) demonstrated that trophoblastic cells obtained from normal pregnant women in the first trimester of pregnancy express endogenously NLRP1 and NLRP3 receptors and also caspase-1. According to these authors, crystals of uric acid can activate the inflammasome in the trophoblast, characterizing a new mechanism of inducing inflammation at the maternal-fetal interface, which leads to placental dysfunction and adverse results such as PE [15]. In a recent study, our group demonstrated that peripheral blood monocytes of preeclamptic women displayed increased gene expression of NLRP3 inflammasome after stimulation with monosodium urate (MSU) [16].

Moreover, PE women show hyperuricemia, a characteristic of the disease, frequently associated with disease severity, by promoting endothelial dysfunction,

damage and inflammation [17]. This fact suggests that uric acid may contribute to PE pathogenesis through its inflammatory effects [18,19], and its crystals of MSU can activate the NLRP3 inflammasome in gout [20] and in PE [16].

Human umbilical vein endothelial cells (HUVEC) are mostly mature endothelial cells, employed frequently in studies of endothelial cell function, blood vessel repair and angiogenesis [21,22]. Literature data suggest that uric acid itself has been also shown to induce vascular endothelial dysfunction via oxidative stress and inflammatory responses. Otani et al. [23] showed that antioxidants improved vascular endothelial dysfunction caused by hyperuricemia in these cells. Besides that, several groups have demonstrated that endothelial cells may also undergo apoptosis in inflammatory conditions. This type of cell death have been observed in hypertension, inflammation, and arteriosclerosis [24,25].

The signaling of inflammasome activation can contribute to vascular inflammation and is important in the sense that, through this pathway, it induces cell death. One type of programmed cell death is apoptosis, which is essential for homeostasis of human tissues, including the placenta. Increased apoptosis of the trophoblast during PE can contribute to suppression of angiogenesis and placental growth, leading to the development of the disease [26].

Vitamin D exerts numerous effects on the organism. This hormone plays an important role in the implantation, placentation, and maintenance of a healthy pregnancy. During human pregnancy the conversion of 25(OH)D to 1,25(OH)2D is increased in the placenta, demonstrating the importance of this organ in the metabolism of vitamin D [27].

Vitamin D deficiency is reported in preeclamptic women [28-30], while other studies show an association between this deficiency and the risk of developing PE [31,32], suggesting that vitamin D supplementation may protect the maternal organism by its modulatory influence on the immune response.

Considering the inflammatory status described in the placenta of pregnancies complicated by PE, as well as activation of apotosis in endothelial cells, this study aimed to evaluate the immunomodulatory action of vitamin D on NLRP1 and NLRP3 inflammasomes in placental explants of preeclamptic and normotensive pregnant women, as well as on apoptosis in HUVEC.

2. METHODS

2.1. Study population and ethical statment

Placentas from 20 preeclamptic women and 10 normotensive (NT) pregnant women were collected in the Obstetrics Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP-Brazil, between August 2018 and February 2020. Women with PE were classified according to the onset of clinical manifestations of the disease at moment of diagnosis as early onset PE - EOPE (< 34 weeks of gestation, n = 10) and late-onset PE - LOPE (≥ 34 weeks of gestation, n = 10), according to the criteria suggested by Huppertz [33]. Gestational age was calculated from the last menstrual period and confirmed by ultrasound dating. The diagnosis of PE was made considering when, without a history of hypertension, a pregnant woman developed hypertension (blood pressure ≥ 140/90 mmHg) with or without proteinuria (≥ 300 mg in a 24-hour urine) after the 20th week of gestation [3]. The NT group did not present a history of hypertensive disorders in pregnancy, and remained normotensive until the time of delivery.

Exclusion criteria included chronic hypertension, multiple gestations, prior preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, cancer, acute infectious disease, cardiovascular, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all women involved in the study (Protocol number: 4.418.043). This work has been carried out under the Code of Ethics of the World Medical Association (Declaration of Helsinki). Also, we inform that all mandatory laboratory health and safety procedures have complied with in the course of conducting any experimental work reported in this paper and all experiments were performed following relevant guidelines and regulations.

2.2.Collection of placental tissue and culture of placental explants with MSU and VD

Placentas were collected from delivery by elective caesarean section. Immediately after delivery, all placentas considered for the study were examined

macroscopically and processed within 10 min, as previously described [34], to constitute placental explants.

A total of 11 mg of human villous tissue was cultured according to Nunes et al. [34] in 24-well plates (SPL Life Sciences, Korea) over 24 h for stabilization. After this time, placental explants from PE and NT women were cultured for 24 h with or without MSU (50 µg/mL), vitamin D (100 nM) and with or without MSU plus VD (Sigma-Aldrich, SL, Missouri, USA).

2.3. HUVEC culture and treatment with MSU and VD

Human umbilical vein endothelial cell line - HUVEC (Lonza, Switzerland) was acquired with a certification proving that the cells were from the designated type. All experiments were performed using cells in the sixth passage and in quintuplicates. HUVEC were cultured until reaching 80% confluence and then incubated for 24 h with or without MSU (50 µg/mL), vitamin D (100 nM) and with or without MSU plus VD (Sigma-Aldrich).

2.4. Expression of transcripts related to inflammation in placental tissue and HUVEC

Placental explants and HUVEC were employed to determine the expression of the genes encoding NLRP1, NLRP3, HMGB1, caspase-1, IL-1β, and IL-18 at the transcriptional level. Total RNA was extracted from the placental explants and cells after culture using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's protocol, and the quantitative polymerase chain reaction (qPCR) was performed according to Weel et al. [35]. Briefly, isolated RNA was treated with DNAse I Amp Grade (Invitrogen). Subsequently, the synthesis of complementary DNA (cDNA) was conducted using the ImProm-II TM Reverse Transcription System, according to the manufacturer's protocol. The qPCR was performed using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA), and the variants of the studied targets were aligned in the MEGA 5.1 program. Each primer was subsequently selected by the software Primer-BLAST. Primers located in exon-exon junctions guarantee the purity of the reaction, namely the absence of any genomic DNA that may contaminate it. The primer sequences used in this study

are shown in Table 1.

Table 1. Primers for NLRP1, NLRP3, HMGB1, caspase-1, IL-1β, IL-18 and GAPDH. Numbers indicate nucleotide positions in the corresponding transcripts.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank
NLRP1	(1728)TCCGGCTCCCATTAGACAGA(1747)	(1810)AGACCCATCCTGGCTCATCT(1791)	NM_033004.3
NLRP3	(2826)GAGGAAAAGGAAGGCCGACA(2845)	(2917)TGGCTGTTCACCAATCCATGA(2897)	NM_004895.4
HMGB1	(1404)TACGAAAAGGATATTGCTGC(1423)	(1505)CTCCTCTTCCTTCTTTTTCTTG(1484)	NM_001313893.1
CASP1	(1065)AGACATCCCACAATGGGCTC(1084)	(1172)TGAAAATCGAACCTTGCGGAAA(1151)	NM_033292.3
IL1B	(544)GAGCAACAAGTGGTGTTCTCC(564)	(653)AACACGCAGGACAGGTACAG(634)	NM_000576.2
IL18	(438)ACTGTAGAGATAATGCACCCCG(459)	(517)AGTTACAGCCATACCTCTAGGC(496)	NM_001562.3
GAPDH	(684)CGTGGAAGGACTCATGACCA(703)	(801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Each reaction was set in duplicate, and the conditions for the qPCR were as follows: initial denaturation at 96 °C for 2 min and then 40 cycles at 95 °C for 15 s and 60 °C for 60 s, followed by a melting curve. Expression values of the analysed transcripts were normalized to that of the enzyme encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve [36]. To analyse the relative expression, after the analysis of gene expression, we chose an RNA sample obtained from each group, which received a relative value of 100. All other samples received values for that sample.

2.5. Cytokine determinations

The concentrations of IL-1β and IL-18 in supernatants of placental explants from NT and PE women, as well as HUVEC supernatants obtained after treatment with or without MSU and VD were determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Assay sensitivity limits were 1.0 pg/mL for IL-1β, and 5.15 pg/mL for IL-18.

2.6. Obtaining homogenate from placental explants and HUVEC lysate for electrophoresis and blotting

Placental explants from three EOPE, three LOPE, and three NT pregnant women were cultured for 24 h in the presence or absence of MSU, VD or MSU plus vitamin D. The fragments previously stored at -80 °C were removed for thawing, and the weight was documented in grams of tissue. These fragments were transferred to the tissue homogenizer tube containing 2 mL of protease inhibitor buffer: 150 mM/L NaCl and 20 mM/L Tris pH 7.5, 5 mM/L EDTA and 1 mM/L L phenylmethylsulphonyl fluoride (pH 7.3). After homogenization in ice bath, the material was placed in test tubes and centrifuged at 3500 rpm for 20 min at 4 ºC. The supernatant was then aliquoted into 0.5 mL microtubes and stored at -80 °C. In the same way, HUVEC cultured for 24 h in the presence or absence of MSU and VD were subjected to cell lysis, and the extract was centrifuged at 1500 g for 10 min. The supernatant obtained from the culture of placental explants and the cell extract were employed to determine the total protein concentration by Lowry's method [37] for NLRP3, NLRP1, caspase-1 and HMGB-1 determinations.

After determining the homogenate protein concentration of placental explants and HUVEC extracts, 30 μg of proteins were treated with Laemli sample buffer (Bio-Rad, Hercules, CA, USA) and β-mercaptoethanol at 95 °C for 5 min, then the proteins were separated on SDS-PAGE and after electrophoresis, transferred to a nitrocellulose membrane. Nonspecific protein binding was blocked by incubating the membranes in 5% skim milk in Tris-HCl buffer containing 0.1% Tween 20 (TBST) for 45 min at room temperature. Membranes were subsequently incubated for 18 h with rabbit polyclonal antigen-specific primary antibodies: anti-NLRP1, anti-NLRP3, anti-caspase-1 and anti-HMGB1 (Novus Biologicals, Littleton, CO, USA) at dilution on manufacturer recommended concentrations containing TBST. After washing, the membranes were incubated with rabbit specific secondary antibody diluted in TBST for 1 h. Immunoreactive components were revealed by the Amersham™ ECL Select™ Western Blotting Detection Reagent luminescent kit (GE Healthcare®, Little Chalfont, United Kingdom).

2.7. Apoptosis Rate in HUVEC by Annexin V-FITC/PI Staining

Annexin V assay were performed with the eBioscience™ Annexin V Apoptosis Detection Kit FITC (Thermo Fisher Scientific, eBioscience, Waltham, Massachusetts, USA). The HUVEC ($2x10^5$ cells) were placed in a 24-well plate and left for 6 h to attach. The cells were then treated with MSU (50 µg/mL), vitamin D (100 nM) or their association for 18h. After the treatment period, cells were trypsinized and centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 10 min at 1200 rpm for culture medium removal. The cell pellet was washed twice with phosphate-buffered saline (PBS) and centrifuged at 10,000 rpm for 30 s, followed by resuspension with 100 µL Annexin V binding buffer and incubation with 2µL Annexin V and 4µL propidium iodide (PI) for 15 min in the dark at room temperature. After the incubation, the prepared cells were then analyzed by flow cytometry in a FACSCantoTMII with FACSDiva (BD Biosciences, Clontech, CA, USA) software. The flow cytometric results were analyzed by the FlowJo software (vX.10.6 version, Tree Stars Inc., Ashland, OR, USA).

2.8. Statistical analysis

The results were evaluated using parametric and non-parametric methods according to the statistical program GraphPad Prism, version 6.01 (GraphPad, CA, USA). The clinical characteristics of preeclamptic and normotensive pregnant women were analysed by Kruskal-Wallis test. Apoptosis, gene and protein expression were evaluated by analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. EOPE is associated with high levels of blood pressure and proteinuria

Table 1 shows the clinical and laboratory characteristics of preecalmptic women (n =20) and NT pregnant women (n =10). Regarding age, there was no statistical difference between the groups. However, gestational age was lower in women with PE compared to NT ones, with a lower gestational age observed in the group of preecalmptic women. In addition, systolic and diastolic blood pressure values were

significantly higher in women with PE compared to NT pregnant women. Proteinuria levels were significantly higher in the group of EOPE compared to the other groups. In LOPE group these values were significantly higher than in NT.

Table 1. Clinical and laboratory characteristics of EOPE, LOPE and normotensive pregnant women.

Parameters	EOPE (< 34w) n = 10	LOPE (≥ 34w) n = 10	Normotensive (NT) n= 10
Age (years)	28 (14 – 40)	24 (16 – 43)	29 (18-41)
Gestational age (weeks)	29 * + (26 – 33)	37 * (34 – 40)	40 (36-41)
Systolic blood pressure (mmHg)	160 * (140 – 200)	155 * (140 – 180)	120 (110-120)
Diastolic blood pressure (mmHg)	110 * (90 – 140)	100 * (90 – 120)	70 (60-80)
Proteinuria (mg/24h)	3105 * + (300-26.052)	535* (300-10.800)	<300

Results expressed as median (minimum-maximum) * ($p < 0.05$) vs NT; + ($p < 0.05$) vs EOPE (Kruskal-Wallis test).

3.2. Gene expression of inflammasome components and inflammatory cytokines is elevated in EOPE and in cultures stimulated with MSU, while treatment with VD reduces inflammation

3.2.1. EOPE shows greater endogenous gene expression of NLRP3 compared to LOPE and NT. VD reduces mRNA levels in this group and in HUVEC stimulated with MSU

Relative quantification of gene expression was performed for NLRP1,

NLRP3, caspase-1, IL-1β, IL-18, and HMGB1 in placental explants of preeclamptic women (EOPE and LOPE) normotensive pregnant women (NT) and in HUVEC, stimulated with MSU and treated or not with VD.

Figure 1 shows that the endogenous gene expression of NLRP3 (A), NLRP1 (B), caspase-1 (C) and HMGB1 (D) was significantly higher in the explants of EOPE compared to NT, as well as increased endogenous expression of NLRP3 and HMGB1 in EOPE explants compared to LOPE. In addition, caspase-1 expression in the LOPE was also significantly higher compared to the NT group. Regarding treatment with vitamin D, all genes had their expression decreased with this treatment in the EOPE group compared to its explant control cultures. Explant cultures of NT pregnant women stimulated with MSU showed increased expression of NLRP3, NLRP1 and caspase-1 compared to unstimulated control.

The MSU+VD explant treatment showed lower gene expression of NLRP3 and caspase-1 in the NT group, NLRP1 in all the studied groups and HMGB1 in the EOPE and NT groups compared to the stimulus with MSU.

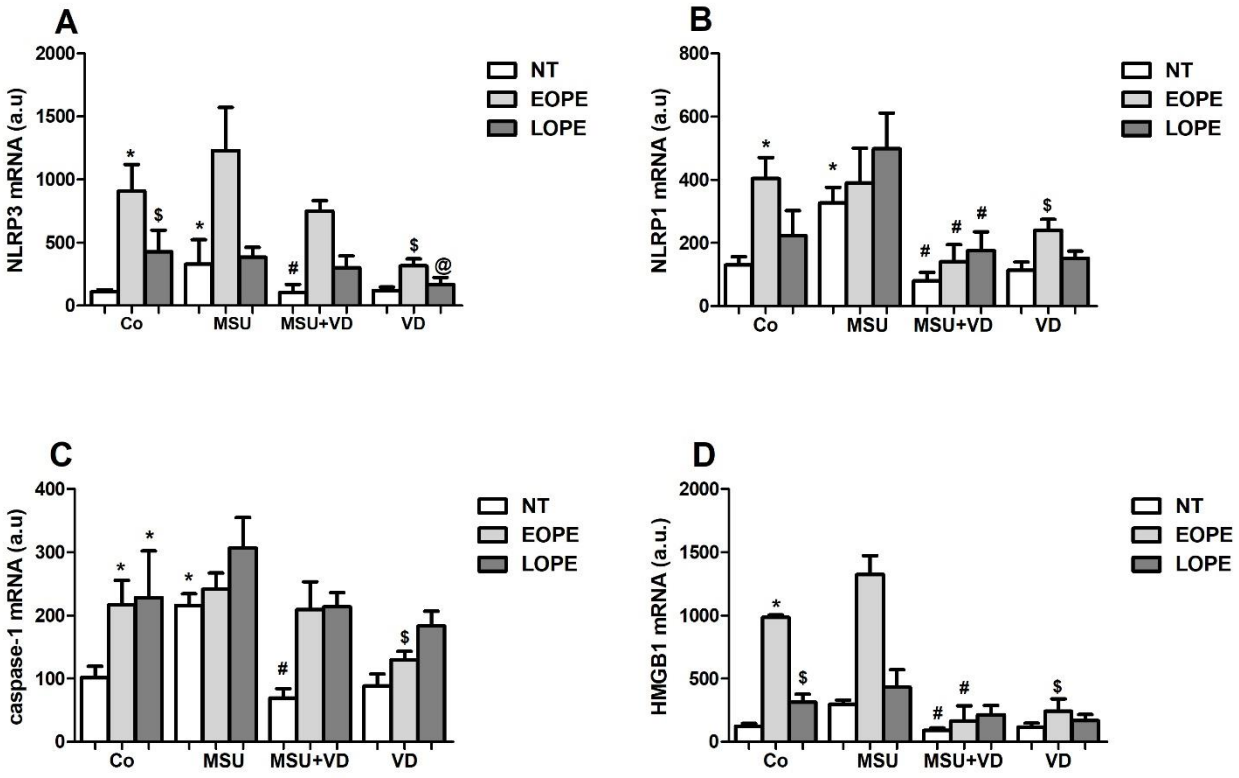

Figure 1. Gene expression of NLRP3 (A), NLRP1 (B), caspase-1 (C) and HMGB1

(D) in placental explants obtained from 10 normotensive pregnant women (NT), 10 pregnant women with early-onset preeclampsia (EOPE) and 10 pregnant women with late-onset preeclampsia (LOPE) cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co NT, $ ($p < 0.05$) vs Co EOPE, @ ($p < 0.05$) vs Co LOPE, # ($p < 0.05$) vs MSU.

Figure 2 shows gene expression of inflammasome components detected in HUVEC. mRNA of NLRP3 (A), NLRP1 (B) and HMGB1 (D) was significantly higher in HUVEC treated with MSU compared to unstimulated cells (Co). The MSU+VD treatments induced lower gene expression of NLRP3, NLRP1, caspase-1 and HMGB1 compared to cell stimulus with only MSU.

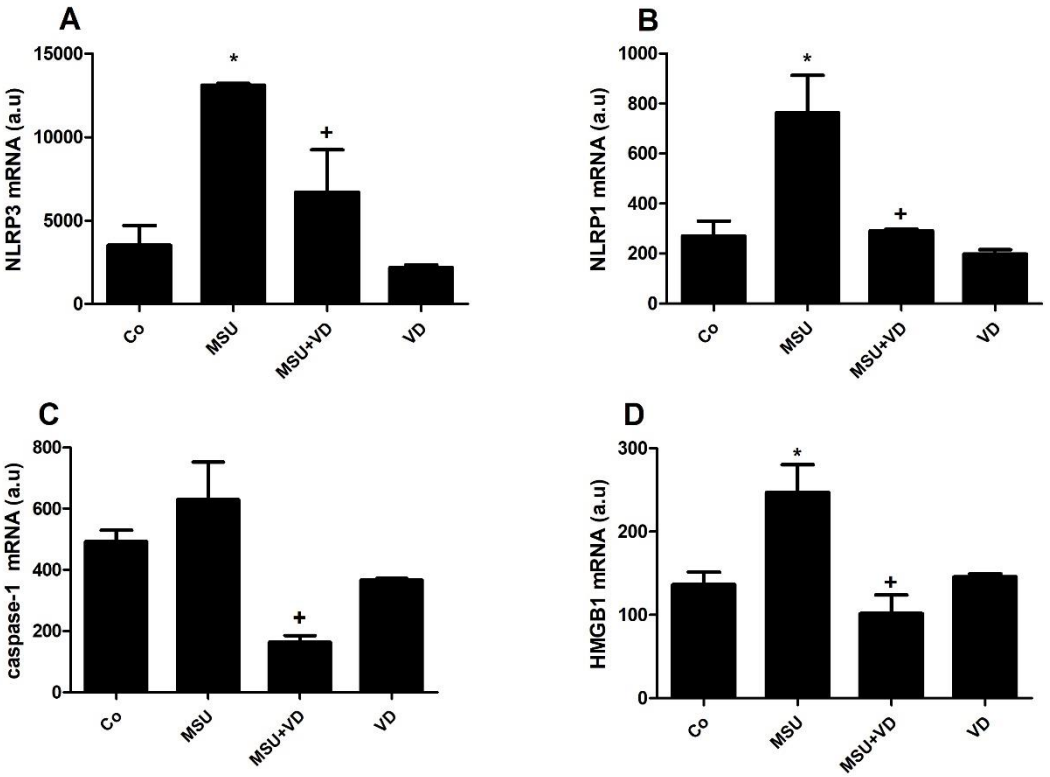

Figure 2. Gene expression of NLRP3 (A), NLRP1 (B), caspase-1 (C) and HMGB1 (D) in HUVEC cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co , + ($p < 0.05$) vs MSU.

3.2.2. Culture with MSU increases expression of inflammatory cytokines in explants and HUVEC, whereas VD decreases the expression of cytokines in EOPE and HUVEC stimulated with MSU

Figures 3 and 4 show the gene expression of IL-1β (A) and IL-18 (B) in explants from EOPE, LOPE and NT groups, and in HUVEC, respectively.

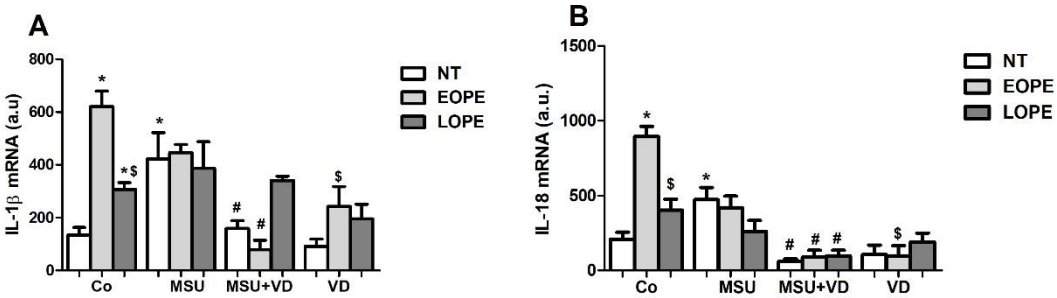

Figure 3. Gene expression of IL-1β (A) and IL-18 (B) in explants of placental tissue obtained from 10 normotensive pregnant women (NT), 10 pregnant women with early-onset preeclampsia (EOPE) and 10 pregnant women with late-onset preeclampsia (LOPE) cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co NT, $ ($p < 0.05$) vs Co EOPE, # ($p < 0.05$) vs MSU.

The basal gene expression of IL-1β and IL-18 was significantly higher in the explants of EOPE compared to LOPE and NT groups. In addition, the endogenous expression of IL-1β in LOPE was significantly higher compared to the NT group. In relation to treatment with vitamin D, both cytokine genes had their expression decreased in the group of EOPE compared to non-treated control (Co). In addition, explant cultures of NT pregnant women stimulated with MSU showed increased expression of both cytokines compared to unstimulated control.

The MSU+VD treatments led to lower gene expression of IL-18 in all studied groups and IL-1β in EOPE and NT groups compared to the stimulus with MSU.

Figure 4 shows gene expression of IL-1β (A) and IL-18 (B). mRNA levels was significantly higher in HUVEC cultures treated with MSU compared to unstimulated cells (Control group). The MSU+VD treatment induced lower gene expression of IL-1β and IL-18 compared to the stimulus with only MSU.

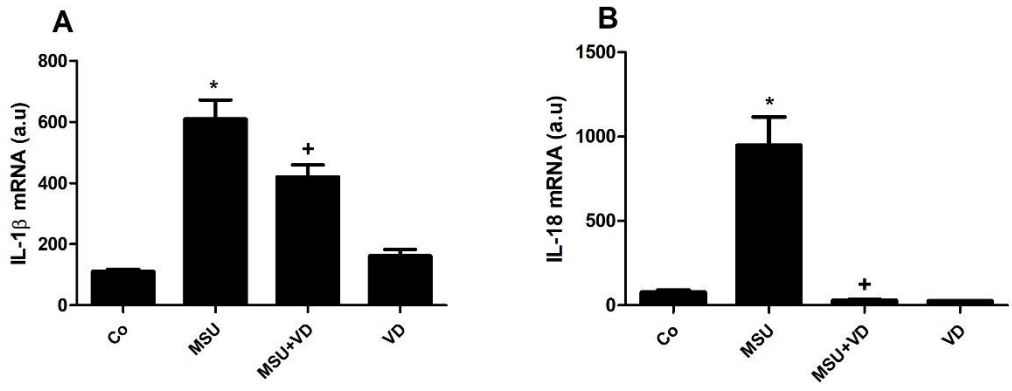

Figure 4. Gene expression of IL-1β (A) and IL-18 (B) in HUVEC cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co , + ($p < 0.05$) vs MSU.

3.3. Endogenous protein expression of IL-1β and IL-18 is higher in EOPE and LOPE versus NT explants, and stimulation with MSU increases cytokines production in HUVEC, while concomitant treatment with MSU+VD decreases these levels

In figures 5 and 6 are shown concentrations of IL-1β (A) and IL-18 (C) in supernatants from placental explants and HUVEC treated with MSU, MSU+VD or VD.

Figure 5 demonstrates that the protein levels of IL-1β and IL-18 was significantly higher in the culture supernatants of placental explants from EOPE and LOPE in relation to NT. Regarding to IL-1β α, the supernatants of the explant cultures of NT pregnant women treated with MSU showed increased expression of these cytokines when compared to control explants. When treated with VD, the supernatants of EOPE explants showed a decrease in the protein expression of IL-1β α when compared to the control, without treatment, as well as the expression of IL-18 in the supernatants of LOPE when treated with VD, compared to the control.

The MSU+VD treatment led to lower IL-1β protein expression compared to MSU treatment in explant cultures of NT, EOPE and LOPE groups. In the NT group, IL-18 levels were significantly reduced in this treatment.

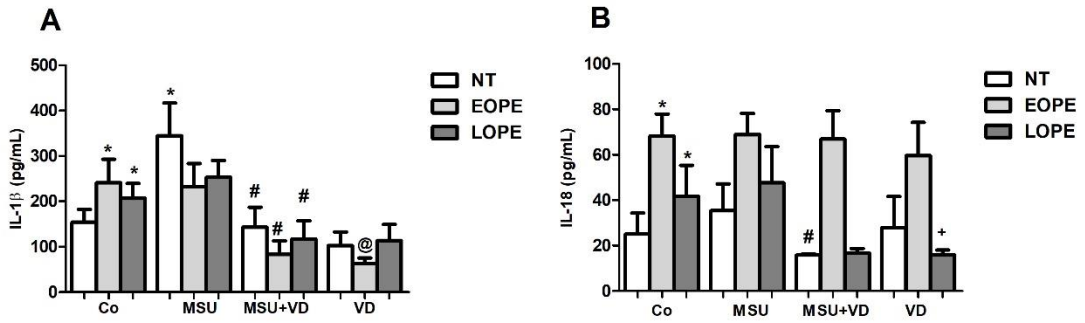

Figure 5. Expression of IL-1β (A) and IL-18 (C) in supernatant from placental explants obtained from 10 normotensive pregnant women (NT), 10 pregnant women with early-onset preeclampsia (EOPE) and 10 pregnant women with late-onset preeclampsia (LOPE) cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co NT,+ ($p < 0.05$) vs Co LOPE, # ($p < 0.05$) vs MSU.

Concentrations of IL-1β (A) and IL-18 (B) was significantly higher in the HUVEC treated with MSU compared to unstimulated cells (Control group). The MSU+VD treatments showed lower concentrations of both cytokines compared to the stimulus with only MSU (Figure 6).

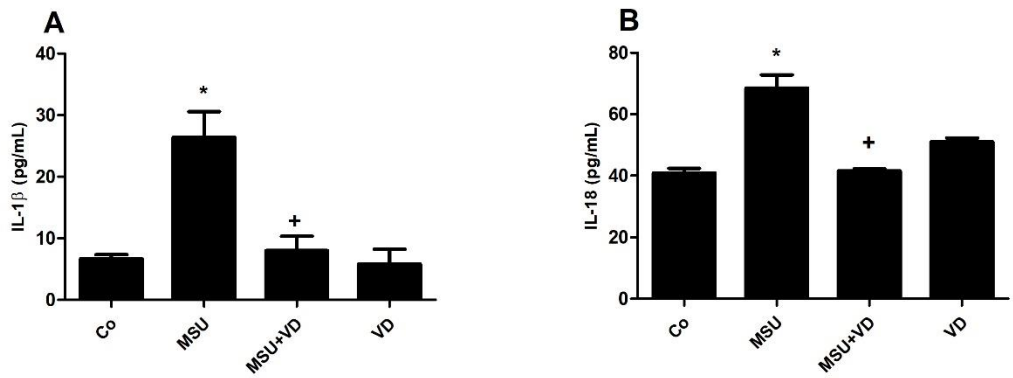

Figure 6. Determination of IL-1β (A) and IL-18 (B) in HUVEC cultured in the absence (Co) or presence of MSU, MSU+VD and VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co , + ($p < 0.05$) vs MSU.

3.4. Protein levels of inflammasomes decrease with concomitant treatment of placental explants with MSU+VD in EOPE, LOPE, NT and in HUVEC

The protein expression analysis of NLRP3, NLRP1, caspase-1 and HMGB1 was performed in placental explants of EOPE (n=3), LOPE (n = 3) and NT (n = 3) groups (Figure 7), and in HUVEC -in triplicates (Figure 8) stimulated with MSU and treated or not with VD.

Figure 7 shows the expression of NLRP3, NLRP1, caspase-1 and HMGB1 in placental explants of EOPE (A-D), LOPE (E-H) and NT (I-L).

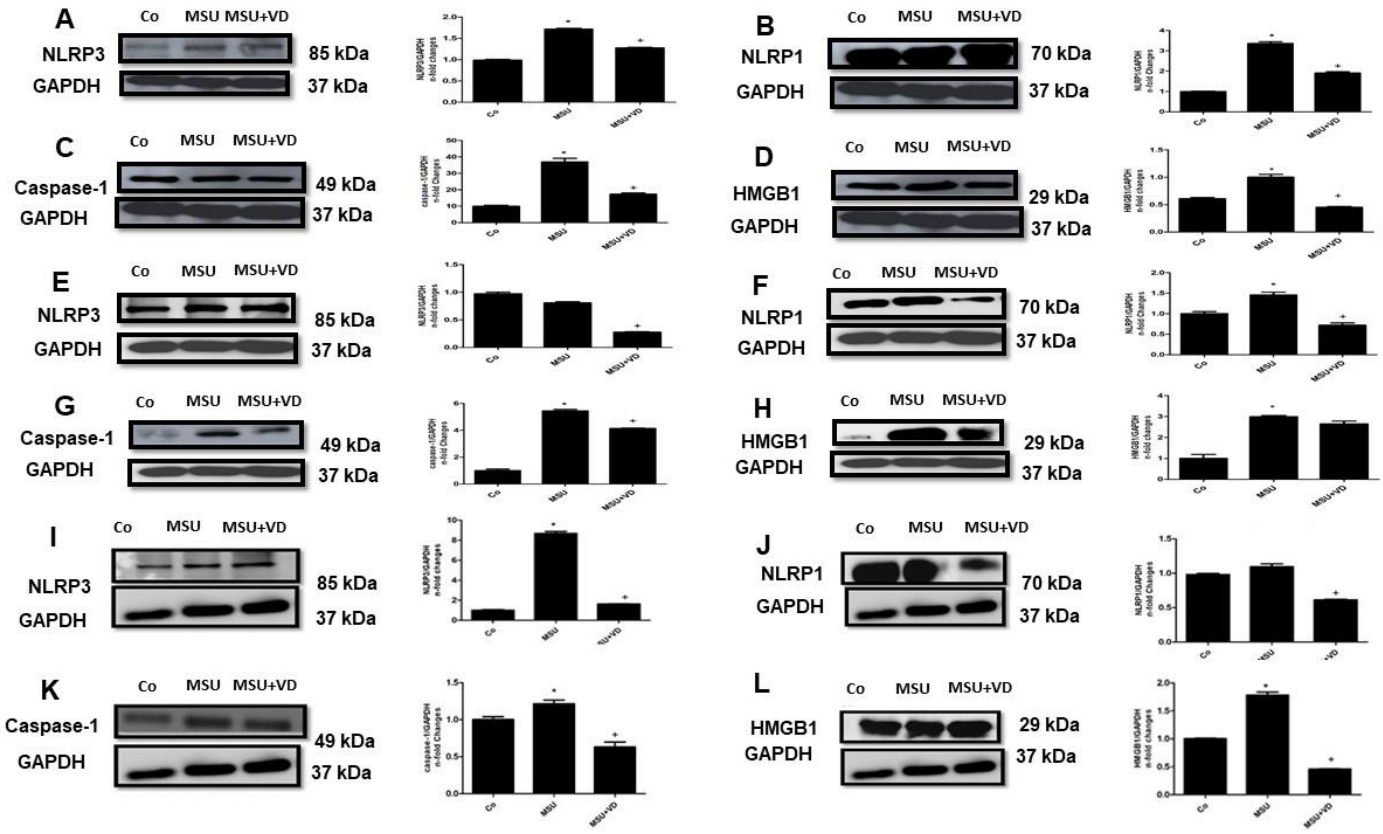

Figure 7. Protein expression of NLRP3, NLRP1, caspase-1 and HMGB1 in placental explants of EOPE (A-D), LOPE (E-H) and NT (I-L). cultured with MSU and treated with VD. Results expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co, + ($p < 0.05$) vs MSU.

Protein expression of NLRP1, caspase-1 and HMGB1 increased significantly with the stimulus with MSU in relation to the control culture (Co) in both LOPE and EOPE groups. Treatment with MSU+VD induced less protein expression of NLRP3, NLRP1, and caspase-1 compared to treatment with MSU alone in LOPE, as well as NLRP3, NLRP1, caspase-1 and HMBG1 in EOPE explants.

In relation to NT group, protein expression of NLRP3, caspase-1 and HMGB1

increased significantly with the stimulus with MSU in relation to the control (Co) culture. Treatment with MSU+VD induced less protein expression of NLRP3, NLRP1, caspase-1 and HMGB1 compared to treatment with MSU only.

Figure 8 shows the protein expression of NLRP3 (A/B), NLRP1 (C/D), caspase-1 (E/F) and HMGB1 (G/H) in HUVEC stimulated with MSU and treated or not with VD.

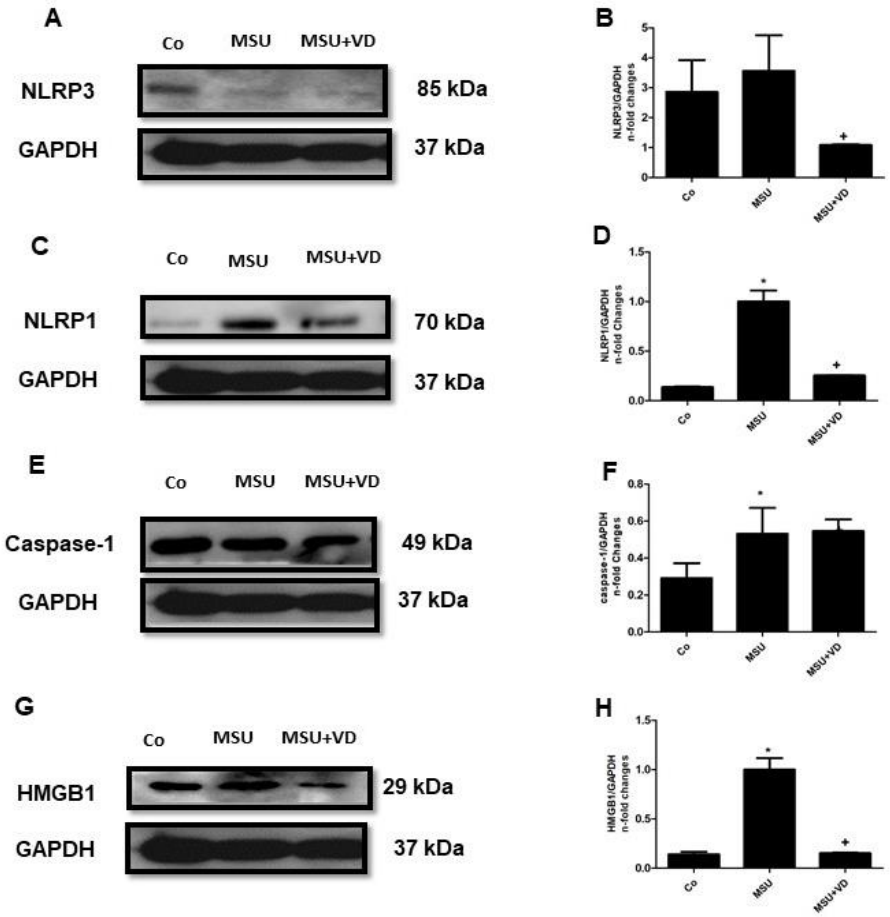

Figure 8. Protein expression of NLRP3 (A/B), NLRP1 (C/D), caspase-1 (E/F) and HMGB1 (G/H) in HUVEC (triplicate) cultured with MSU and treated with VD. Results expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co, + ($p < 0.05$) vs MSU.

Protein expression of NLRP1, caspase-1 and HMGB1 increased significantly with the stimulus with MSU in relation to the control (Co) culture. Treatment with MSU+VD induced less protein expression of NLRP3, NLRP1 and HMGB1 compared to treatment with MSU only.

3.5. MSU reduces cell viability and induces apoptosis in HUVEC while concomitant treatment with VD mantains cell viability and decrease apoptosis

The HUVEC cell viability was reduced after the stimulus with MSU compared to the control culture, while treatment with MSU+VD showed a maintenance in cell viability (Figure 9-A). The apoptosis index increased influenced by MSU treatment and decreased with concomitant culture with MSU+VD (Figure 10B). Figure 10C shows apoptosis/necrosis ratio, which also increased with MSU stimulus. Despite the tendency to induce lower apoptosis/necrosis ratio, MSU+VD treatment didn't show statistical significance compared with MSU treatment. Figure 10 D-F is a representative histogram of apoptotic/necrotic index in HUVEC treated with or without MSU and VD for 24 h detected by annexin V/PI flow cytometry.

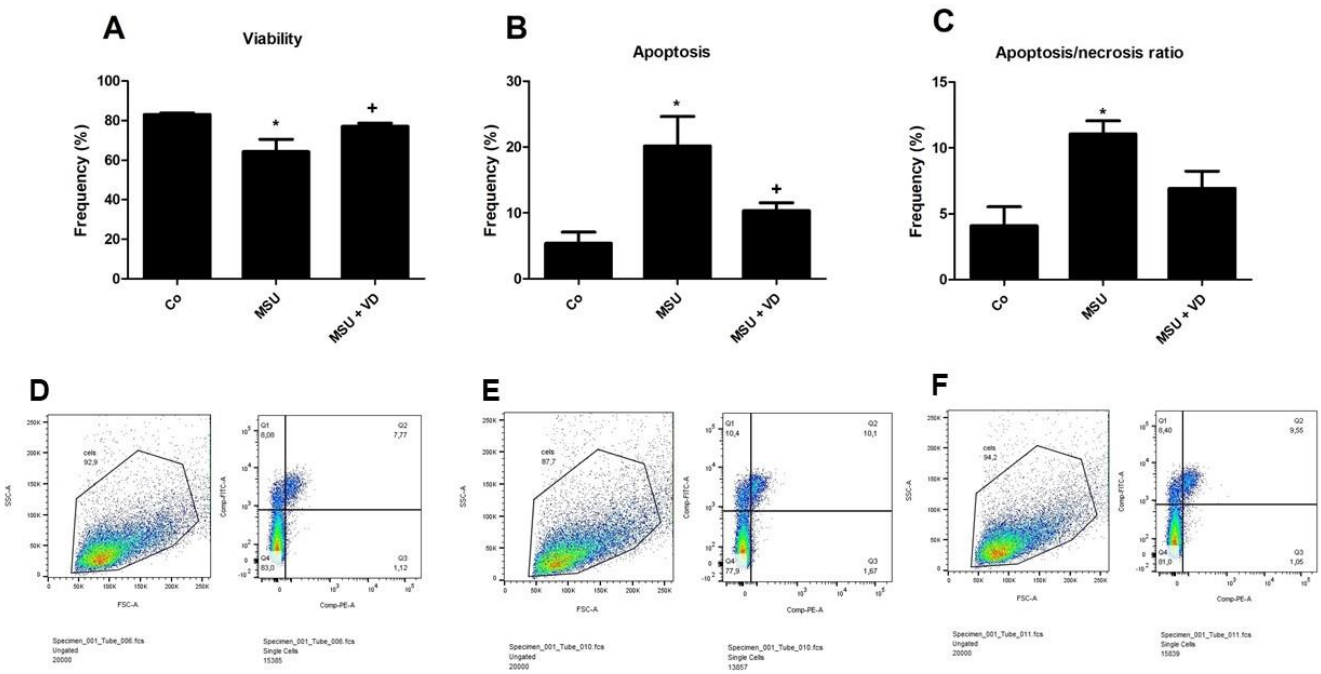

Figure 9. MSU reduces cell viability and increases apoptosis/necrosis rate in the HUVEC (A-C). Representative apoptotic/necrotic index in HUVEC detected by Annexin V/PI flow cytometry (D-F). The samples were assayed in three technical and biological replicates. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co, + ($p < 0.05$) vs MSU.

4. DISCUSSION

The present study show the analysis, at the transcriptional and protein levels, of components related to inflammation, such as the inflammasomes NLRP1 and NLRP3, HMGB1, IL-1β and IL-18 in placental explants obtained from preeclamptic and normotensive pregnant women. We also evaluated the modulatory effect of VD on these inflammasomes in explants, as well as on apoptosis induced by MSU in HUVEC. We detected increase in the basal gene and protein expression of NLRP1/NLRP3 inflammasomes as well as in the inflammatory cytokines IL-1β, HMGB1 and IL-18 expressed in placental explants of EOPE women, compared with LOPE and NT groups. These results are in line with other authors showing higher gene expression of NLRP3, caspase-1, IL-1β and HMGB1 mRNA in placenta as well as increased protein levels of caspase-1, IL-1β and HMGB1 in placental homogenate obtained from preeclamptic women, indicating the involvement of NLRP3 inflammasome in the pathogenesis of PE [35]. Excessive placental inflammation affects the mother and the fetus and is associated with obstetric complications, such as preterm birth, fetal growth restriction and PE [35,38,39], and NLRP3 inflammasome is intimaly involved in the inflammatory response of these conditions [40].

The higher endogenous gene expression of NLRP1/NLRP3 inflammasomes, as well as higher mRNA levels for IL-1β, IL-18 and HMGB1 were more evident in placental explants of EOPE group, compared with LOPE and NT groups. These results confirms previous study of Weel et al [41] showing that placenta from EOPE women is more compromised than in LOPE and NT pregnant women, with more severe lesions and imbalance between inflammatory/regulatory cytokines as well as in angiogenic factors. These results corroborate the theory that EOPE is considered a fetal disorder that is typically associated with placental dysfunction [42].

The molecular mechanism involved in placental NLRP1/NLRP3 activation in PE is unknown. It has been proposed that the inappropriate inflammatory response observed in PE may have its origin in the placenta. A potential mechanism in PE is the shedding of syncytiotrophoblast micro-vesicles and nano-vesicles from

the placenta into the maternal circulation exerting proinflammatory, antiendothelial and procoagulant activity *in vitro*, all of which are features of the PE syndrome [43,44]. It is possible that these microparticles can act as DAMPs that induce hyperactive inflammasome activity, resulting in the exaggerated inflammatory state in PE [45]. In PE, many such DAMPs are known to contribute to both local placental and systemic inflammation and endothelial dysfunction. Thus, the great inflammation that has beginning in placenta from preeclamptic women can reach circulating leukocytes, leading to these cells activation. Studies developed by our research group have showed that monocytes of preeclamptic women with PE show greater endogenous gene expression of the NLRP1, NLRP3, caspase-1, HMGB1, as well as high production of IL-1β and IL-18 in relation to NT pregnant women, suggesting the participation of these inflammasomes in the systemic inflammation that characterizes PE. The stimulation of these cells with MSU or hyaluronan induced higher expression of the NLRP1/NLRP3 inflammasomes, showing that these DAMPs are able to activate this molecular complex and act in the release of inflammatory cytokines [16,46,47].

In this study, placental explants cultured with MSU showed higher gene expression of NLRP1, NLRP3 and HMGB1 as well as inflammatory cytokines in NT pregnant women and HUVEC, whereas VD and concomitant treatment with MSU+VD decreased significantly these effects. Although the results of NLRP1 and NLRP3 inflammasomes activation after MSU stimulation didn't reach statistical significance in placental explants from EOPE and LOPE pregnant women, the treatment with VD and MSU+VD led to decrease in mRNA and protein expression of these inflammasomes, HMGB1, IL-1β and IL-18 in EOPE and LOPE and NT groups. These data confirm the inhibitory effect of VD on NLRP1 and NLRP3 inflammasome activation, as well as reducing gene and protein expression of inflammatory cytokines by monocytes from preeclamptic women and THP-1 cells stimulated with hyaluronan, an agonist of NLRP3 activation [48]. Other study demonstrated that VD inhibit the ROS-NLRP3-IL-1β signalling axis in human corneal epithelial cells, cultured in an inflammatory environment [49]. The regulatory effect of VD on inflammatory cytokines expression and secretion by placental trophoblastic cells from preeclamptic women, cultured with different

concentrations of this hormone, was described by Noyola-Martínez et al., 2013 [50]. The authors emphasised the role of calcitriol in controlling the immune response at the maternal-fetal interface, particularly in pathologies of pregnancy, such as PE.

In addition to inflammasomes, gene expression of HMGB1 is elevated in explants of preeclamptic women with EOPE and on HUVEC stimulated with MSU. HMBG1 acts as a mediator molecule involved in the pathogenesis of several inflammatory diseases [51,52]. Recent study showed higher levels of HMGB1 in the maternal circulation of preeclamptic women compared to normotensive pregnant women [46]. Chen et al. [44] also reported an increase in HMGB1 expression in the syncytiotrophoblast of placenta from LOPE and EOPE women compared to NT pregnancies.

Preeclampsia is haracterized by changes in vitamin D metabolism compared to NT pregnancies [53]. Several studies have demonstrated a significant relationship between maternal vitamin D deficiency and the incidence of preeclampsia [54,55]. Moreover, vitamin D supplementation studies showed protective effects on preeclampsia incidence [54].

The *in vitro* treatment of HUVEC with MSU was employed in an attempt to mimetize the effect of hyperuricemia, characteristic in PE on endothelial cells, evaluating the effect of this DAMP on cell viability and apoptosis induction, as well as the modulatory effect of VD on these aspects. The results showed that MSU was able to decrease the viability of HUVEC and increase the rate of apoptosis, while the addition of VD increased viability, decrease the rate of apoptosis, confirmig the protective role of VD on endothelial cells. Otani et al., demonstrated that treatment of HUVEC with MSU reduced cell viability and promoted hypoxia-induced cell death, while the antioxidant treatment with ascorbic acid had an inhibitory effect, suggesting the possibility of antioxidants treatments [23]. Brodowski et al. tried an intervention using VD to observe if this hormone was able to improve HUVEC characteristics [55]. Previous studies showed improvement in migration, proliferation and tubule formation using VD as a terapheutic model [57-59].

Together, the results presented here corroborate with the literature, reinforcing the hypothesis that NLRP1 and NLRP3 hyperactivation as well as apoptosis play a role in the pathophysiology of PE, which can be mitigated with the

administration of anti-inflammatory and antioxidant substances, such as vitamin D. Thus, studies on the molecular mechanism of VD modulation of inflammasome activation and endothelial disfunction in PE are the subject of future studies by our group.

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Capítulo 4

Os resultados referentes à esse capítulo serão enviados para publicação em periódico internacional

VITAMIN D MANTAINS VIABILITY AND DECREASES APOPTOSIS IN HUVEC AND MODULATES INFLAMMATION IN PLACENTAL EXPLANTS FROM PREECLAMPTIC WOMEN CULTURED WITH TNF-α

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ABSTRACT

This study aimed to evaluate the effect of vitamin D (VD) on apoptosis in Human Umbilical Vein Endothelial Cells (HUVEC) and inflammatory markers in placental explants from preeclamptic (PE) and normotensive (NT) pregnant women. Placental explants from 10 late-onset PE (LOPE), 10 early-onset PE (EOPE) and 10 NT pregnant women were cultured with or without *tumor necrosis factor alpha* (TNF-α) and VD. Protein expression of interleukin-1 beta (IL-1β), IL-18, TNF-α and *tumor necrosis factor related apopotosis-inducing ligant* (TRAIL) were detected by ELISA. Gene and protein expression of *high-mobility group box-1* (HMGB1) were determined by qPCR or Western blotting, and *Annexin V-fluorescein isothiocyanate* (FITC)/*Propidium iodide* (PI) staining was employed to determine apoptosis/necrosis in HUVEC. Compared to NT group, the endogenous levels of IL-1β, TNF-α and IL-18 was higher in PE group. Stimuli with TNF-α increased cytokine levels in NT, TNF-α in EOPE/LOPE, IL-18 in LOPE, and all cytokines in HUVEC. TNF-α+VD treatment decreased cytokine levels in explant supernatants and HUVEC. TRAIL were higher in EOPE versus NT, while TNF-α increased this receptor in NT compared to control. In HUVEC, TNF-α was able to increase TRAIL versus control, and TNF-α+VD decreased levels compared to only TNF-α stimulus. Protein expression of HMGB1 were higher in cultures with TNF-α and decreased after treatment of TNF-α+VD in all groups and gene and protein expression in HUVEC. Gene expression were elevated in EOPE versus NT and LOPE, and TNF-α increased HMGB1 in NT versus control, as well as TNF-α+VD decreased mRNA levels in EOPE. This stimulus decreased HUVEC viability, increased apoptosis/necrosis ratio, while the addition of VD increased viability and decreased apoptosis/necrosis. The results demonstrated that the *in vitro* treatment with VD may play an immunomodulatory role in the placental inflammation and decreased apoptosis in HUVEC.

Key words: Preeclampsia, placental explants, HUVEC, TNF-α, cytokines, HMGB1, vitamin D.

1. INTRODUCTION

The pathophysiology of preeclampsia (PE) is not yet fully understood, but it is already well known that early-onset PE (<34 weeks of gestation - EOPE) and late-onset PE (≥34 weeks - LOPE) should be treated as distinct entities. The two pathologies share some etiological and prognostics characteristics in common, but differ in relation to risk factors, leading to different outcomes [1]. Nevertheless, PE is a disease characterized by generalized systemic inflammatory response and vascular endothelial damage associated with placental dysfunction [2].

Human umbilical vein endothelial cells (HUVEC) are used frequently in studies of endothelial cell function, blood vessel repair and angiogenesis.[3,4]. Several groups have demonstrated that endothelial cells may also undergo apoptosis in inflammatory conditions. This type of cell death have been observed in various conditions[5,6].

Apoptosis is an intrinsic cellular mechanism that leads to cell self-destruction. This cellular process is induced by specific stimuli and occurs in cells that participate in important pathways in the organism [7]. Many aspects of apoptosis distinguish from necrosis, among them, the most important is that in apoptosis, cell removal depends on an internal machinery of the cell, while in necrosis it is an accidental process, stimulated by an unplanned external event. A second important point is that apoptosis follows a very characteristic morphological pattern, with condensation of nuclear heterochromatin in an increasing deposit on the nuclear membrane and, subsequently, in individual or multiple dense bodies, which are eliminated by phagocytic cells [8].

Recently, some authors have discussed apoptosis in human trophoblasts and its possible implication in normal development, remodeling, and aging of the placenta. Studies show that apoptosis in the trophoblast can change during certain pathological conditions, such as PE or fetal growth restriction [9]. TNF-α was the first cytokine identified to be capable of inducing apoptosis in human trophoblasts. In combination with interferon- gamma (IFN-γ), TNF-α stimulated apoptosis in syncytium and cytotrophoblasts [10,11].

TRAIL (apoptosis-inducing ligand related to tumor necrosis factor) is the third death receptor in the apoptosis regulation system in the immune system [12]. During PE, the increase in TRAIL expression by trophoblastic cells is probably a mechanism against apoptotic signals from lymphocytes and maternal natural killer cells [13]. Chaemsaithong et al. observed that plasma levels of soluble TRAIL are lower in women with PE compared to normotensive women [14].

Bai et al. found evidences about a new mechanism by which TRAIL and one of the TRAIL ligands, death receptor 5 (DR5), detected in trophoblastic cells, may be activated in conditions of excessive TNF-α [15]. Stimulation with cytokines such as TNF-α can lead to the redirection of DR5 to the cell surface, and consequently increase the susceptibility of trophoblastic cells to TRAIL. It is suggested that excessive stimulation with TNF-α can cause cellular damage, as occurs in PE [12].

Considering inflammatory status in PE pregnant women, an important cytokine, High mobility group box 1 (HMGB1), could be considered as a “danger signal”, because its action as pro-inflammatory mediator [16]. Plasma level of this protein is increased in PE pregnancies compared to NT pregnant women [17] and may contribute to the exacerbation of the inflammatory response in this pathology [18], also HMGB1 is expressed in syncytiotrophoblast and is present at higher levels in the decidua of women with PE [19].

Vitamin D have an important role in the implantation, placentation, and maintenance of a healthy pregnancy. During human pregnancy the conversion of 25(OH)D to 1,25(OH)2D is increased in the placenta, demonstrating the importance of this organ in the metabolism of vitamin D [20]. Vitamin D supplementation at late pregnancy also reduced mean arterial pressure and inflammation in a rat model of preeclampsia [21,22]. Sablok et al. reported that vitamin D supplementation in pregnant women reduced the risk of maternal preterm labor and helped to improve neonatal birthweight [23] and Tian et al. showed improvement of placenta function related to the inhibition of apoptosis by VD treatment [24].

Considering the inflammatory status described in the placenta of pregnancies complicated by PE, as well as activation of apotosis in endothelial cells, this study aimed to evaluate the immunomodulatory action of vitamin D on

cytokines and TRAIL in placental explants from preeclamptic and NT pregnant women, as well as on apoptosis in HUVEC.

2. METHODS

2.2. Patients

We collected placentas from 20 preeclamptic women and 10 normotensive (NT) pregnant women attended in the Obstetrics Unit of Botucatu Medical School, Sao Paulo State University, Botucatu, SP-Brazil, between August 2018 and February 2020. Women with PE were stratified according to the onset of disease clinical manifestations at moment of diagnosis as: EOPE - early onset PE (< 34 weeks of gestation, n = 10) and LOPE -late-onset PE (≥ 34 weeks of gestation, n = 10), according to Huppertz [25]. Gestational age was calculated from the last menstrual period and confirmed by ultrasound dating. The diagnosis of PE was closed considering hypertension (blood pressure ≥ 140/90 mmHg) followed or not by proteinuria (≥ 300 mg in a 24-hour urine) after the 20th week of gestation [26]. The NT group did not present history of hypertensive disorders in pregnancy.

Exclusion criteria included: chronic hypertension, multiple gestations, prior preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, cancer, acute infectious disease, cardiovascular, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all pregnant women involved in the study (Protocol number: 4.418.043). This work has been carried out under the Code of Ethics of the World Medical Association (Declaration of Helsinki). All mandatory laboratory health and safety procedures have complied with in the course of conducting any experimental work reported in this paper and all experiments were performed following relevant guidelines and regulations.

2.3. Obtaining placental tissue and separating the explants for culture with TNF-α and VD

Placentas were collected from delivery after elective caesarean section. Immediately after delivery, all placentas were examined macroscopically and

processed within an interval of maximum 10 min, as previously described [27], to constitute placental explants. A total of 11 mg of human villous tissue was cultured according to Nunes et al. [27] in 24-well plates (SPL Life Sciences, Korea) over 24 h for stabilization. After this time, placental explants from EOPE, LOPE and NT pregnant women were cultured for 24 h with or without TNF-α (20 pg/mL), vitamin D (100 nM) and with or without TNF-α plus VD (Sigma-Aldrich, SL, Missouri, USA).

2.4. In vitro assay and treatment with TNF-α and VD

Human umbilical vein endothelial cells (HUVEC) (Lonza, Switzerland) were cultured at 37°C in 5% CO_2 in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal bovine serum (SFB) (Gibco, CA, USA), 50μg/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL amphotericin B (Gibco, CA, USA). HUVEC were cultured until reaching 80% confluence ans then incubated for 24 h with or without TNF-α (20 pg/mL), vitamin D (100 nM) and with or without TNF-α plus VD (Sigma-Aldrich).

HUVEC was acquired with a certification proving that the cells were from the designated type. All experiments were performed using cells in the sixth passage and in quintuplicates.

2.5.Cytokine and TRAIL determinations

The concentrations of cytokines IL-1β, TNF-α, IL-18 and the receptor TRAIL in supernatants of placental explants from NT and PE women, as well as HUVEC supernatants obtained after treatment with or without TNF-α and VD were determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Assay sensitivity limits were 1.0 pg/mL for IL-1β, 1.6 pg/mL for TNF-α, 5.15 pg/mL for IL-18 and 2.86 pg/mL for TRAIL.

2.6. Gene expression of HMGB1 in placental tissue and HUVEC

Placental explants and HUVEC were employed to determine the expression of the genes encoding HMGB1 at the transcriptional level. Total RNA was extracted

from the placental explants and cells after culture using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's protocol, and the quantitative polymerase chain reaction (qPCR) was performed according to Weel et al. [28]. Briefly, isolated RNA was treated with DNAse I Amp Grade (Invitrogen). Subsequently, the synthesis of complementary DNA (cDNA) was conducted using the ImProm-II TM Reverse Transcription System, according to the manufacturer's protocol. The qPCR was performed using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA), and the variants of the studied targets were aligned in the MEGA 5.1 program. Each primer was subsequently selected by the software Primer-BLAST. Primers located in exon-exon junctions guarantee the purity of the reaction, namely the absence of any genomic DNA that may contaminate it. The primer sequences used in this study are shown in Table 1.

Table 1. Primers for HMGB1 and GAPDH. Numbers indicate nucleotide positions in the corresponding transcripts.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank
HMGB1	(1404)TACGAAAAGGATATTGCTGC(1423)	(1505)CTCCTCTTCCTTCTTTTTCTTG(1484)	NM_001313893.1
GAPDH	(684)CGTGGAAGGACTCATGACCA(703)	(801)GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Each reaction was set in duplicate, and the conditions for the qPCR were as follows: initial denaturation at 96 °C for 2 min and then 40 cycles at 95 °C for 15 s and 60 °C for 60 s, followed by a melting curve. Expression values of the analysed transcripts were normalized to that of the enzyme encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve [29]. To analyse the relative expression, after the analysis of gene expression, we chose an RNA sample obtained from each group, which received a relative value of 100. All other samples received values for that sample.

2.7. Obtaining homogenate from placental explants and HUVEC lysate for electrophoresis and blotting

Placental explants from three EOPE, three LOPE, and three NT pregnant women were cultured for 24 h in the presence or absence of TNF-α or TNF-α plus vitamin D and previously stored at -80 °C were removed for thawing, and the placental fragment sample was weighed and documented in grams of tissue. These fragments were then transferred to the tissue homogenizer container containing 2 mL of protease inhibitor buffer: 150 mM/L NaCl and 20 mM/L Tris pH 7.5, 5 mM/L EDTA and 1 mM/L L phenylmethylsulphonyl fluoride (pH 7.3). After homogenization in an ice bath, the material was placed in test tubes and centrifuged at 3500 rpm for 20 min at 4 ºC. The supernatant was then aliquoted into 0.5 mL microtubes and stored at -80 °C. In the same way, HUVEC cultured for 24 h in the presence or absence of TNF-α and VD were subjected to cell lysis. The cell extract was centrifuged at 1500 g for 10 min. The supernatant obtained from the culture of placental explants and the cell extract were used to determine the total protein concentration by Lowry's method [30] for HMGB-1 determinations.

After determining the homogenate protein concentration of placental explants and HUVEC extracts, 30 μg of proteins were treated with Laemli sample buffer (Bio-Rad, Hercules, CA, USA) and β-mercaptoethanol at 95 °C for 5 min, then the proteins were separated on SDS-PAGE and after electrophoresis, transferred to a nitrocellulose membrane. Nonspecific protein binding was blocked by incubating the membranes in 5% skim milk in Tris-HCl buffer containing 0.1% Tween 20 (TBST) for 45 min at room temperature. Membranes were subsequently incubated for 18 h with rabbit polyclonal antigen-specific primary antibody anti-HMGB1 (Novus Biologicals, Littleton, CO, USA) at dilution on manufacturer recommended concentrations containing TBST. After washing, the membranes were incubated with rabbit specific secondary antibody diluted in TBST for 1 h. Immunoreactive components were revealed by the Amersham™ ECL Select™ Western Blotting Detection Reagent luminescent kit (GE Healthcare®, Little Chalfont, United Kingdom).

2.8. Apoptosis and necrosis in HUVEC were determined by Annexin V-

FITC/PI Staining

Annexin V assay were performed with the eBioscience™ Annexin V Apoptosis Detection Kit FITC (Thermo Fisher). The HUVEC ($2x10^5$ cells) were placed in a 24-well plate and left for 6 h to attach. The cells were then treated with TNF-α (20 µg/mL), vitamin D (100 nM) or their association for 18h. After the treatment period, cells were trypsinized and centrifuged (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) for 10 min at 1200 rpm for culture medium removal. The cell pellet was washed twice with phosphate-buffered saline (PBS) and centrifuged at 10,000 rpm for 30 s, followed by resuspension with 100 µL Annexin V binding buffer and incubation with 2µL Annexin V and 4µL propidium iodide (PI) for 15 min in the dark at room temperature. After the incubation, the prepared cells were then analyzed by flow cytometry in a FACSCantoTMII with FACSDiva (BD Biosciences, Clontech, CA, USA) software. The flow cytometric results were analyzed by the FlowJo software (vX.10.6 version, Tree Stars Inc., Ashland, OR, USA).

2.9. Statistical analysis

The results were evaluated using parametric and non-parametric methods according to the statistical program GraphPad Prism, version 6.01 (GraphPad, CA, USA). The clinical characteristics of PE and NT pregnant women were analysed by Kruskal-Wallis test. Apoptosis, gene and protein expression were evaluated by analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1.Early-onset preeclampsia is related to unfavorable clinical outcomes

Table 1 shows the clinical and laboratory characteristics of EOPE, LOPE and NT pregnant women. There was no statistical difference between the groups regarding age. However, gestational age was lower in preeclamptic women compared to normotensive ones, with a lower gestational age being observed in the group of EOPE pregnant women. In addition, blood pressure were significantly higher in PE groups compared to NT pregnant women. Proteinuria levels were

significantly higher in the EOPE group compared to the other groups. Additionally, LOPE group presented these values significantly higher than NT.

Table 1. Clinical and laboratory characteristics of EOPE, LOPE and normotensive pregnant women.

Parameters	EOPE (< 34w) n = 10	LOPE (≥ 34w) n = 10	Normotensive (NT) n= 10
Age (years)	28 (14 – 40)	24 (16 – 43)	29 (18-41)
Gestational age (weeks)	29 (26 – 33)	37 + (34 – 40)	40 (36-41)
Systolic blood pressure (mmHg)	160 * (140 – 200)	155 * (140 – 180)	120 (110-120)
Diastolic blood pressure (mmHg)	110 * (90 – 140)	100 * (90 – 120)	70 (60-80)
Proteinuria (mg/24h)	3105*+ (300-26.052)	535* (300-10.800)	<300

Results expressed as median (minimum-maximum) * ($p < 0.05$) vs NT; + ($p < 0.05$) vs EOPE (Kruskal-Wallis test).

3.2. Expression of inflammatory cytokines is elevated in EOPE and in cultures stimulated with TNF-α, while treatment with VD reduces inflammation also in HUVEC

Expression of IL-1β, TNF-α, and IL-18, in supernatant from placental explants of preeclamptic women (EOPE and LOPE), normotensive pregnant women (NT) and HUVEC were determined, stimulated with TNF-α and treated or not with VD.

Figure 1 shows that the protein expression of IL-1β (A), TNF-α (B) and IL-

18 (C) was significantly higher in the explants of PE compared to NT: IL-1β and IL-18 higher in EOPE compared to NT and TNF-α in LOPE compared to EOPE. Also, significant diference was observed in IL-1β production between LOPE and NT.

In addition, stimuli with TNF-α increased all cytokines levels in NT group compared to control. In the same way, this stimuli enhanced TNF-α in LOPE and EOPE compared to control, and IL-18 in LOPE.

The TNF-α+VD treatments induced lower IL-1β, TNF-α, and IL-18 levels in all studied groups compared to the stimulus with TNF-α.

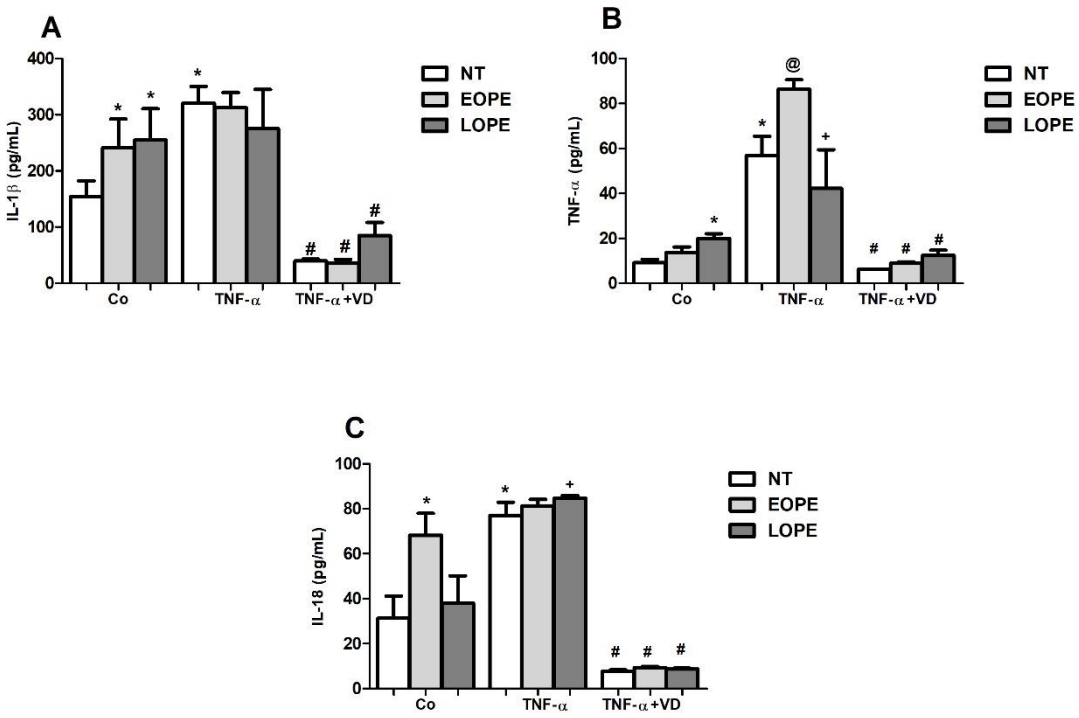

Figure 1. Cytokine levels of IL-1β (A), TNF-α (B), and IL-18 (C) in supernatant from placental explants obtained from 10 normotensive pregnant women (NT), 10 pregnant women with early-onset preeclampsia (EOPE) and 10 pregnant women with late-onset preeclampsia (LOPE) cultured in the presence or absence of TNF-α and TNF-α+VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co NT, @($p < 0.05$) vs Co EOPE, + ($p < 0.05$) vs Co LOPE, # ($p < 0.05$) vs TNF-α.

Figure 2 shows cytokine levels in HUVEC. IL-1β (A), TNF-α (B), and IL-18 (C) was significantly higher in the cultures treated with TNF-α compared to

unstimulated cells. The TNF-α+VD treatments showed lower cytokine levels compared to the stimulus with only TNF-α.

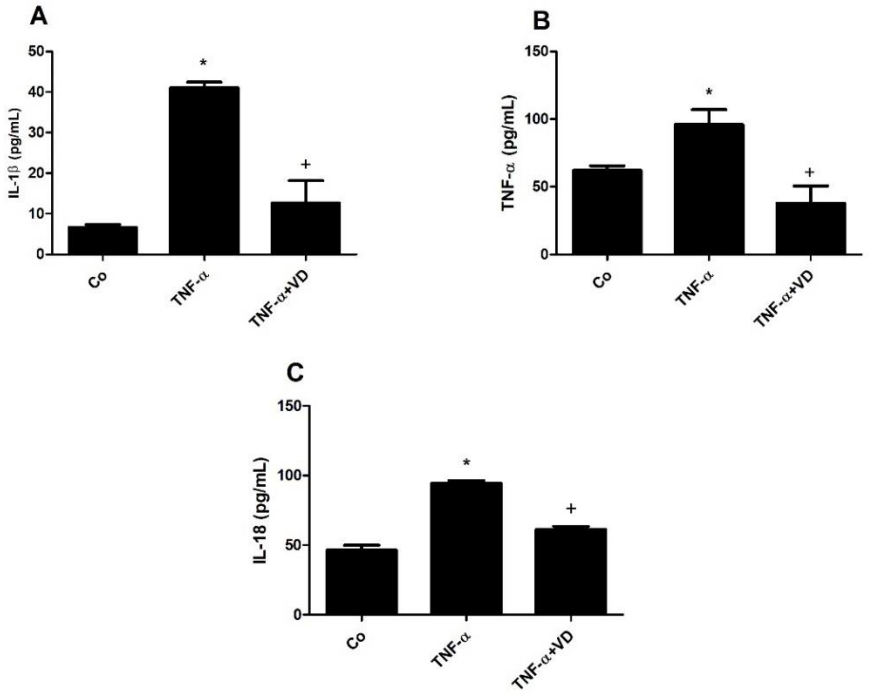

Figure 2. Levels of IL-1β (A), TNF-α (B), and IL-18 (C) in supernatant from HUVEC cultured in the presence or absence of TNF-α and TNF-α VD. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co , + ($p < 0.05$) vs TNF-α.

3.3.TRAIL levels are elevated in EOPE while VD treatment improves this levels and culture with TNF-α increases levels of this receptor in supernatant from explants of NT pregnant women

Figures 3A shows TRAIL levels in supernatants from placental explants from EOPE, LOPE and NT pregnant women. The endogenous protein expression of TRAIL was significantly higher in the explants of EOPE compared to NT. In addition, stimuli with TNF-α showed increased expression of TRAIL compared to unstimulated control in NT group. The TNF-α+VD treatments showed lower levels of TRAIL in EOPE compared to the stimulus with TNF-α.

Figure 3B shows protein expression of TRAIL in HUVEC supernatant. Treatment with TNF-α increased TRAIL levels compared to unstimulated cells. The TNF-α+VD treatment decreased TRAIL expression compared to the stimulus with only TNF-α.

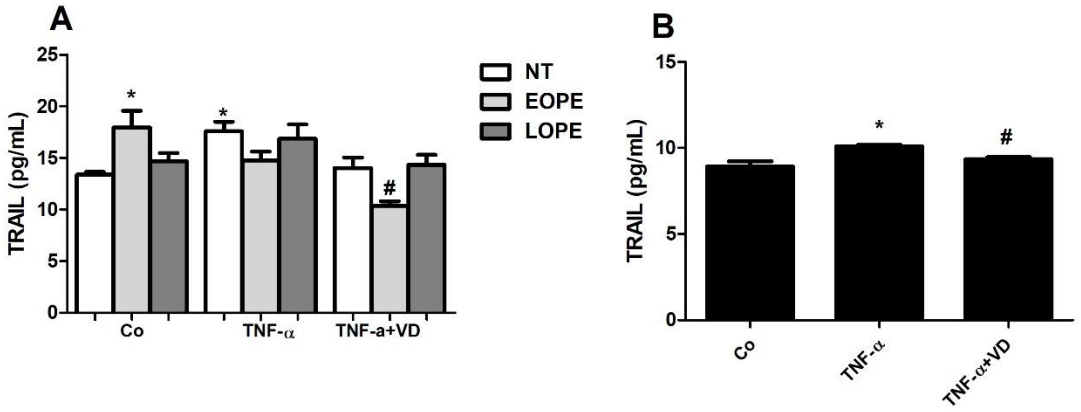

Figure 3. Protein levels of *tumor necrosis factor related apopotosis-inducing ligant* (TRAIL) in supernatant from explants of placental tissue obtained from 10 normotensive pregnant women (NT), 10 pregnant women with early-onset preeclampsia (EOPE) and 10 pregnant women with late-onset preeclampsia (LOPE) cultured in the presence or absence of TNF-α and TNF-α+VD. Results are expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co NT/Co, # ($p < 0.05$) vs TNF-α.

3.4.HMGB1 gene and protein expression are increased after stimuli with TNF-α and decreaded after VD treatment in EOPE, LOPE, NT and HUVEC

Figure 4 (A-C) represents results of HMGB1 gene and protein levels of *high-mobility group box-1* (HMGB1) in explants of EOPE (A), LOPE (B), and NT pregnant women (C) cultured in the presence or absence of TNF-α and TNF-α+VD. Protein expression is increased after stimuli with TNF-α and decreaded after concomitant treatment with TNF-α +VD in all groups. In figure 4D are shown results of gene expression of HMGB1. Endogenous gene expression are higher in EOPE versus NT and LOPE, and the TNF-α stimuli enhanced gene expression of HMGB1 in NT group compared to unstimulated group (Co), as well as concomitant treatment of TNF-α+VD decreased HMGB1 mRNA levels in EOPE versus only TNF-α treatment.

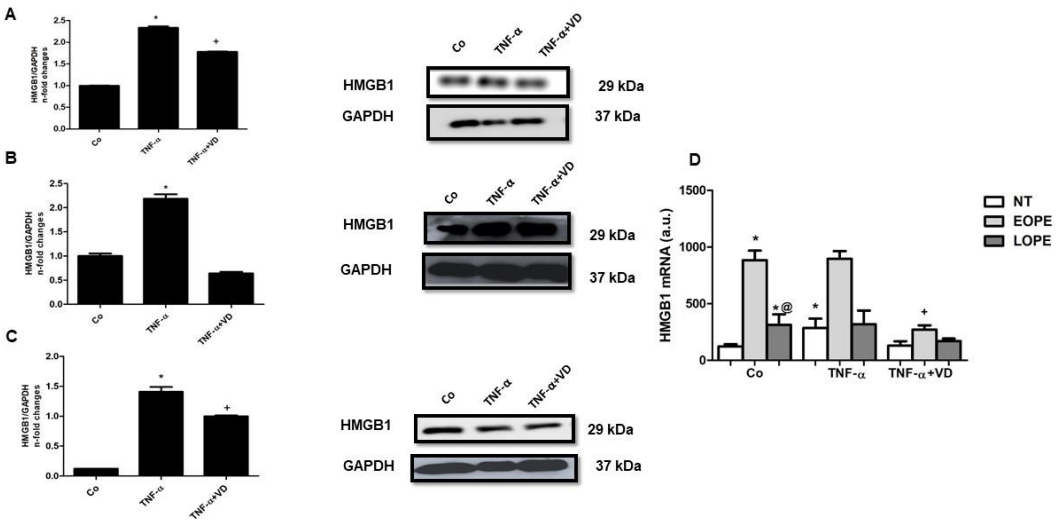

Figure 4. Protein levels of *high-mobility group box-1* (HMGB1) in explants of placental tissue obtained from 10 pregnant women with early-onset preeclampsia (EOPE) (A), 10 pregnant women with late-onset preeclampsia (LOPE) (B), and 10 normotensive pregnant women (NT) (C) and mRNA levels (D) of HMGB1 in these groups cultured in the presence or absence of TNF-α and TNF-α+VD. Results are expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co NT/Co, + ($p < 0.05$) vs TNF-α, @ vs Co EOPE.

Figure 5 demonstrates protein and mRNA levels of *high-mobility group box-1* (HMGB1) in HUVEC cultured in the presence or absence of TNF-α and TNF-α+VD. In both cases, TNF-α stimuli increases protein and gene expression, and in opposite, treatment with TNF- α+VD decreased HMGB1 protein and mRNA.

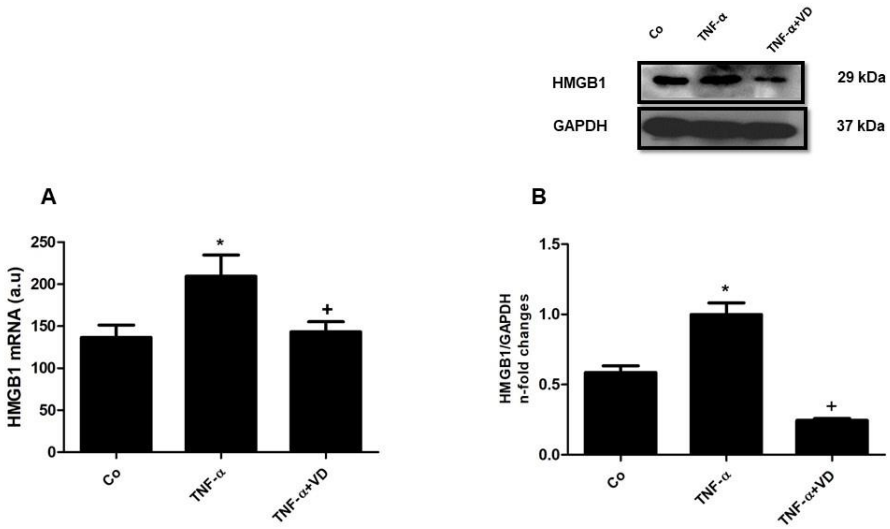

Figure 5. Protein and mRNA levels of *high-mobility group box-1* (HMGB1) in supernatant of HUVEC cultivated in presence or absence of TNF-α and TNF-α+VD. Results are expressed as mean ± SD. ANOVA followed by Bonferroni multiple comparisons test. * ($p < 0.05$) vs Co, + ($p < 0.05$) vs TNF-α.

3.5. TNF-α reduce cell viability and induce apoptosis/necrosis in HUVEC while concomitant treatment with VD mantains cell viability and decreased apoptosis/necrosis

The HUVEC cell viability showed in figure 7 was reduced after the stimulus with TNF-α (Figure 6A) compared to control, while treatment with TNF-α+VD showed a mantainance in cell viability. The apoptosis and necrosis index (Figure 6B and 6D) increased influenced by TNF-α treatment and decreased with concomitant culture with TNF-α +VD. Figure 6C show apoptosis/necrosis ratio, which also increased with TNF-α stimulus, and decreased with TNF-α +VD treatment. Figure 6 E-G is a representative histogram of apoptotic/necrotic index in HUVEC treated with ou without TNF-α and VD for 24 h detected by annexin V/PI flow cytometry.

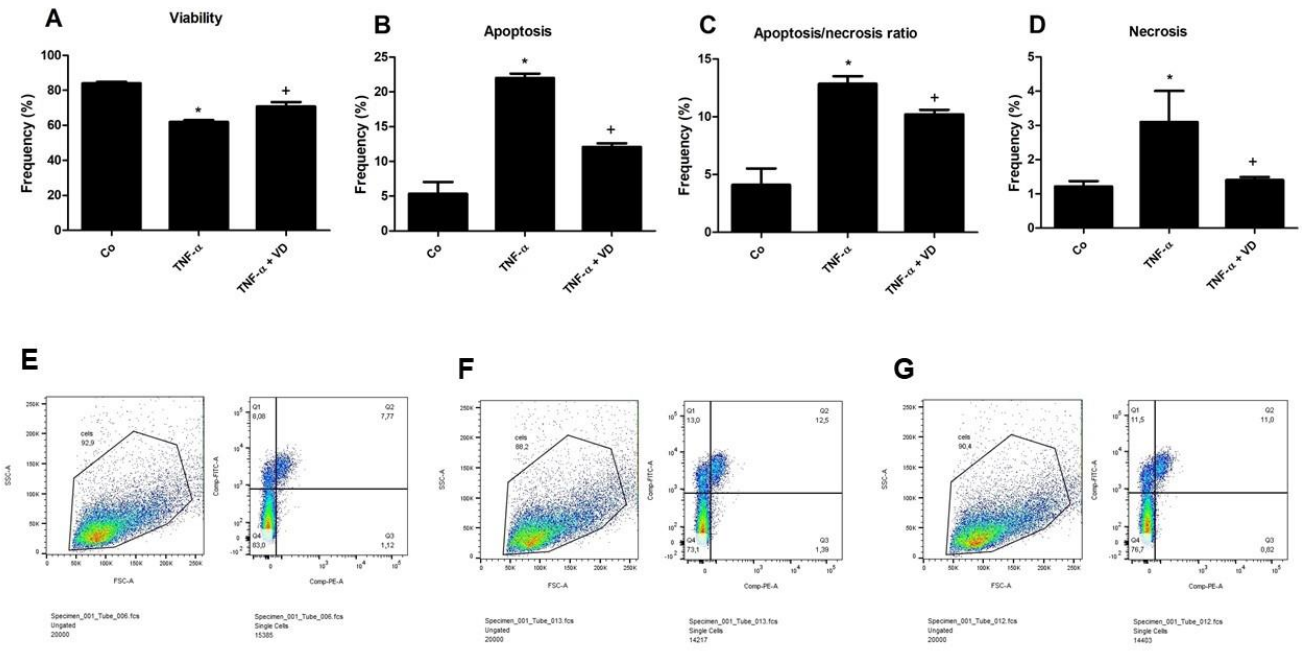

Figure 6. TNF-α reduce cell viability and increase apoptosis/necrosis rate in the HUVEC (A-D). Representative apoptotic/necrotic index in HUVEC detected by Annexin V/PI flow cytometry (E-G). The samples were assayed in three technical and biological replicates. Results are expressed as mean ± SD. ANOVA followed by the Bonferroni multiple comparison test. * ($p < 0.05$) vs Co, + ($p < 0.05$) vs TNF-α.

4. DISCUSSION

Taken together, the results in this manuscript demonstrated the immunomodulatory effect of vitamin D on cytokines and *tumor necrosis factor related apopotosis-inducing ligant* (TRAIL) in placental explants PE and NT pregnant women, as well as on apoptosis in endothelial cells.

In relation to NT group, the endogenous protein expression of IL-1β, TNF-α and IL-18 was higher in explants of preeclamptic women. Treatment with TNF-α increased these cytokines in NT group, and this stimulus increased TNF-α levels in EOPE and LOPE, as well as IL-18 in LOPE. Additionally, in HUVEC supernatants, we also observed increased levels of all cytokines after TNF-α as well as after concomitant treatment of TNF-α+VD, all cytokine levels decreased in explants supernatants and HUVEC.

Previous study of our group showed higher expression of TNF-α and IL-1β in placental villi and increased levels of these cytokines in placental homogenates from preeclamptic women [28]. These results are in agreement with literature, suggesting that ischemia and hypoxia, resulting from the inappropriate trophoblast invasion, lead to overexpression and increased production of proinflammatory cytokines in placentas from preeclamptic women [31,32].

Our results showed higher levels of IL-18 in explants of preeclamptic women, while previous study conducted by Liu et al. reported decreased levels of IL-18 in placenta from preeclamptic women compared with healthy pregnant women [33]. The low expression of IL-18 in preeclamptic placenta might be attributed to a regulatory effect exerted by IL-18 binding protein (IL-18BP), an inhibitory molecule of IL-1 family regulatory proteins [34].

TRAIL levels were higher in EOPE than in NT pregnant women, while TNF-α increased this receptor in NT group compared to control. In HUVEC, TNF-

α was able to increase TRAIL compared to control, and TNF-α+VD decreased TRAIL compared to only addition of TNF-α. This result matchs with the presence of an intravascular inflammation typical of preeclampsia. The higher levels of TRAIL was already detected in miscarriage being considered a potential important biomarker in different physiopathological settings. [35].

Xiao et al. demonstrated that TRAIL regulated miR-146a-CXCR/EGFR axis to promote the invasion of trophoblast cells. Its decreased levels in preeclamptic patients suggest that low levels of TRAIL might contribute to the pathogenesis of preeclampsia [36].

In this work, we demonstrated higher levels of HMGB1 in cultures treated with TNF-α that decreased after treatment of TNF-α+VD in all groups. The same results, as well as gene expression were detected in HUVEC. Gene expression of HMGB1 were elevated in EOPE versus NT and LOPE pregnant women, and TNF-α stimuli increased HMGB1 in NT compared to control, as well as TNF-α+VD decreased mRNA levels in EOPE pregnant women.

HMBG1 acts as a mediator molecule involved in the pathogenesis of several inflammatory diseases. [37,38]. Recent studies have shown greater protein expression in the maternal serum of preeclamptic women PE compared to normotensive women [39]. Our results corroborate with these authors and with Chen et al., who reported increased protein expression of HMGB1 in the syncytiotrophoblast of placenta in with severe and early-onset PE compared to normotensive pregnancies [16].

Treatment with TNF-α was able to decrease the viability of HUVEC and increase apoptosis/necrosis ratio, while the addition of VD increased viability and decreased apoptosis/necrosis. Brodowski et al., used an intervention to improve HUVEC characteristics [40]. Previous studies showed improvement in migration, proliferation and tubule formation using VD as a terapheutic model [41-43]. Tian et al. found that the administration of VD in rats in early gestation before preeclampsia led to improvements of structure impairment in placenta and in the main preeclamptic symptoms. The decrease in oxidative stress induced by VD treatment was accompanied by autophagy activation and apoptosis attenuation [24].

The results presented in this work demonstrated the presence of higher

inflammatory environment in placenta from preeclamptic women, which can be mitigated by *in vitro* treatment with vitamin D. The possible molecular mechanisms involved in apoptosis as well as the immunomodulatory role played by VD in endothelial cells was also suggested.

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"Não basta a leitura sem a unção, não basta a especulação sem a devoção, não basta a pesquisa sem maravilhar-se; não basta a circunspecção sem o júbilo, o trabalho sem a piedade, a ciência sem a caridade, a inteligência sem a humildade, o estudo sem a graça".

São Boaventura

Capítulo 5

Conclusões gerais

Em conclusão, o presente estudo demonstrou que o tratamento *in vitro* de explantes placentários de mulheres com pré-eclâmpsia com vitamina D regula negativamente a ativação endógena dos inflamassomas NLRP1 e NLRP3 e citocinas pró-inflamatórias. Esses resultados sugerem que a VD pode desempenhar um papel imunomodulador na inflamação placentária de mulheres com pré-eclâmpsia e diminuir o estresse oxidativo induzido *in vitro* em explantes placentários. Além disso, a cultura com VD diminuiu o estresse oxidativo e a peroxidação lipídica, bem como aumentou a biodisponibilidade de NO em HUVEC. Esses achados são consistentes com estudos anteriores que sugerem que a superprodução de radicais livres e a peroxidação lipídica são fatores importantes na patogênese da pré-eclâmpsia. O estresse oxidativo poderia contribuir para os mecanismos citotóxicos na PE, induzindo dano celular, levando à lesão das células endoteliais, inflamação e desequilíbrio angiogênico.

Os resultados apresentados neste trabalho corroboram com os dados presentes na literatura, mostrando o papel das citocinas inflamatórias na PE, que podem ser atenuadas com a administração de substâncias antiinflamatórias, como a vitamina D, após estímulos com MSU e TNF-α, bem como mecanismos moleculares como a apoptose em células endoteliais.

Pesquisas futuras são necessárias para compreender o mecanismo da VD na imunomodulação da inflamação placentária e sobre as características da pré-eclâmpsia. Assim, mais estudos sobre o mecanismo molecular envolvido na ação da VD na ativação do inflamassoma e disfunção endotelial na PE serão o assunto de estudos futuros por nosso grupo.

Capítulo 6

Termo de consentimento livre e esclarecido (TCLE) e comprovante do comitê de ética em pesquisa (CEP)

TCLE

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012 – GRUPO CONTROLE

CONVIDO, a senhora _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D e silibinina sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.
Sua participação nesta pesquisa se dará por ser uma gestante saudável, sem nenhum tipo de doença. Todos os dados que obtivermos com sua participação são meramente para compararmos com os dados de outros participantes que possuem a doença que estamos estudando.
Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença.
O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.
Informamos que o material biológico colhido da Senhora ,não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.
Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.
A Senhora não terá nenhum benefício em participar desta pesquisa, pois como já explicamos seus dados serão meramente para comparar com os dados de uma pessoa em tratamento de doença.

Fique ciente de que sua participação neste estudo é voluntária e que mesmo após ter dado seu consentimento para participar da pesquisa, você poderá retirá-lo a qualquer momento, sem qualquer penalidade.
Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue ao Senhor (a) devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.
Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:
Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO EM PARTICIPAR de forma voluntária, estando ciente que todos os meus dados estarão resguardo através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados e revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,_____/___/_____

_____ _____

Pesquisador Participante da Pesquisa

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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012
(Participante maior de 18 anos)

CONVIDO, a senhora _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D e silibinina sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.
Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.
Informamos que o material biológico colhido da Senhora, não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.
Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.
Fui informada que não terei benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informada também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de participar da pesquisa, sem prejuízo do atendimento médico que necessitar e, que meu nome não aparecerá quando os resultados forem divulgados.

Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue a Senhora devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término

da pesquisa.
Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descritos:
Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO EM PARTICIPAR de forma voluntária, estando ciente que todos os meus dados estarão resguardados através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados em revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,_____/___/_____

_____ _____
Pesquisador Participante da Pesquisa

Maria Terezinha S. Peraçoli – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu – UNESP
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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012
(Para Responsável Legal de participantes de 12anos a 17anos/11meses e 29 dias)

CONVIDO, o Senhor (a) _____responsável pelo menor _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D e silibinina sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.
Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do sangue ou um pedaço da placenta após o parto da sua filha _____, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente
Informamos que o material biológico colhido de sua filha não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para o (a) senhor (a) assine nova autorização para utilização desse material.
Solicito também seu consentimento para consultar o prontuário médico de sua filha, para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.
Fui informada que minha filha não terá benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informado (a) também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de consentir que ela participe da pesquisa, sem prejuízo do atendimento médico que ela necessitar e, que o nome dela não aparecerá quando os resultados forem divulgados.
Informo que a participação do seu filho (a) neste estudo é voluntária e que mesmo após o senhor ter dado o consentimento para que ele participe da pesquisa, o senhor poderá retirar o consentimento a qualquer momento, sem qualquer prejuízo na continuidade do seu tratamento.
Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue ao Senhor (a) devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o

término da pesquisa.
Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:
Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO na qualidade de "Representante Legal" a participação de meu (minha) filho(a) de forma voluntária, estando ciente que todos os seus dados estarão resguardados através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados em revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,_____/___/_____

_____
Pesquisador

_____
Representante Legal pelo Participante da Pesquisa

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Virginia Juliani Gomes – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu - UNESP

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OBS: Esse Termo de Consentimento Livre e Esclarecido, deverá vir acompanhado do Termo de Assentimento Livre e Esclarecido para participantes de pesquisa de 12anos a 17anos/11meses e 29 dias).

TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO (TALE)
RESOLUÇÃO 466/2012
Para menores entre 12 a 17 anos/11meses e 29 dias

CONVIDO, você_____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":

Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia Projeto 2: Efeito imunomodulador da vitamina D e silibinina sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia

Projeto 3: Efeito imunomodulador da vitamina D sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia

Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia

que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.

Caso concorde em participar da pesquisa você deverá assinar este Termo de Assentimento e seu Representante Legal assinará o Termo de Consentimento Livre e Esclarecido para participantes de pesquisa entre 11 a 17 anos/11/meses e 29 dias

Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença.

O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.

Informamos que o seu material biológico colhido não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.

Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.

Fui informada que não terei benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informada também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de participar da pesquisa, sem prejuízo do atendimento médico que necessitar e, que meu nome não aparecerá quando os resultados forem divulgados.

Fique ciente, que a participação neste estudo é voluntária e que mesmo após ter dado consentimento para participar da pesquisa, você poderá retirar a qualquer momento, sem qualquer prejuízo na continuidade do tratamento, ou qualquer outra atividade em que você esteja participando.
Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue a você devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.
Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:
Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO em participar de forma voluntária, estando ciente que todos os meus dados estarão resguardos através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados e revistas científicas.

Botucatu,_____/___/_____

_____ _____
Pesquisador Participante da Pesquisa

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Fone: (14)3880-0431 vi.juliani@hotmail.com

OBS: Este Assentimento Livre e Esclarecido deverá vir acompanhado do Termo de Consentimento Livre e Esclarecido de para participantes de pesquisa de 12anos a 17anos/11meses e 29 dias).

CEP

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Modulação da resposta inflamatória sistêmica na pré-eclâmpsia
Subprojeto 1: MODULAÇÃO M1/M2 EM MONÓCITOS DE GESTANTES PORTADORAS DE PRÉ-ECLAMPSIA
Subprojeto 2:Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Subprojeto 3: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Subprojeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia

Pesquisador: Maria Terezinha Serrão Peraçoli
Área Temática:
Versão: 5
CAAE: 74067417.0.0000.5411
Instituição Proponente: Departamento de Microbiologia e Imunologia
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 4.418.043

Apresentação do Projeto:
A presente Emenda refere-se alterações nos Subprojeto 1 e Subprojeto 2, segundo o documento Carta_de_resposta_a_pendencias.pdf, postada na PB (Plataforma Brasil) em 18 de novembro de 2020.

No Subprojeto 1, não foi possível utilizar a linhagem celular THP-1, pois após várias tentativas de cultivo dessa célula em laboratório foi impossível manter a viabilidade celular. Sendo assim, foi preciso substituir as células THP-1 na metodologia por células de vinte mulheres saudáveis, que serão coletadas no ambulatório de Ginecologia e Obstetrícia. Considerando que haverá coleta de sangue de mulheres saudáveis foram incluídos TCLEs e TALE.

No Subprojeto 2, devido a resultados promissores com o uso da silibinina (outro agente

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imunomodulador), decidiu-se utilizá-la também nesse projeto em andamento, uma vez que foram coletadas células suficientes para dar continuidade nos experimentos, não sendo necessário coletar maior quantidade de sangue periférico das gestantes. Dessa forma, houve apenas alteração no título e na análise laboratorial de material previamente coletado.

De acordo com retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil):

SUBPROJETO 1: A pré-eclâmpsia (PE) é uma síndrome específica da gestação que se caracteriza por um estado de má adaptação da tolerância imunológica, identificado por estresse oxidativo e ativação anormal do sistema imune inato. No plasma de gestantes portadoras de PE encontram-se níveis elevados de estruturas moleculares associadas ao estresse e morte celular, denominados padrões moleculares associados ao dano (DAMPs) tais como, proteína de choque térmico (Hsp70), high mobility group box 1 (HMGB1), Hialurona (HA) e Ácido Úrico, que parecem contribuir para a patogênese dessa doença. As DAMPs podem ativar monócitos desviando-os para perfil proinflamatório M1. O desbalanço entre citocinas pró e antiinflamatórias na PE pode ser dependente da deficiência de fatores reguladores capazes de modular essa resposta inflamatória como a vitamina D, ou ainda esse desbalanço poderia ser regulado através da administração de flavonóides com propriedades anti-inflamatórias como a silibinina. O objetivo desse trabalho é estimular e tratar monócitos com moléculas moduladoras da ativação ou regulação, para melhor compreensão das vias de ativação dos monócitos na inflamação sistêmica observada na PE e, possivelmente, propor formas alternativas para o tratamento dessa importante síndrome da gestação. Monócitos de gestantes portadoras de PE serão cultivados na presença ou ausência de hialurona de alto peso molecular (hmw-HA), vitamina D e silibinina e os monócitos de mulheres não grávidas serão cultivadas na presença ou ausência de urato monossódico (MSU), hialurona de baixo peso molecular (lmw-HA), HSP70, HMGB1, vitamina D ou silibinina, por diferentes períodos: a) por 30 min para análise da expressão de proteínas de vias de sinalização p38 MAPK, ERK1/2, IKB-, STAT1, STAT6, e NF-kBp65 por citometria de fluxo, b) por 18h para análise da expressão dos receptores TLR4, RAGE, CD64, CD44, CD163 e CD14 por citometria de fluxo e para a determinação da concentração das citocinas por IL-1, IL-6, IL-8, IL-10, IL-12p70 e TNF- por CBA e IL-23 por ELISA. Os resultados serão analisados por meio de testes paramétricos ou não-paramétricos com nível de significância de 5%.

SUBPROJETO 2: Estudos sugerem que na pré-eclâmpsia (PE) ocorre um estado de má adaptação da

tolerância imunológica, caracterizada por ativação anormal do sistema imune inato e adaptativo. As células T reguladoras (Treg) são uma população de células T responsáveis pela manutenção da tolerância e controle da inflamação, enquanto células Th17 medeiam diferentes tipos de reações inflamatórias, possuindo ações opostas. Na PE, a ocorrência de resposta inflamatória sistêmica parece ser devida ao desbalanço das subpopulações de células T CD4+ causado por aumento do número de células Th17 e diminuição das células T reguladoras em comparação às gestantes normotensas. Portanto, o balanço entre células Treg e Th17 pode ser crítico para a tolerância ao feto e para prevenção da PE. O presente estudo pretende avaliar
o efeito imunomodulador da vitamina D e da silibinina sobre as subpopulações de células T (Th17 e Treg) em gestantes préeclâmpticas. Serão estudadas 20 gestantes portadoras de PE e 20 normotensas, pareadas pela idade gestacional. Sangue periférico será obtido das gestantes de ambos os grupos para determinação da concentração plasmática de vitamina D [25(OH)D]. O efeito modulador da vitamina D e da silibinina sobre as subpopulações de células T CD4+ (Th1, Th2, Th17 e Treg) será avaliado quanto à expressão dos ativadores de transcrição para as células Th1 (STAT1 e STAT4), Th2 (STAT6), Th17 (STAT3) e Treg (STAT5) e dos fatores de transcrição intracitoplasmáticos para células Th1 (Tbet), Th2 (GATA-3), Th17 (RORt e Runx1) e Treg (FoxP3) característicos de cada subpopulação de células T, além dos receptores de IL-23 (IL-23R) e de vitamina D (VDR) por meio das técnicas de citometria de fluxo e qPCR. O perfil de citocinas pró e anti-inflamatórias produzido por essas subpopulações celulares após os tratamentos com a vitamina D e silibinina, Th1 (IFN- e TNF-), Th2 (IL-4), Th17 (IL-6, IL-17 e IL-22) e Treg (IL-10 e TGF-) no sobrenadante de cultura de células mononucleares de gestantes pré-eclâmpticas e normotensas por CBA (citometria de fluxo) e Elisa.

Objetivo da Pesquisa:

Segundo informações retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil), os objetivos do estudo são:

SUBPROJETO 1: Estimular e tratar monócitos com moléculas moduladoras da ativação ou regulação, para melhor compreensão das vias de ativação dos monócitos na inflamação sistêmica observada na PE, e possivelmente, propor formas alternativas para o tratamento dessa importante síndrome da gestação.

SUBPROJETO 2: Avaliar o efeito modulador da vitamina D e da silibinina sobre as subpopulações de células T (Th17 e T reguladoras - Treg) em gestantes pré-eclâmpticas.

A presente Emenda não se refere aos Subprojetos 3 e 4.

Avaliação dos Riscos e Benefícios:

De acordo com informações retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil), tem-se:

RISCOS: Nos Subprojetos 1 e 2, no momento da coleta de sangue pode haver dor da picada de agulha e, raramente, formação de um pequeno hematoma no local.

BENEFÍCIOS: Os resultados do presente projeto contribuirão para melhor conhecimento dos mecanismos imunes envolvido na fisiopatologia da pré-eclâmpsia e poderão, no futuro estabelecer estratégias diferentes de prevenção e tratamento dessa patologia obstétrica.

A presente Emenda não se refere aos Subprojetos 3 e 4.

Comentários e Considerações sobre a Pesquisa:

Trata-se de Emenda ao projeto original intitulado "Modulação da resposta inflamatória sistêmica na préeclâmpsia", sob responsabilidade da Profa. Maria Terezinha Serrão Peraçoli.

Segundo o item "Justificativa da Emenda" nas "Informações básicas do projeto" da PB, houve alterações nos subprojetos 1 e 2, informações confiramdas pela pesquisadora em documento postado em 18 de novembro de 2020 (Carta_de_resposta_a_pendencias.pdf).

No SUBPROJETO 1, não foi possível utilizar a linhagem celular THP-1, pois após várias tentativas de cultivo dessa célula em laboratório foi impossível manter a viabilidade celular. Sendo assim, foi preciso substituir as células THP-1 na metodologia por células de mulheres saudáveis, que serão coletadas no ambulatório de Ginecologia e Obstetrícia.
Considerando que haverá coleta de sangue de mulheres saudáveis foram incluídos TCLEs e TALE.

No SUBPROJETO 2, devido a resultados promissores com o uso da silibinina (outro agente imunomodulador), decidiu-se utilizá-la também nesse projeto em andamento, uma vez que foram coletadas células suficientes para dar continuidade nos experimentos, não sendo necessário coletar maior quantidade de sangue periférico das gestantes. Dessa forma, houve apenas alteração no título e na análise laboratorial de material previamente coletado.

Considerações sobre os Termos de apresentação obrigatória:

Vide campo de "Conclusões ou Pendências e Lista de Inadequações".

Conclusões ou Pendências e Lista de Inadequações:

Após análise em REUNIÃO EXTRAORDINÁRIA, o Colegiado deliberou APROVAÇÃO da Emenda.

Considerações Finais a critério do CEP:

Conforme deliberação do Colegiado, em REUNIÃO EXTRAORDINÁRIA do Comitê de Ética em Pesquisa FMB/UNESP, realizada em 23/11/2020, a Emenda encontra-se APROVADA. O Pesquisador deverá enviar Relatório Final de Atividades ao final da pesquisa.

Atenciosamente,

Comitê de Ética em Pesquisa FMB/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1635771_E2.pdf	18/11/2020 14:12:48		Aceito
Outros	Carta_de_resposta_a_pendencias.pdf	18/11/2020 14:11:00	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_1_novo.docx	16/11/2020 16:28:19	Maria Terezinha Serrão Peraçoli	Aceito
Folha de Rosto	folha_de_rosto.pdf	24/09/2020 14:07:40	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_2novo.docx	23/09/2020 15:56:00	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_responsavel_novo.docx	23/09/2020 15:23:04	Maria Terezinha Serrão Peraçoli	Aceito

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TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_maior_novo.docx	23/09/2020 15:22:48	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_GN_novo.docx	23/09/2020 15:22:37	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TALE_novo.docx	23/09/2020 15:22:24	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_grupo_controle_NG.docx	23/09/2020 15:17:11	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_3_incluida_UNIMED.docx	02/05/2019 10:56:58	Priscila Rezeck Nunes	Aceito
Outros	Oficio_UNIMED.docx	02/05/2019 10:56:42	Priscila Rezeck Nunes	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	biorrepositorio_CEP.pdf	16/08/2017 11:44:20	Maria Terezinha Serrão Peraçoli	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_Anuencia.pdf	03/08/2017 10:32:11	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_4.docx	03/08/2017 10:26:47	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_3.docx	03/08/2017 10:26:35	Maria Terezinha Serrão Peraçoli	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não