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AVALIAÇÃO DA AÇÃO FARMACOLÓGICA DO NEBIVOLOL E SUAS REPERCUSSÕES EM MODELO *IN VITRO* DE PRÉ-ECLÂMPSIA

Botucatu-SP
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Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para a obtenção do título de Mestra no Programa de Pós-Graduação em Farmacologia e Biotecnologia.

Orientadora: Prof. Dra. Valéria Cristina Sandrim

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EPÍGRAFE

Slow down, you crazy child
You're so ambitious for a juvenile
But then if you're so smart
Tell me why are you still so afraid?

Where's the fire?
What's the hurry about?
You better cool it off before you burn it out
You got so much to do and only so many hours in a day

(...)

Slow down, you're doing fine
You can't be everything you wanna be before your time

Vienna – Billy Joel

PREFÁCIO

É com grande satisfação que apresentamos este estudo, que traz resultados em busca de respostas para um importante desafio médico: a Pré-eclâmpsia (PE). Esta condição obstétrica representa um sério risco à saúde das gestantes e requer esforços contínuos na busca por novas abordagens terapêuticas.

O trabalho está dividido em dois capítulos. O Capítulo 1 aborda o artigo publicado na revista Cells, intitulado "*Nebivolol increases nitric oxide synthase via β3 adrenergic receptor in endothelial cells following exposure to plasma from preeclamptic patients*." Neste estudo, o foco foi avaliar como o nebivolol pode afetar a produção de óxido nítrico em células endoteliais quando expostas ao plasma de pacientes com PE.

No Capítulo 2, apresentamos um artigo inédito intitulado "*Shear Stress and nebivolol in Preeclampsia: Modulating the NO pathway*." Neste estudo, o objetivo foi investigar como a tensão de cisalhamento (*shear stress*) e o nebivolol podem influenciar a via do óxido nítrico; produção de espécies reativas de oxigênio (ROS); expressão de moléculas de adesão e a atividade antioxidante.

Este trabalho é um importante passo rumo ao entendimento mais profundo da PE e suas implicações. Esperamos que este estudo contribua para avanços significativos no tratamento e manejo dessa condição, trazendo benefícios concretos para as gestantes.

LISTA DE ABREVIAÇÕES E SIGLAS

ACOG - American College of Obstetrics and Gynecology, Colégio Americano de Obstetras e Ginecologistas

ADMA - asymmetric dimethylarginine, dimetilarginina assimétrica

BSA - bovine serum albumin, albumina sérica bovina

CD - Cluster of differentiation, grupamento de diferenciação

ECs - endothelial cells, células endoteliais

EDRF - endothelium-derived relaxing factor, fator de relaxamento derivado do endotélio

EDTA - ácido etilenodiamino tetra-acético

eNOS - endothelial nitric oxide synthase, óxido nitrico sintase endotelial

FSI - Fragile states index, índice de estado frágil

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase, gliceraldeído 3-fosfato desidrogenase

HRP - horseradish peroxidase, peroxidase de rábano

HUVECs - Human umbilical vein endothelial cells, células endoteliais de veia umbilical humana

LDS - Lithium dodecyl sulfate, Dodecil sulfato de lítio

NaCl - cloreto de sódio

NED - N-(1-naphthyl)-ethylenediamine dihydrochloride, Dicloridrato de N-(1-naftil)-etilenodiamina

NO - nitric oxide, óxido nítrico

NOS - nitric oxide synthase, óxido nitrico sintase

OOBr - Observatório Obstétrico Brasileiro

PBS - phosphate buffered saline, tampão salino tamponado com fosfato

PVDF - polyvinylidene fluoride, fluoreto de polivinilideno

RMM - razão de mortalidade materna

RNS - espécies reativas de nitrogênio

ROS - espécies reativas de oxigênio

TBS - tris-buffered saline, solução salina tris-tamponada

TCEP - (tris(2-carboxyethyl)phosphine), (tris(2-carboxietil)fosfina)

LISTA DE FIGURAS

RESUMO

A pré-eclâmpsia (PE) é marcada por disfunção endotelial significativa, onde uma redução na biodisponibilidade do óxido nítrico (NO, do inglês *nitric oxide*) é particularmente significativa. Nesse contexto, esta dissertação está dividida em dois capítulos que abordam as repercussões da doença em modelo *in vitro* de PE. O Capítulo 1 apresenta o artigo publicado na revista Cells, intitulado "*Nebivolol increases nitric oxide synthase via β3 adrenergic receptor in endothelial cells following exposure to plasma from preeclamptic patients*." Neste estudo, exploramos a ação do fármaco nebivolol em reestabelecer os níveis de NO em células endoteliais (CEs) expostas ao plasma de pacientes com PE. Encontramos que, as CEs incubadas com plasma de mulheres com PE apresentam menores níveis de NO em comparação com o grupo de células que recebeu o plasma de gestantes saudáveis. Entretanto, o tratamento com nebivolol reestabelece estes níveis, e essa ação está relacionada parcialmente pela ativação dos receptores do tipo β3 adrenérgicos e pela NO sintase endotelial (eNOS). Nossos resultados sugerem que o nebivolol atua na síntese de NO através da ativação da eNOS e dos receptores β3 adrenérgicos no endotélio. No entanto, mais estudos serão necessários para compreender esta molécula. No Capítulo 2, apresentamos um artigo inédito intitulado "*Shear Stress and nebivolol in Preeclampsia: Modulating the NO pathway*." Neste estudo, o objetivo foi investigar como a tensão de cisalhamento (*shear stress*) e o nebivolol podem influenciar a via do NO; produção de espécies reativas de oxigênio (ROS); expressão de moléculas de adesão e a atividade antioxidante. Encontramos que, o plasma de PE aumenta a fosforilação da enzima eNOS no resíduo Serina1177, e o tratamento com nebivolol resulta em uma elevação adicional dessa fosforilação comparado ao grupo de CEs incubadas apenas com plasma de PE. As CEs tratadas com plasma de PE também demonstram maior capacidade antioxidante e níveis reduzidos de ROS em comparação com as CEs tratados com plasma de gestante saudáveis. Além disso, o plasma de PE diminui a expressão das moléculas de adesão ICAM-1 e VCAM-1, que desempenham papéis essenciais na adesão celular. A intervenção do nebivolol influencia a via do NO e a liberação de espécies reativas de oxigênio (ROS), ao mesmo tempo que regula positivamente os marcadores de adesão. O estudo ressalta o potencial do plasma de PE em modular a função endotelial e destaca o nebivolol como uma possível ação farmacológica. Em conjunto, os dois estudos apresentam resultados importantes em relação à disfunção endotelial na PE e lançam luz sobre um novo fármaco capaz de modular marcadores presentes na doença, como o NO.

ABSTRACT

Preeclampsia (PE) is characterized by significant endothelial dysfunction, where a reduction in the bioavailability of nitric oxide (NO) is particularly significant. In this context, this dissertation is divided into two chapters that address the implications of the disease in an in vitro model of PE. Chapter 1 presents the article published in the journal Cells, titled "Nebivolol increases nitric oxide synthase via β3 adrenergic receptor in endothelial cells following exposure to plasma from preeclamptic patients." In this study, we explore the action of the drug nebivolol in reestablishing NO levels in endothelial cells (ECs) exposed to plasma from PE patients. We found that ECs incubated with plasma from women with PE have lower NO levels compared to the group of cells that received plasma from healthy pregnant women. However, treatment with nebivolol restores these levels, and this action is partially related to the activation of β3 adrenergic receptors and endothelial nitric oxide synthase (eNOS). Our results suggest that nebivolol acts on NO synthesis through the activation of eNOS and β3 adrenergic receptors in the endothelium. However, further studies will be necessary to understand this molecule. In Chapter 2, we present an unpublished article titled "Shear Stress and nebivolol in Preeclampsia: Modulating the NO pathway." In this study, the objective was to investigate how shear stress and nebivolol can influence the NO pathway, production of reactive oxygen species (ROS), expression of adhesion molecules, and antioxidant activity. We found that PE plasma increases the phosphorylation of the eNOS enzyme at Serine1177, and treatment with nebivolol results in additional elevation of this phosphorylation compared to the group of ECs incubated only with PE plasma. ECs treated with PE plasma also demonstrate greater antioxidant capacity and reduced levels of reactive oxygen species (ROS) compared to ECs treated with plasma from healthy pregnant women. Furthermore, PE plasma decreases the expression of adhesion molecules ICAM-1 and VCAM-1, which play essential roles in cell adhesion. Nebivolol intervention influences the NO pathway and the release of ROS while positively regulating adhesion markers. The study highlights the potential of PE plasma in modulating endothelial function and underscores nebivolol as a potential pharmacological intervention. Taken together, the two studies present important results regarding endothelial dysfunction in PE and shed light on a new drug capable of modulating markers present in the disease, such as NO.

SUMÁRIO

INTRODUÇÃO

1. Mortalidade materna: uma perspectiva global e o cenário brasileiro

Em todo o mundo, estima-se que ocorreram cerca de 287 mil mortes maternas em 2020, a maioria delas em países de baixa e média renda e de causas evitáveis (SAY et al., 2014). A definição de mortalidade materna abrange especificamente a morte de uma mulher durante a gestação ou até 42 dias após o parto, devido a causas relacionadas ou agravadas pela própria gravidez ou seu manejo, excluindo óbitos acidentais ou incidentais (WHO, 2015). Essa definição permite uma identificação precisa das mortes maternas, levando em consideração suas causas diretas ou indiretas (WHO, 2015). A razão de mortalidade materna (RMM), um dos indicadores globais mais importantes em saúde, é calculada como o número de mortes por causas relacionadas à gestação, parto e puerpério (até 42 dias após o parto) a cada 100 mil nascidos vivos (WORLD HEALTH ORGANIZATION, 2019).

As desigualdades no acesso à serviços de saúde de qualidade são evidenciadas pelo alto número de mortes maternas em determinadas regiões do mundo, ressaltando a disparidade entre os países ricos e pobres. Em 2020, a RMM em países de baixa renda foi de 430 por 100 mil nascidos vivos, em contraste com apenas 12 por 100 mil nascidos vivos em países de alta renda. Essa diferença alarmante é ainda mais evidente quando observamos o Índice de Estados Frágeis (FSI, do inglês *Fragile States Index*), que classifica nove países em "alerta muito alto" ou "alerta alto", com taxas de mortalidade materna variando de 30 (República Árabe da Síria) a 1223 (Sudão do Sul). Além disso, as mulheres em países de baixa renda enfrentam um risco maior de morte materna ao longo da vida. Essas discrepâncias reforçam a necessidade urgente de abordar as barreiras socioeconômicas que impedem o acesso equitativo aos cuidados de saúde materna e implementar estratégias eficazes para reduzir a mortalidade materna em todo o mundo (WORLD HEALTH ORGANIZATION, 2019).

No Brasil, análises feitas pelo Observatório Obstétrico Brasileiro (OOBr) com base em dados do Ministério da Saúde revelaram um aumento significativo nos óbitos de gestantes em 2021, quase dobrando em relação a 2019 (OOBR, 2022). No ano de 2021, a taxa de mortalidade materna foi de 110 mortes a cada 100 mil nascidos vivos, o que é equivalente à mesma taxa registrada em 1998 (OOBR, 2022). Em comparação, em 2019 a taxa foi de 57,9 mortes por 100 mil nascidos vivos e em 2020 foi de 71,9 mortes (OOBR, 2022). O Brasil estabeleceu como meta, em conjunto com as Nações Unidas, a redução

da taxa de mortalidade materna para 30 mortes por 100 mil nascidos vivos até 2030 (OOBR, 2022). Em países desenvolvidos, essa taxa varia em torno de 10 mortes por 100 mil nascidos vivos (WORLD HEALTH ORGANIZATION, 2019). Esses números destacam a urgência de medidas e investimentos para melhorar a saúde materna no país e alcançar as metas estabelecidas.

As mulheres em todo o mundo enfrentam riscos de complicações durante e após a gravidez e o parto, algumas das quais podem ser fatais (WORLD HEALTH ORGANIZATION, 2019). A maioria dessas complicações ocorre durante a gravidez e muitas delas são evitáveis ou tratáveis com intervenções adequadas (WORLD HEALTH ORGANIZATION, 2019). Entre as complicações mais prevalentes, que contribuem para aproximadamente 75% de todas as mortes maternas, estão: hemorragia (principalmente após o parto); infecções (geralmente pós-parto); hipertensão arterial durante a gravidez (pré-eclâmpsia e eclâmpsia); complicações do parto e aborto inseguro (WORLD HEALTH ORGANIZATION, 2019).

A ONU enfatiza a importância de garantir que todas as mulheres tenham acesso a cuidados de saúde de qualidade durante a gravidez, no momento do parto e no pós-parto (WORLD HEALTH ORGANIZATION, 2019). A saúde materna e a saúde do recém-nascido estão intrinsecamente ligadas, e é crucial que todos os partos sejam realizados por profissionais de saúde qualificados (WORLD HEALTH ORGANIZATION, 2019). A gestão adequada e o tratamento oportuno podem fazer a diferença entre a vida e a morte para as mulheres e seus recém-nascidos.

2. Pré-eclâmpsia

A pré-eclâmpsia (PE) afeta cerca de 5-7% das mulheres grávidas globalmente, resultando em mais de 70 mil mortes maternas e 500 mil mortes fetais a cada ano (RANA et al., 2019). No Brasil, a incidência da PE é de 1,5% e 0,6% para a eclâmpsia, quadro mais grave da doença (RAMOS; SASS; COSTA, 2017). A PE é definida, pelo Colégio Americano de Obstetras e Ginecologistas (ACOG, do inglês *American College of Obstetrics and Gynecology*), por um novo início de hipertensão (pressão arterial sistólica ≥140 mmHg e/ou pressão arterial diastólica ≥90 mmHg) em duas medições com 4h de intervalo em repouso, e frequentemente acompanhada por proteinúria e comprometimento de órgãos-alvo como cérebro e fígado (ACOG, 2020).

Nesse contexto, os fatores determinantes da síndrome incluem histórico familiar, predisposição genética, número de gestações, idade materna, uso de fertilização *in vitro*

e condições médicas maternas, como hipertensão pré-existente, diabetes, doença renal crônica (DRC) e obesidade (BARTSCH et al., 2016; HUTCHEON; LISONKOVA; JOSEPH, 2011). Existem condições que estão associadas ao aumento da massa placentária, como gestações multifetais e mola hidatiforme, as quais também aumentam o risco de PE (KARUMANCHI, 2016). Indivíduos que foram frutos de gestações afetadas pela PE possuem um risco maior de vivenciarem ou serem pais com uma gravidez complicada pela PE, e esse risco persiste mesmo após suas primeiras gestações (ESPLIN et al., 2001; SKJAERVEN et al., 2005). Estudos indicam que cerca de 55% da doença apresentam base hereditária, com as contribuições genéticas maternas e fetais correspondendo a aproximadamente 30-35% e 20% do risco, respectivamente (GRAY; SAXENA; KARUMANCHI, 2018).

A fisiopatologia da PE se apresenta como uma síndrome de dois estágios (ROBERTS; HUBEL, 2009). O primeiro estágio é descrito como a redução da perfusão placentária, considerada a origem da doença e este se traduz, em algumas, mas não em todas as mulheres, no segundo estágio, a síndrome materna multissistêmica (REDMAN; SACKS; SARGENT, 1999; ROBERTS; GAMMILL, 2005). O primeiro estágio ocorre devido à remodelação incompleta das artérias espiraladas uterinas, que são responsáveis pelo suprimento do espaço interviloso. Dessa forma, a chegada de sangue à placenta fica comprometida, levando à isquemia placentária (ROBERTS et al., 1989). Além disso, em uma gestação saudável, o diâmetro luminal vascular é aumentado em quatro vezes, e a parede do vaso é modificada devido à perda de músculo liso e da lâmina elástica interna (ROBERTS; GAMMILL, 2005). No entanto, na PE, essa remodelação vascular não ocorre de forma completa, restringindo-se às porções superficiais do vaso, encontradas na decídua (ROBERTS; GAMMILL, 2005). Logo, o segundo estágio é estabelecido, no qual ocorre a liberação, pela placenta, de diversas moléculas, incluindo fatores anti-angiogênicos, na corrente sanguínea materna, levando à disfunção endotelial generalizada, hipertensão e lesão de órgãos-alvo (KARUMANCHI, 2016; MAYNARD; KARUMANCHI, 2011).

Diante do diagnóstico e dos possíveis desdobramentos negativos da doença, o controle clínico da paciente é sinônimo de prevenção da mortalidade materna e perinatal (RAMOS; SASS; COSTA, 2017). Por isso, entre as orientações para o gerenciamento da PE, destaca-se os tratamentos farmacológicos e não farmacológicos (REGITZ-ZAGROSEK et al., 2011). As abordagens não farmacológicas incluem repouso hospitalar ou domiciliar e acompanhamento laboratorial (ACOG, 2020). Por sua vez, a abordagem

farmacológica faz uso dos anti-hipertensivos para controlar os valores de pressão arterial e a presença ou não de sinais e sintomas relacionados aos níveis pressóricos (ACOG, 2020). As classes de agentes anti-hipertensivos mais utilizados são os simpatolíticos de ação central, α2-agonistas (metildopa e clonidina); bloqueadores de canais de cálcio (nifedipino e anlodipino); vasodilatores periféricos, recomendados em associação com medicamentos para controle pressórico (hidralazina) e antagonistas de adrenoreceptores β (metoprolol e carvedilol) (LEFEVRE; KRUMM, 2019; PODYMOW; AUGUST, 2008; WEBSTER et al., 2017). Apesar da diversidade de intervenções disponíveis, todas elas são apenas paliativas, destinadas a aliviar as consequências da doença. O tratamento, contudo, só é alcançado por meio do parto (IVES et al., 2020).

2.1. Disfunção endotelial e a importância do óxido nítrico

Entre as diversas manifestações da disfunção endotelial na PE, as espécies reativas de oxigênio (ROS) e de nitrogênio (RNS), como o óxido nítrico (NO, do inglês *nitric oxide*), superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e peroxinitrito ($ONOO^-$) desempenham papel fundamental como moléculas sinalizadoras e reguladoras dentro das repercussões da doença (AOUACHE et al., 2018). A sinalização de ROS e RNS são controladas diretamente pela defesa antioxidante do organismo, que sequestra estas moléculas oxidantes em condições fisiológicas (DI MEO et al., 2016). Na gestação saudável, é bem estabelecido que a produção de ROS ocorre em concentrações elevadas e é necessária para regulação da homeostase vascular (DUHIG; CHAPPELL; SHENNAN, 2016). No entanto, um cenário completamente diferente se manifesta quando há o desequilíbrio entre a capacidade antioxidante e as espécies pró-oxidantes, resultando no estresse oxidativo observado na PE (HUBEL, 1999; MYATT; CUI, 2004).

A descoberta de um fator de relaxamento derivado do endotélio (EDRF, do inglês *endothelium-derived relaxing factor*) que, posteriormente, foi identificado como óxido nítrico, revolucionou o conhecimento sobre diversos processos biológicos no organismo (FURCHGOTT; ZAWADZKI, 1980; IGNARRO et al., 1987). O NO é uma molécula multifacetada, protagonista de ações vasodilatadoras, anti-plaquetárias e angiogênicas (ALHEID; FRÖLICH; FÖRSTERMANN, 1987; FURCHGOTT et al., 1984; ZICHE et al., 1994). Além disso, é sintetizado pelo substrato L-arginina por uma família de enzimas dependentes de cálcio (Ca^{2+})/calmodulina (CaM), chamadas de óxido nítrico sintase (NOS, do inglês NO synthase EC 1.14.13.39); sendo a mais importante, nesse contexto, a NOS endotelial (eNOS, do inglês endothelial NO synthase) (MURAD, 1994). É

produzido, predominantemente pelo endotélio em resposta à estímulos mecânicos e químicos que incluem a tensão de cisalhamento endotelial, o chamado *shear stress*, e a ativação via receptores por agonistas, respectivamente (FULTON et al., 2008; TSAO et al., 1995).

O *shear stress* é uma força mecânica que afeta as camadas adjacentes dos vasos sanguíneos, atuando em diferentes velocidades ou direções (HOEFER; DEN ADEL; DAEMEN, 2013). O *shear stress* laminar (unidirecional e constante) é conhecido por exercer um efeito protetivo contra lesões ateroscleróticas, enquanto o *shear stress* oscilatório (irregular e turbulento) está associado a efeitos relacionados à aterosclerose (DIMMELER et al., 1996; KU et al., 1985; LEVESQUE; NEREM; SPRAGUE, 1990; MALEK; ALPER; IZUMO, 1999). O fluxo laminar aumenta a expressão gênica da eNOS, além de elevar a capacidade das células endoteliais de liberar NO (UEMATSU et al., 1995). Essa ação está relacionada diretamente com a fosforilação da eNOS no resíduo Serina1177 (Ser1177), o que resulta na ativação dessa enzima (FISSLTHALER et al., 2000; GO et al., 1998). Da mesma maneira, a produção de NO é regulada pela ação de agonistas (FLEMING; BUSSE, 1999). Um agonista é uma molécula capaz de se ligar e ativar funcionalmente um receptor e neste caso aumentar a concentração de Ca^{2+} livre e a associação do Ca^{2+}/CaM resultando na liberação de NO (ZHAO; VANHOUTTE; LEUNG, 2015). Nesse contexto, o fármaco nebivolol se destaca pelos seus efeitos agonistas no endotélio, através da ativação do adrenoreceptor do tipo β3. Esse processo resulta na ativação da eNOS e consequentemente liberação de NO (BUENO-PEREIRA et al., 2022; DESSY et al., 2005).

Nessa perspectiva, a diminuição da biodisponibilidade de NO é identificada como um fator adicional relevante na disfunção endotelial característica da PE (DAVIDGE; STRANKO; ROBERTS, 1996; SANDRIM et al., 2008; SELIGMAN et al., 1994). Essa redução acontece devido ao microambiente de estresse oxidativo, característico da doença, que faz com que o NO gerado reaja com O_2^- e forme $ONOO^-$, molécula encontrada em altas concentrações na vasculatura de mulheres pré-eclâmpticas (BECKMAN; KOPPENOL, 1996; LOWE, 2000; ROGGENSACK; ZHANG; DAVIDGE, 1999). Outro motivo dessa redução é o aumento dos níveis de dimetilarginina assimétrica (ADMA, do inglês *asymmetric dimethylarginine*), molécula produzida de maneira endógena e reconhecida por ser por ser inibidora competitiva da NOS quando está em altas concentrações (CHEN; XIA; ZHAO, 1997; HOLDEN et al., 1998). Da mesma maneira, a enzima arginase (EC 3.5.3.1) compete pelo mesmo substrato da NOS,

L-arginina, para formar uréia e L-ornitina (ASH, 2004). Em altas concentrações como as observadas no plasma, plaquetas e vasculatura de mulheres com PE, a enzima se liga ao substrato comum da NOS e consequentemente reduz os níveis de NO (JAE et al., 2009; PIMENTEL et al., 2013; SANKARALINGAM; XU; DAVIDGE, 2010).

3. Justificativa

In suma, apesar dos inúmeros estudos sobre a PE, incluindo a descoberta de novos biomarcadores e possíveis novos tratamentos, a forma de intervenção farmacológica mais utilizada ainda são anti-hipertensivos (ACOG, 2020). Investigar os mecanismos pelos quais a disfunção endotelial se desenvolve e como esse aspecto afeta as células endoteliais em modelo *in vitro* é de suma importância para propor tratamentos farmacológicos mais efetivos. Inicialmente, incubamos as células endoteliais em condições estáticas com plasma de mulheres com PE, submetidas à intervenção farmacológica do nebivolol. O objetivo foi avaliar a dependência da ativação do receptor β3 na produção intracelular de óxido nítrico (NO) (Capítulo 1). No segundo momento, incubamos as células endotelias em condições de *shear stress* com plasma de mulheres com PE, submetidas à intervenção farmacológica do nebivolol. O objetivo foi investigar a ativação da eNOS, em níveis de fosforilação e de quantificação das concentrações de nitrito/nitrato, além de avaliar o papel do nebivolol em restabelecer os níveis de NO e ROS, em modular as moléculas de adesão endotelial e a atividade antioxidante, nesse contexto (Capítulo 2). Acreditamos que os dados obtidos pelo modelo *in vitro* podem ser uma ferramenta valiosa para orientar estudos clínicos com potenciais fármacos destinados ao tratamento da PE. É importante ressaltar que, embora o conhecimento sobre a disfunção endotelial na doença e os mecanismos subjacentes estejam avançando, o desenvolvimento de medicamentos ainda não acompanha esse progresso. A falta de investimentos das indústrias farmacêuticas nessa área provavelmente se deve ao elevado risco associado ao tratamento de gestantes.

CAPÍTULO 1

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Communication

Nebivolol Increases Nitric Oxide Synthase via β_3 Adrenergic Receptor in Endothelial Cells Following Exposure to Plasma from Preeclamptic Patients

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Abstract: Background: Low bioavailability of nitric oxide (NO) is related to the pathophysiology of preeclampsia (PE). In the present study, we investigated the effect of nebivolol (NEB), a β_3-receptor agonist with vasodilator properties, on the NO synthesis in endothelial cells incubated with plasma from preeclamptic patients. Methods and results: Human umbilical vein endothelial cells (HUVECs) were incubated with plasma from healthy pregnant (HP) and PE women; NO quantification was assessed by a fluorescence compound. We found that endothelial cells incubated with plasma from women with PE show lower NO levels compared with the HP group ($p < 0.0001$). However, NEB treatment increases NO levels, partially, mediated by β_3 adrenergic receptors ($p < 0.0001$) and through eNOS activation ($p < 0.0001$). Conclusions: Our results suggest that NEB acts in NO synthesis through eNOS activation and β_3 adrenergic receptors in the endothelium. However, further studies will be needed to understand this molecule.

Keywords: Nebivolol; nitric oxide; adrenoceptor; preeclampsia; endothelial dysfunction

1. Introduction

Nebivolol (NEB) is a third-generation beta-blocker combining highly selectivity for the β_1 adrenoceptors and the ability to release nitric oxide (NO) through the endothelium [1]. It is a racemic mixture of two enantiomers, D- and L-nebivolol, where L-nebivolol behaves as a β_3 adrenergic receptor agonist, increasing Ca^{+2} efflux into endothelial cells by activating eNOS and consequently increasing NO bioavailability [2].

Preeclampsia (PE) is associated with a decrease in the bioavailability of NO [3]; the incubation of plasma or serum from women with PE on endothelial cells promotes changes in endothelial cell functions [4].

Therefore, in the present study, we evaluated the effect of NEB addition on NO levels in endothelial cells incubated with plasma from healthy pregnant (HP) women and with PE in the presence of NEB. Additionally, we investigated the molecular mechanisms of NEB in NO synthesis through activation of endothelial nitric oxide synthase and β_3 adrenoceptors.

2. Methods

The study was approved by the Research Ethics Committee of the Faculty of Medicine of Ribeirão Preto, Brazil (CAAE 37738620.0.0000.5440 approved on 19 October 2020 FMRP–USP) following the principles of the Helsinki Declaration, and all subjects gave written informed consent. Diagnosis criteria of PE were defined by the American College of Obstetricians and Gynecologists [5]. For each pool, 10 plasma samples from healthy pregnant (HP) women and 10 samples from pregnant women with PE were selected.

Human umbilical vein endothelial cells (HUVEC) (EA.hy 926) were cultured until reaching 80–90% of confluence. HUVEC were incubated in supplemented DMEM with 10% (v/v) plasma from PE or HP and 10 µM of NEB, 100 µM of L-name (NOS inhibitor), 3µM of L-748,337 (**β**3 antagonist), for 4 h. Cells were used until the 10th passage.

We used the DAF-FM compound for intracellular NO quantification (5 µM) (Invitro-gen, Thermo Fisher Scientific, Carlsbad, CA, USA). The fluorescence signal was measured (excitation 495 nm, emission 535 nm) in a multifunctional plate reader (Synergy 4, BioTek, Winooski, VT, USA).

Replicates of 5 per group combined with treatments were performed in each experi-ment. When we compare three or more groups, we used One-Way ANOVA followed by the Holm-Sidak test. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) and for all tests, we considered a p-value ≤ 0.05 (two-tailed) significant.

3. Results

The general clinical parameters of women in the HP and PE groups whose plasma samples were collected and pooled for the *in vitro* studies are shown in Table 1.

Table 1. Clinical parameters of HP and PE pregnant women enrolled in the study.

Parameters	HP (*n* = 10)	PE (*n* = 10)
Maternal age (years)	24 ± 1	26 ± 1
SBP (mmHg)	110 ± 3	137 ± 6 *
DBP (mmHg)	71 ± 3	85 ± 4 **
GA at sampling (weeks)	36.25 ± 0.4	32.75 ± 1.7
BMI before pregnancy (Kg/m^2)	25 ± 1	30 ± 1 **
GA at delivery (weeks)	40 ± 1	32 ± 1 ***
Newborn weight (g)	3175 ± 166	2053 ± 358 *

Values are the means ± S.D. or percentage. HP, Healthy Pregnant; PE, preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age. Values are mean ± standard error of the mean. Parametric variables were compared by Student t-test and non-parametric by Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs healthy pregnant.

No differences were identified in the maternal age parameter between the groups. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) is increased in the PE group when compared to the HP group (both $p < 0.001$). Furthermore, the index of body mass (BMI) before pregnancy is also increased in the PE group when compared with the HP group ($p < 0.001$), the gestational age (GA) at delivery, and the weight of the newborn were lower in PE compared to the HP group ($p < 0.001$ and $p < 0.05$, respectively). In addition, no differences were identified in the GA at sampling parameter between the groups. The PE samples were composed of 40% late-onset and 60% early-onset, according to GA at sampling (data not shown).

Endothelial cells incubated with HP plasma induce higher NO levels when compared with the PE group ($p < 0.05$) (Figure 1). In this group, NEB treatment increased NO levels ($p < 0.0001$) through eNOS activation, as we can identify in the group incubated with L-name (NOS inhibitor) ($p < 0.0001$) (Figure 1). To verify if NEB's action was mediated by the β_3 adrenergic receptor it was used the β_3 antagonist and we found that it reduces NO levels when compared to cultures without antagonist ($p < 0.0001$) (Figure 1).

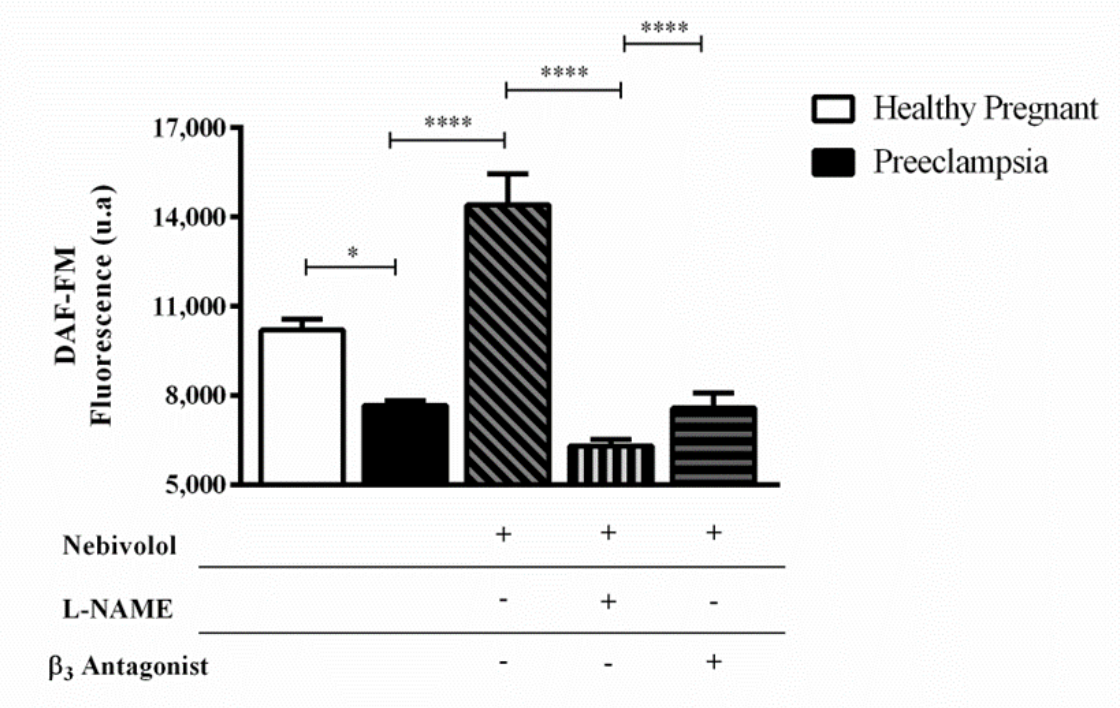

Figure 1. NO fluorescence intensity measured by DAF-FM. HUVECS were incubated with 10% (v/v) plasma from healthy pregnant (HP, white column) or preeclampsia (PE, black column) (n=10 per group) and treatments for 4 hours. Values are means ± S.E.M. Comparisons between groups were realized, assed by One-Way ANOVA followed by Holm-Sidak test. * ($p < 0.05$) HP vs PE; **** ($p < 0.0001$) PE vs PE + Nebivolol; PE + Nebivolol vs PE + L-NAME + Nebivolol; PE + Nebivolol vs PE+ β_3 antagonist + Nebivolol.

4. Conclusions

Our results suggest that NEB acts through eNOS activation and β3 adrenergic receptors in the endothelial cells following exposure to plasma from preeclamptic patients. The manuscript data lead us to further investigate other mechanisms involving the NO pathway and how nebivolol acts on them in a preeclampsia context. We believe that a further understanding of these mechanisms using our strategy model can offer, in the future, alternative management of preeclampsia and, consequently, reduce the repercussions of the disease in patients.

Author Contributions:

Conceptualization, T.O.B.P.; P.R.N; M.B.M.; A.L.V.d.R; V.C.S

Data curation, T.O.B.P.; P.R.N; M.B.M.; A.L.V.d.R; V.C.S

Formal Analysis, T.O.B.P

Investigation, T.O.B.P, and A.L.V.d.R

Methodology, T.O.B.P.; P.R.N; M.B.M.; A.L.V.d.R;
Visualization, T.O.B.P
Funding acquisition, Project administration, Resources, Supervision, Validation, V.C.S
Writing - original draft, T.O.B.P
Writing - review & editing, T.O.B.P.; P.R.N; M.B.M.; A.L.V.d.R; V.C.S.

All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guide-lines of the Declaration of Helsinki, and approved by the Research Ethics Committee of the Faculty of Medicine of Ribeirão Preto, Brazil (CAAE 37738620.0.0000.5440 approved on 19 October 2020 FMRP–USP).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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

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CAPÍTULO 2

Shear Stress and Nebivolol in Preeclampsia: Modulating the NO Pathway

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Abstract

Preeclampsia (PE) is marked by significant endothelial dysfunction, where a reduction in nitric oxide (NO) bioavailability holds particular significance. This study aimed to investigate the influence of treating endothelial cells (ECs) with plasma obtained from preeclamptic patients, both in the presence and absence of nebivolol. To conduct the study, ECs were submitted to shear stress and exposed to a 10% (v/v) pool of PE and healthy pregnant women (HP) plasma for 4 hours. We evaluate the relative protein expression of t-eNOS, peNOS (Ser1177), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control; we measured the levels of nitrite/nitrate oxide (NOx); Reactive oxygen species (ROS) levels and adhesion molecules CD54 (ICAM, intracellular adhesion molecule 1), CD106 (VCAM, vascular cell adhesion molecule 1) and CD105 [Endoglin (ENG)] were assessed by flow cytometry and antioxidant capacity by Ferric Reducing Antioxidant Power (FRAP). We found a higher peNOS (Ser1177)/t-eNOS ratio in the PE-treated ECs compared to the HP-treated cells. In parallel, nebivolol treatment resulted in further elevation of the peNOS(Ser1177)/t-eNOS ratio compared to ECs treated with plasma alone. Additionally, PE-treated cells combined with nebivolol, increased levels of total NOx when compared with plasma alone. PE-treated ECs also demonstrate higher antioxidant capacity and reduced levels of ROS compared to HP-treated ECs. Additionally, PE plasma decreases the expression of adhesion molecules ICAM-1 and VCAM-1, which play roles in cell adhesion. Nebivolol intervention, influences the NO pathway and ROS release, while also upregulating adhesion markers. The study underscores the potential of PE plasma to modulate endothelial function and highlights nebivolol as a potential pharmacological intervention.

Keywords: preeclampsia; endothelial dysfunction; nitric oxide; shear stress; nebivolol

1. Introduction

Shear stress is a hemodynamic force that affects the adjacent blood vessels, occurring at different speeds or directions, and strongly regulates endothelial function and dysfunction (ANDO; KAMIYA, 1993). Laminar shear stress (LSS) is characterized by a steady and protective endothelial flow that activates endothelial nitric oxide synthase (eNOS), leading to the release of nitric oxide (NO) (DIMMELER et al., 1996; UEMATSU et al., 1995). On the other hand, oscillatory shear stress (OSS) is defined as irregular and fluctuating blood flow leading to endothelial dysfunction and consequently reducing levels of NO bioavailability (MALEK; ALPER; IZUMO, 1999). However, many studies that investigate endothelial dysfunction *in vitro*, do not consider biomechanical force. This is often because they primarily use static cultures of endothelial cells (ECs) (VIANA-MATTIOLI et al., 2023).

Considering this, *in vitro* models have become indispensable for comprehending their implications, providing controlled experiments and valuable insights (BOUÏS et al., 2001). Notably, when ECs were incubated with plasma from women with PE, a higher arginase (eNOS competitor) expression was observed (SANKARALINGAM; XU; DAVIDGE, 2010). Moreover, serum samples from PE women inhibited tubule formation reflecting the anti-angiogenic state of the disorder (VIRTANEN et al., 2016). Our group showed reduced NO levels in ECs treated with PE plasma when compared with plasma from healthy pregnant women (CALDEIRA-DIAS et al., 2021). Nevertheless, none of these studies considered shear stress to evaluate EC function in the PE context. The only study was limited to evaluating NO production, prostacyclin, and endothelin secretion in the cell culture supernatant exposed to LSS (BAKER et al., 1996). Additional research will be imperative into these parameters, thereby advancing our understanding of endothelial dysfunction in PE and suggesting potential pharmacological interventions.

Preeclampsia (PE), one of the main multifaceted pregnancy-related disorders, poses a significant challenge to maternal and fetal health worldwide (ACOG, 2020). Defined as the onset of hypertension in the 20th week of gestation, this condition strikes expectant mothers without warning and remains one of the leading causes of maternal mortality and morbidity globally (ACOG, 2020). Affecting approximately 5-7% of pregnancies, PE has far-reaching consequences, not only impacting the health of the mother but also posing risks to the developing fetus (ACOG, 2020; RANA et al., 2019). PE is associated with circulation factors released from the placenta into the maternal systemic circulation accompanied by great endothelial dysfunction that disrupts both the

production and breakdown of NO, resulting in a reduction of NO bioavailability (DAVIDGE; STRANKO; ROBERTS, 1996; REDMAN; SARGENT, 2005; SANDRIM et al., 2008; SELIGMAN et al., 1994). NO is an endothelium-derived relaxing factor, that exerts its action by diffusing through smooth muscle cells and activating the cyclic GMP-dependent pathway (LOWE, 2000). This molecule is produced from L-arginine by three calcium-dependent enzyme isoforms known as nitric oxide synthase (NOS), with endothelial eNOS being the most important in this context (FÖRSTERMANN; SESSA, 2012).

Nebivolol is a third-generation β1-antagonist with vasodilating properties (GAO et al., 1991). The drug is a racemic mixture of two isomers, D-nebivolol an antagonist of β1 receptors in the conductive tissue of the heart along the myocytes; while L-nebivolol, due to its agonist effect on β3-adrenergic receptors, can increase the bioavailability of NO in the endothelium (EVANGELISTA et al., 2007; IGNARRO, 2008). Our group showed for the first time that ECs exposed to plasma from PE pregnant women demonstrated higher NO levels when incubated with nebivolol (BUENO-PEREIRA et al., 2022). Also, nebivolol decreases reactive oxygen species (ROS) and superoxide concentration in ECs exposed to an oxidative stress environment (PASINI et al., 2005). In this context, nebivolol exhibits promising pharmacological attributes associated with the NO pathway, suggesting its potential effects in addressing the repercussions of PE.

Therefore, this study aimed to elucidate the underlying NO-dependent pathway in ECs exposed to shear stress and PE plasma. Additionally, evaluate ECs adhesion markers and antioxidant capacity. Through the integration of these methodologies, our main objective is to attain a comprehensive understanding of PE repercussions, while concurrently exploring the feasibility of nebivolol potential pharmacological intervention.

2. Material and methods

2.1. Study Population

This study was approved by the Research Ethics Committee of the Faculty of Medicine of Botucatu, Sao Paulo – UNESP (reference 4.961.945/ approved on 09 September 2021). The approval follows the Helsinki Declaration, which outlines ethical principles for medical research involving human subjects, including research on identifiable human material and data (WORLD MEDICAL ASSOCIATION, 2013). Accordingly, 10 plasma samples were collected from healthy pregnant women, and 10

plasma samples were selected from pregnant women with PE (with maternal ages of 24.8±2.0 years and 24.7±0.7 years, respectively). The gestational age at the time of sampling was 32.9±1.4 weeks for healthy pregnant women and 33.8±1.2 weeks for those with PE, following the diagnostic criteria established by the American College of Obstetricians and Gynecologists (ACOG, 2020). Inclusion criteria for the PE group required pregnant women with single pregnancies, gestational age between 30 and 34 weeks, and the absence of any other clinical or obstetric pathologies. For the HP group, the inclusion criteria comprised women with single pregnancies, gestational age between 30 and 34 weeks, no diagnosis of PE, and no clinical or obstetric pathologies. Both PE and HP women were excluded from the study if they presented any obstetric or clinical complications, such as twin pregnancies, previous PE, chronic arterial hypertension, diabetes, kidney diseases, infectious diseases, fetal malformation, seropositivity for human immunodeficiency virus (HIV), or reported drug and alcohol use. All pregnant women participating in the study signed an informed consent form.

Next, for blood collection, peripheral blood was drawn from pregnant women (20 mL) via venipuncture and placed into sterile tubes containing 158 units of Sodium Heparin (BD Vacutainer, Franklin Lakes, USA). The tubes were used to store plasma samples from both PE pregnant women at the time of disease diagnosis and HP women matched by gestational age. Subsequently, the peripheral blood samples were centrifuged to separate the plasma and serum, which were then stored at -80 °C until incubation with ECs.

2.2. Cell culture

Human umbilical vein ECs (HUVEC-EA.hy 926) obtained from the Rio de Janeiro cell bank (BCRJ, Brazil) were cultured until they reached 70-80% confluence. The cells were then treated with supplemented culture medium DMEM (Gibco, Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mmol/L L-Glutamine, 4500mg/L glucose, 1 mM sodium pyruvate, 1500mg/L sodium bicarbonate (Sigma-Aldrich, Saint Louis, MO, USA), and maintained at 37 °C and 5% CO2. For subsequent experiments, ECs were seeded based on the specified cell number for each assay. Subsequently, HUVECs were incubated with or without 10 µM of nebivolol (Sigma-Aldrich, Saint Louis, MO, USA) and a 10% (v/v) pool of plasma for 4 hours.

2.3. Shear Stress Model

ECs were cultured in the periphery of modified 100mm culture dishes that were made by bonding the bottoms of 60mm culture dishes upside down into the center of 100mm culture dishes using transparent adhesive silicone (TekBond-Saint Gobain, Embu das Artes, Brazil). Dishes were sterilized under UV light for 15 minutes. Confluent monolayers were subjected to orbital shear stress for 72 hours at 37˚C in the presence of CO2 using an orbital shaker (AO-330D, Gehaka, Sao Paulo-SP, Brazil) positioned inside an incubator as previously described (LEY et al., 1989). The cells are constrained to the periphery of the modified Petri dishes, and the shear stress is estimated to match the maximum shear stress experienced by the vessel wall, following the formula:

$$t(r) = r \sqrt{pn(2\pi f)3}$$

where 'r' represents the rotor radius (orbital diameter in mm), 'p' denotes the media density in g/mL, 'n' stands for the media viscosity in dynas s/cm2, 'f' is the rotation frequency in rpm, and 't' indicates the shear stress in dynas. Based on the shaker specifications, we reached a shear stress t(r)= 8,28 dynas s/cm2 at a rotating frequency of 200 rpm, which is within the range of physiological arterial shear stress (6–40 dynes/cm2) (DELA PAZ et al., 2012). ECs from the same passage, which were not subjected to shear stress, were kept in the same incubator and served as the static control. The alignment of ECs was visualized using a Carl Zeiss Axiovert 100 Inverted Microscope. Supplementary Figure S1 shows the validation of this method.

2.4. Western Blot

After appropriate treatment, ECs were washed in PBS, and protein extracts were performed using lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1 % NP-40, and 1 mM EDTA), supplemented with protease and phosphatase inhibitor plus sodium orthovanadate (Sigma-Aldrich, Missouri, EUA). After, the sample was subjected to one pulse of sonication for 5 seconds, followed by a 30-minute incubation on ice. Next, give a nitrogen shock and let thaw on ice slowly, then centrifuge the samples at 10000xg, 4ºC for 10 minutes. Protein concentration was determined using the Bradford method after clearing by centrifugation (KRUGER, 1994). An equal volume of 4x LDS sample buffer (Thermo Fisher Scientific, Massachusetts, EUA) and 10x Bond-Breaker TCEP solution (Thermo Fisher Scientific, Massachusetts, EUA) were added to 30 µg samples and boiled for 10 min at 70ºC. Aliquots of the samples were loaded into polyacrylamide gel

electrophoresis (NuPage 4-12% gel) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA), which were blocked with 5% nonfat dry milk dissolved in tris-buffered saline (TBS)-Tween-20 (0.05%). Afterward, the samples were incubated overnight at 4°C with either 5% bovine serum albumin (BSA) containing t-eNOS, peNOS (Ser1177) primary antibody (Cell Signalling Technology, Massachusetts, USA) at a 1:500 dilution, or with a 3% nonfat dry milk containing a 1:3,000 dilution of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as positive control (Cell Signalling Technology, Massachusetts, USA). After washing in TBST, membranes were incubated with proper Anti-rabbit IgG, HRP-linked secondary antibody (Cell Signalling Technology, Massachusetts, EUA), at 1:200,000 dilutions, in 3% nonfat dry milk overnight. The immunoreactive bands were visualized using the Luminol SuperSignal West Femto Maximum Sensitivity Substrate reagent (Pierce Biotechnology, Massachusetts, USA) in a G-BOX photodocumenting system (Syngene International Limited). All analyses were conducted using ImageJ software (NIH, National Institutes of Health).

2.5. Measurement of nitrite/nitrate oxide (NOx)

NOx was assessed using Griess reagents, as previously described (GRANGER et al., 1996). In brief, a 50 µL aliquot of cell culture supernatant was incubated for 3 hours at 37°C in a microplate with 50 µL of sulfanilamide (2%), 50 µL of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NED), and 100 µL of vanadium chloride III (5%) solution in the absence of light for 3 hours. A calibration curve (100-1,56 µmol/L) was generated by incubating prepared nitrite solutions with deionized water and Griess reagents. The absorbance was then measured at 535 nm using a spectrophotometer (SYNERGY 4, BIOTEK, Winooski, VT, USA). Values were represented as a percentage regarding control.

2.6. Flow cytometry

Fluorescence of 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA, reactive oxygen species (ROS) indicator) (Cayman Chemical Company, Michigan, EUA), CD54 (ICAM, intracellular adhesion molecule 1), CD106 (VCAM, vascular cell adhesion molecule 1) and CD105 [Endoglin (ENG)] (BD Bioscience, New York City, EUA) were evaluated soon after cells collection. The cell concentration was adjusted to $1,5x10^5$ cells/ml and distributed in Falcon cytometer tubes (BD Bioscience, New York City,

EUA). The cells were incubated with antibodies, with respective fluorochromes: DCFH (FITC, fluorescein isothiocyanate) (15 minutes), anti-CD54 (BB515), anti-CD106 [peridinin chlorophyll protein (PerCP)-Cy5.5] and anti-CD105 [allophycocyanin (APC)] 20 minutes in the dark at room temperature. After centrifugation, the cells were washed with wash buffer (BD Biosciences, New York City, EUA) and centrifuged again at 1500 rpm for 5 min. For the delineation of the gates, control tubes were incubated with specific isotype antibodies for each fluorochrome (FITC, BB515, PerCP-Cy5.5, and APC) in each test performed. Regarding the measurements, 20 000 events for each sample were required on a FACSCantoTM II flow cytometer (BD Biosciences, New York City, EUA) with FACSDIVA software (BD Biosciences, New York City, EUA). The results were analyzed in the software FLOWJO, version vX.10.6 (FlowJo, LLC, Ashland, OR). Analyses of the fluorescence intensity were represented as median fluorescence intensity (MFI).

2.7. Total Antioxidant Capacity

The assessment of total antioxidant capacity was conducted utilizing the iron reduction assay, specifically the Ferric Reducing Antioxidant Power (FRAP) method. This approach hinges on the swift reduction of iron within ferric tripyridyl triazine (FeIII-TPTZ) by the antioxidants inherent in the samples. This reduction leads to the formation of ferrous tripyridyl triazine (FeII-TPTZa), characterized by its intense blue color (BENZIE; STRAIN, 1996).

In summary, a working reagent was performed, comprising 300 mmol/L acetate buffer, 10 mmol/L TPTZ/HCl solution, and 20 mmol/L ferric chloride. Within a 96-well plate, 10 µL of the sample was combined with 290 µL of the prepared working solution. A calibration curve employing ferrous sulfate, ranging from 0.0312 to 1 mmol, was established. Following a 5-minute incubation period, the spectrophotometer (Synergy 4, Biotek) was employed to measure absorbance at 593 nm. The resulting data are presented in µmol/L units.

2.8. Statistical analysis

To compare the two groups, we performed multiple t-tests. When comparing three or more groups we used one-way ANOVA followed by the Bonferroni multiple comparisons test. Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism for Windows, version 6.01; (GraphPad, San Diego, CA).

3. Results

Nebivolol activates eNOS via phosphorylation at Ser1177

To assess the effect of nebivolol in the eNOS pathway, ECs exposed to shear stress were incubated with the drug and compared to a static group of ECs. Our results showed a higher peNOS(Ser1177)/t-eNOS ratio in the nebivolol-treated ECs (0.99 arbitrary unit) compared to the control ECs (0.79 arbitrary unit) (Figure 1A, B). To further explore the impact of plasma, we exposed ECs to plasma from both HP and PE groups. Surprisingly, the analysis revealed a significantly higher peNOS(Ser1177)/t-eNOS ratio in the PE-treated ECs (0.20 arbitrary unit) compared to the HP-treated cells (Figure 1A, B). Finally, the PE-treated ECs were exposed to nebivolol, resulting in further elevation of the peNOS(Ser1177)/t-eNOS ratio (0.46 arbitrary unit) compared to ECs treated with plasma alone (Figure 1A, B). The peNOS(Ser1177)/GAPDH ratio, with GAPDH used as a positive control, was assessed, and similar results, as described above, were obtained (Figure 1C).

In the same set of experiments, we evaluate t-eNOS/GAPDH ratio and we detected a difference between the control group and ECs nebivolol- treated (Figure 1A, C). Curiously, HP-treated cells showed higher levels of t-eNOS/GAPDH ratio when compared with PE-treated cells (Figure 1A, C). Likewise, PE-treated combined with nebivolol demonstrated increased levels of t-eNOS/GAPDH ratio when compared with ECs treated with plasma alone (Figure 1A, C).

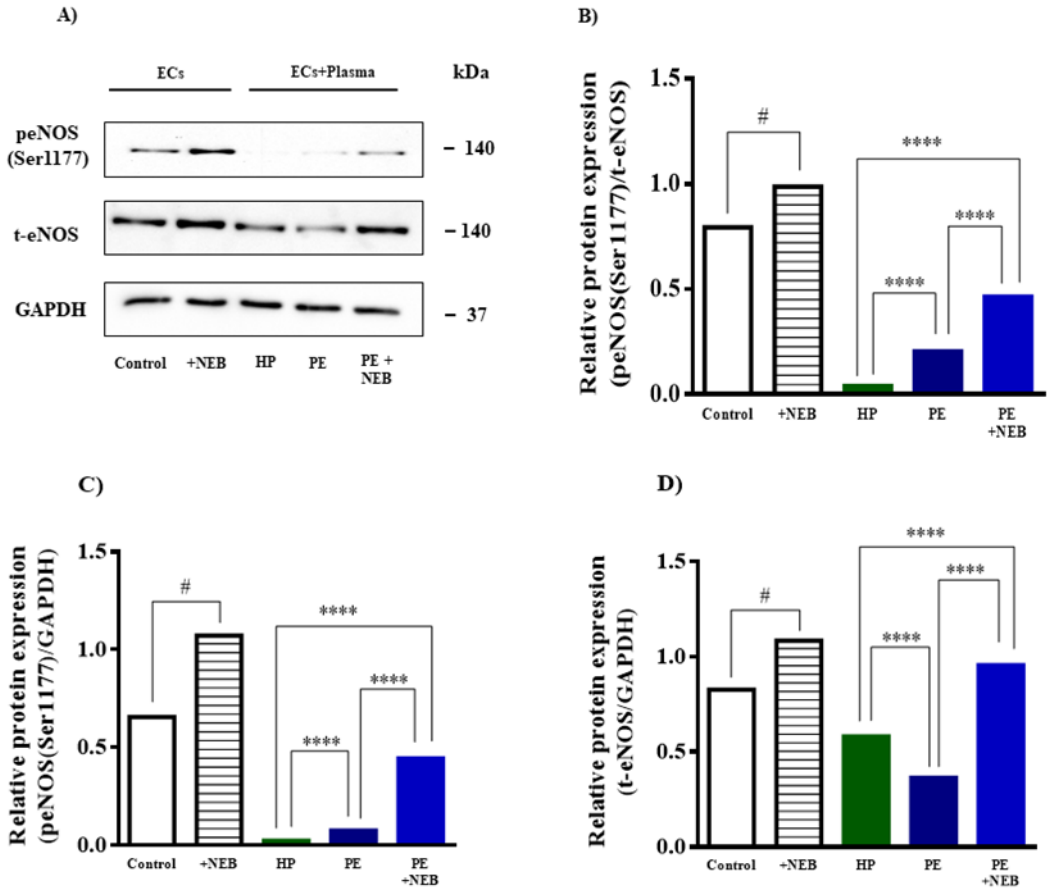

Figure 1. Representative western blots showing eNOS phosphorylation at Ser1177 (top blot), total eNOS (top blot), and GAPDH (bottom blot; loading control) in ECs under various conditions: control (blank), nebivolol-treated (hatched), incubated with plasma from healthy pregnant women (HP, green), (PE, navy blue) or with (PE+NEB, medium blue) nebivolol (**A**, **B**, **C** and **D**). The images of blots were processed by adjusting brightness and contrast, and these adjustments were uniformly applied across the entire image and control samples. Comparisons between the groups were performed by One-Way ANOVA followed by Bonferroni multiple tests. #$p<0.0001$ represents the statistical difference between the control and nebivolol-treated. ****$p<0.0001$ represents the statistical difference between HP, PE, and PE+NEB groups.

Nebivolol treatment enhances total NOx levels

To evaluate the role of nebivolol in total NOx levels, ECs exposed to shear stress were incubated with the drug. The results demonstrated that ECs nebivolol-treated exhibited higher levels of supernatant total NOx compared to the control (Supplementary Figure S2).

Additionally, PE-treated cells present lower NOx levels when compared with the HP group (Figure 2A). However, nebivolol increased these levels when compared with PE plasma alone (Figure 2B).

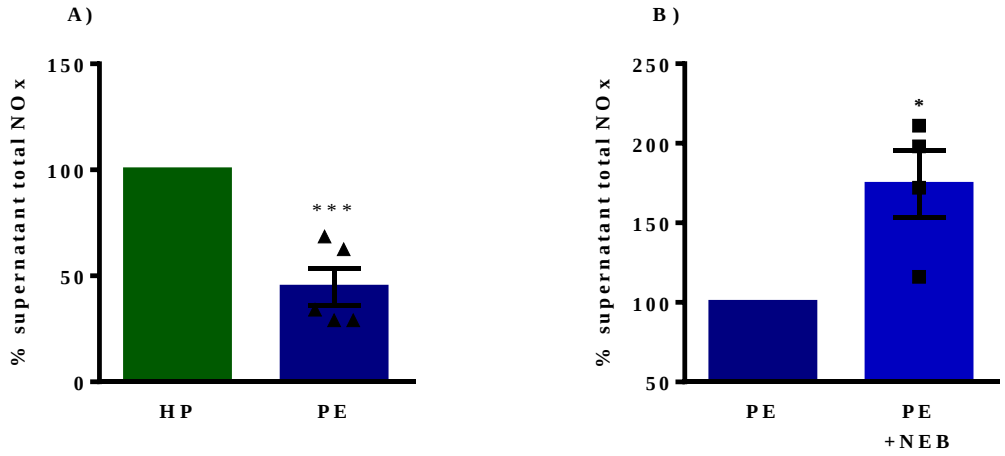

Figure 2. The levels of nitrite/nitrate oxide (NOx) in ECs treated with (A) HP plasma and PE plasma (B) PE plasma, with or without nebivolol. Data were measured as percentages relative to the HP group (A) and the PE group (control set as 100%) (B). Comparisons between the groups were performed by t test. Data are presented as mean ± SEM. *p<0.05 and ***p<0.0001 indicates a statistically significant difference in NOx levels.

PE plasma reduces ROS levels, while nebivolol elevates

Control and HP-treated ECs elevated total ROS levels when compared with PE-treated ECs (Figure 3). Similarly, nebivolol treatment increases these levels when compared with ECs exposed only to PE plasma (Figure 3).

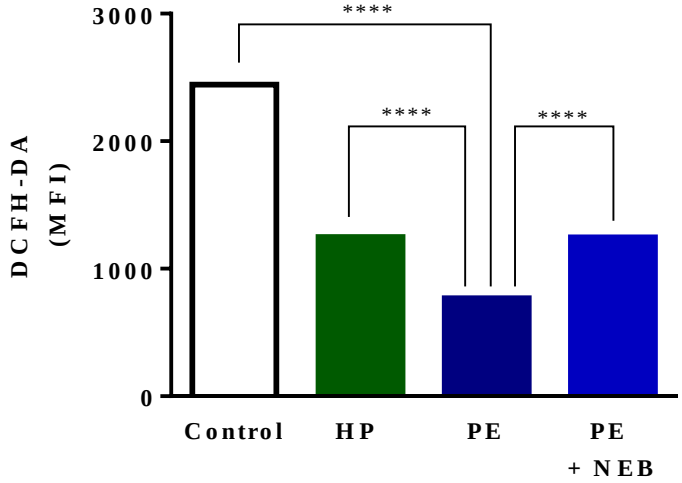

Figure 3. Median fluorescence of intensity (MFI) of DCFH-DA incubation for 15 minutes in ECs under various conditions: control (blank), incubated with plasma from healthy pregnant women (HP, green), and preeclamptic plasma without (PE, navy blue) or with

(PE+NEB, medium blue) nebivolol (A). Comparisons between the groups were performed by One-way ANOVA followed by Bonferroni multiple tests. ****p<0.0001 vs PE group.

Nebivolol treatment slightly upregulated endothelial cell adhesion markers

The control group showed reduced ICAM-1/CD54 expression when compared with PE-treated ECs (Figure 4). Conversely, ECs exposed to HP plasma showed higher ICAM-1/CD54 expression compared with the same group (Figure 4). In comparison to PE-treated ECs, both control and HP-treated ECs exhibited elevated VCAM-1/CD106 expression (Figure 5). Subsequently, the control group showed reduced ENG/CD105 expression when compared with PE-treated ECs (Figure 6). Similarly, ECs exposed to HP plasma showed lower ENG/CD105 expression compared with the same group (Figure 6).

Curiously, nebivolol treatment slightly enhanced ICAM-1/CD54, VCAM-1/CD106, and ENG/CD105 expression when compared with ECs incubated solely with PE plasma (Figures 4, 5, and 6).

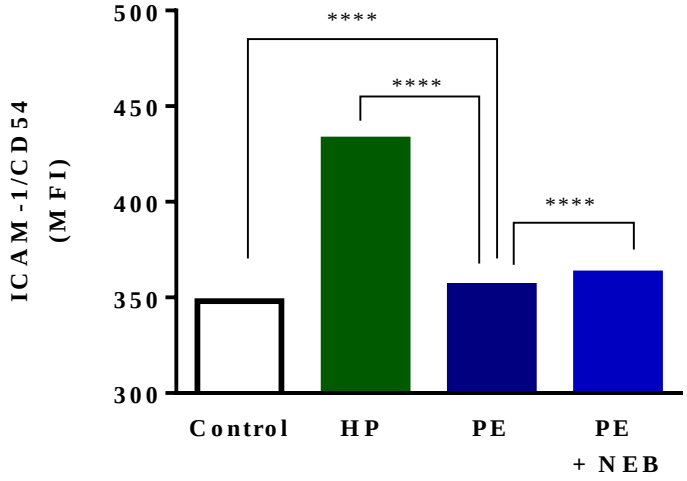

Figure 4. Median fluorescence of intensity (MFI) of ICAM-1/CD54 antibody incubation for 20 minutes in ECs under various conditions: control (blank), incubated with plasma from healthy pregnant women (HP, green) and preeclamptic plasma without (PE, navy blue) or with (PE+NEB, medium blue) nebivolol. Comparisons between the groups were performed by One-way ANOVA followed by Bonferroni multiple tests. **p<0.001 and ****p<0.0001 vs PE group.

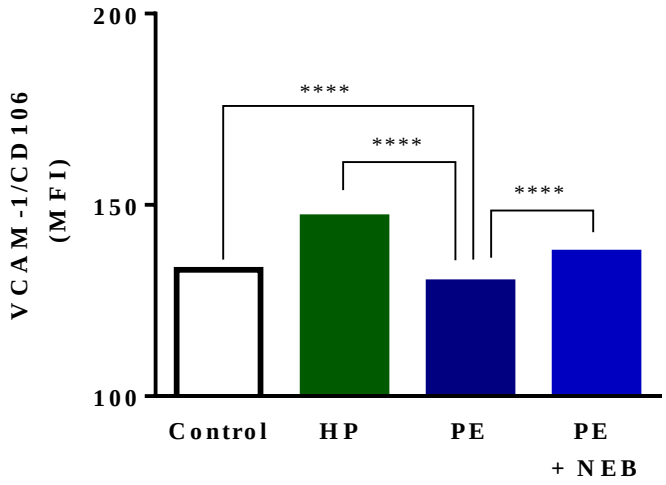

Figure 5. Median fluorescence of intensity (MFI) of VCAM-1/CD106 antibody incubation for 20 minutes in ECs under various conditions: control (blank), incubated with plasma from healthy pregnant women (HP, green), and preeclamptic plasma without (PE, navy blue) or with (PE+NEB, medium blue) nebivolol. Comparisons between the groups were performed by One-way ANOVA followed by Bonferroni multiple tests. *p<0.05, ***p<0.0001 and ****p<0.0001 vs PE group.

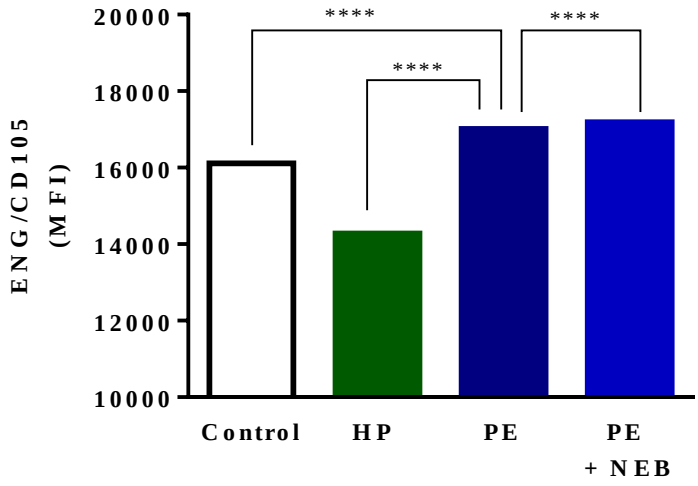

Figure 6. Median fluorescence of intensity (MFI) of ENG/CD105 antibody incubation for 20 minutes in ECs under various conditions: control (blank), incubated with plasma from healthy pregnant women (HP, green), and preeclamptic plasma without (PE, navy blue) or with (PE+NEB, medium blue) nebivolol. Comparisons between the groups were performed by One-way ANOVA followed by Bonferroni multiple tests. ****p<0.0001 vs PE group.

PE plasma showed a higher antioxidant capacity, while nebivolol decrease these levels

ECs treated with PE plasma showed higher antioxidant capacity when compared with HP-treated ECs and control (Figure 7). In contrast, nebivolol treatment in the same group reduces this capacity (Figure 7).

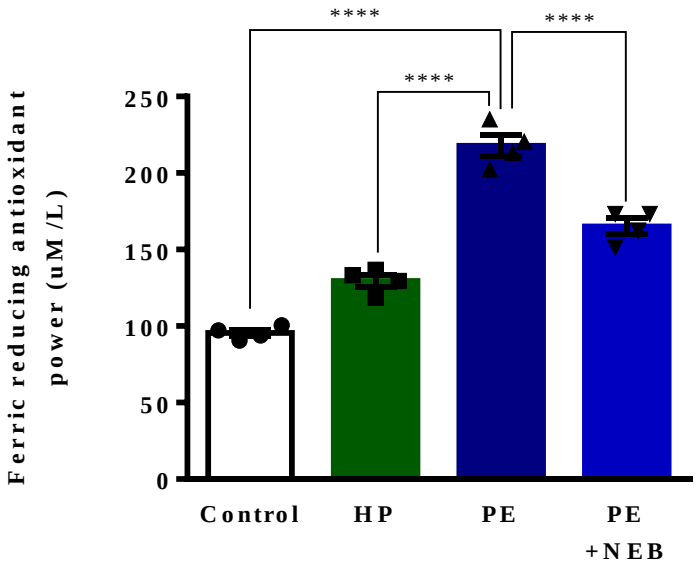

Figure 7. Ferric reducing antioxidant power assessed by FRAP. ECs under various conditions: control (blank), incubated with plasma from healthy pregnant women (HP, green), and preeclamptic plasma with (PE+NEB, medium blue) or without (PE, navy blue) nebivolol. Data are presented as mean ± SEM. Comparisons between the groups were performed by One-way ANOVA followed by Bonferroni multiple tests. ****$p<0.0001$ vs PE group.

4. Discussion

This study presents the initial evidence that under shear stress, ECs treated with PE plasma exhibit elevated phosphorylation of eNOS at residue Ser1177. Furthermore, PE-treated ECs demonstrate increased total antioxidant capacity and reduced total ROS in comparison to HP-treated ECs. Additionally, PE plasma decreases the expression of ICAM-1 and VCAM-1 glycoproteins. Curiously, nebivolol intervention influences the NO pathway and total ROS release, while also upregulating endothelial cell adhesion markers. These findings collectively underscore the potential of PE plasma to effectively modulate the NO pathway, impacting eNOS phosphorylation and NO metabolite release, while highlighting nebivolol as a pharmacological promise intervention with synergistic enhancements.

It is well-established that the application of LSS in ECs promotes activation of the phosphatidylinositol3-kinase (PI3K) and the subsequent activation of the serine kinases, which phosphorylate eNOS on Ser1177 and increases eNOS activity (DIMMELER et al., 1999; FISSLTHALER et al., 2000; GO et al., 1998). Our data corroborate these previous findings, as we demonstrated the presence of phosphorylation at residue Ser1177 in ECs exposed to LSS compared to static ECs. In the context of eNOS expression in PE samples, there remains a consistent observation: eNOS expression and activity are consistently elevated in the placental vascular endothelium of women affected by PE when compared to healthy pregnant patients (MYATT et al., 1997; SHAAMASH et al., 2001). Also, bovine microvascular ECs have shown an increase in eNOS expression when treated with PE plasma (BAKER; DAVIDGE; ROBERTS, 1995; DAVIDGE; BAKER; ROBERTS, 1995). Only one study, applying shear stress, demonstrated that ECs exposed to both PE and HP plasma showed similar supernatant NOx levels, suggesting no significant variations (BAKER et al., 1996). Our findings show that ECs treated with PE plasma increase eNOS phosphorylation at residue Ser1177 and decrease NOx levels when compared with the HP group. These findings suggest that PE plasma exerts a regulatory influence on eNOS activation; however, when assessing NO levels (NOx) it seems to exhibit a less pronounced impact compared to HP plasma.

Oxidative stress is characterized as a disparity between oxidants and antioxidants, resulting in the disturbance of redox signaling and regulation, along with potential molecular damage (SIES, 2015). This phenomenon involves ROS and reactive nitrogen species (RNS) production, the most common molecules being superoxide, hydrogen peroxide, NO, and peroxynitrite (AOUACHE et al., 2018). In PE, a decrease in NO bioavailability by scavenging ROS impairs endothelium-dependent vasodilation, establishing a greater endothelial dysfunction (LOWE, 2000). Nevertheless, our previous studies have demonstrated that exposure of static ECs to PE plasma did not induce effects in ROS parameters. Conversely, when subjected to HP plasma, elevated levels of ROS were observed (CALDEIRA-DIAS et al., 2019, 2021; NUNES et al., 2022). In the present data, we demonstrate lower levels of total ROS in ECs exposed to shear stress and incubated with PE plasma when compared with the HP group. Interestingly, this outcome is correlated with an elevated antioxidant capacity observed in the supernatant of the PE group. This finding implies a compensatory mechanism, wherein ECs enhance their antioxidant capacity to counteract heightened levels of ROS. Also, LSS plays a protective effect under endothelial dysfunction provided by PE plasma.

The ROS production also modulates other molecules, such as ICAM-1/CD54 and VCAM-1/CD106 (ZHANG et al., 2009). The intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) serve as cell surface glycoproteins and adhesion receptors, known for several actions, such as their role in leukocyte recruitment to inflamed sites and modulating the barrier function of ECs (OPPENHEIMER-MARKS et al., 1991; OSBORN et al., 1989; TRIET M. BUI, HANNAH L. WIESOLEK, 2014). Within the context of endothelial dysfunction inherent to the pathophysiology of PE, levels of soluble ICAM-1 and VCAM-1 exhibit elevation in PE serum samples as compared to HP plasma (AUSTGULEN et al., 1997; KIM et al., 2004). Furthermore, *in vitro* studies showed higher levels of ICAM-1 and VCAM-1 in static ECs exposed to PE plasma when compared to the HP group (ENDRESEN et al., 1998; HALLER et al., 1997; ZENG; FAN; JIANG, 2000). However, our present data demonstrate lower expression of ICAM-1 and VCAM-1 in the PE group when compared with ECs exposed to HP plasma. These findings can be elucidated by suggesting a correlation between the levels of ROS and the expression of ICAM-1 and VCAM-1, both regulated by ROS-dependent mechanisms (CHEN et al., 2008). Given the decreased ROS levels due to an enhanced antioxidant capacity in the PE group, these glycoproteins are consequently downregulated. Another hypothesis involves an impairment of the endothelial barrier in ECs exposed to PE plasma, which may contribute to the downregulation of these receptors.

The Endoglin (ENG)/CD105 is a type I transmembrane glycoprotein predominantly expressed in ECs playing an important role as a receptor for the cytokines transforming growth factor-beta 1 and 3 (TGF-β1 and TGF-β3), which are related to cell proliferation and apoptosis (NASSIRI et al., 2011; ST-JACQUES et al., 1994). Elevated levels of both membrane and soluble ENG (sENG) have been observed in cases of PE and have garnered attention as a potential diagnostic tool for identifying the syndrome (NIKUEI et al., 2017; VENKATESHA et al., 2006). Our results showed, for the first time that ECs exposed to PE plasma present higher membrane ENG expression when compared with the HP group. However, further investigation into the levels of sENG, TGF-β1, and TGF-β3 is warranted within this model, as these molecules play a crucial role in the repercussions of PE and are connected to glycoprotein ENG.

The utilization of third-generation β-antagonists, which possess vasodilating properties, in the treatment of diseases associated with endothelial dysfunction holds promise as a pharmacological intervention (GOLDSTEIN, 1997; PELLER et al., 2015).

Nebivolol presents vasodilatory properties that are endothelium-dependent and are associated with the activation of eNOS at Ser1177 residue (BOWMAN; CHEN; FORD, 1994; GAO et al., 1991; LADAGE et al., 2006). In the current study, ECs exposed to nebivolol exhibit greater eNOS activation and consequently NOx release in comparison to ECs without the drug. In this context, vasodilatory pathways are being proposed, with the most significant one in this scenario being the β3-adrenoreceptor pathway (DESSY et al., 2005). In light of this, our research group has pioneered the demonstration that static ECs exposed to PE plasma and nebivolol exhibit elevated levels of NO, mediated through β3 adrenergic receptors, in comparison to the PE group without the drug (BUENO-PEREIRA et al., 2022). Under these circumstances, when considering LSS, nebivolol-induced eNOS activation was observed and consequently increased NOx release. These findings suggest that even under an endothelial dysfunction environment present in PE, nebivolol acts as a beneficial treatment eNOS-related pathway.

Additionally, the role of nebivolol in the modulation of oxidative stress and antioxidant capacity is currently under discussion. Clinical trials involving patients with hypertension treated with nebivolol present slightly reduced parameters associated with oxidative stress – ROS –, as well as either no change or even diminished antioxidant capacity when compared to the healthy control group (BARROSO et al., 2022; PETER et al., 2006; ZEPEDA et al., 2012). Furthermore, hypertension *in vitro* studies showed a decrease in oxidative stress by nebivolol, however, none of these studies considered shear stress (COMINACINI et al., 2003; FRATTA PASINI et al., 2005; MASON et al., 2005). Our present study demonstrated for the first time that nebivolol increases ROS levels and decreases antioxidant capacity when compared to ECs treated only with PE plasma. A potential explanation for these findings involves the hypothesis that (i) nebivolol increases NOx; when these molecules encounter an oxidative stress environment induced by PE plasma, they react with it, subsequently leading to an elevation in ROS. This phenomenon is attributed to a decreased antioxidant capacity by the drug (ii) Employing shear stress as a mechanical force in conjunction with PE plasma as an endothelial activator already modulates ROS release and antioxidant capacity. Consequently, the concentration of the drug is insufficient to alter these parameters. Although, further studies are needed to clarify the role of nebivolol in PE oxidative stress.

Also, the effects of nebivolol were investigated on ROS-dependent molecules, such as ICAM-1 and VCAM-1. The lack of *in vitro* studies related to these markers and nebivolol is a fact. Only one study investigated the RNA expression of ICAM-1 in static

ECs exposed to oxidative stress and treated with nebivolol, leading to the conclusion that the drug decreased these levels when compared to the control (GARBIN et al., 2008). The present data showed that nebivolol increases ICAM-1 and VCAM-1 protein levels when compared with to ECs treated only with PE plasma. Considering the increased ROS levels resulting from reduced antioxidant capacity in the ECs treated with both PE plasma and nebivolol, these glycoproteins are subsequently upregulated. Furthermore, nebivolol might be inducing the elevation of ICAM-1 and VCAM-1 as a response to the endothelial barrier impairment present in the PE group. Finally, nebivolol treatment slightly upregulated ENG expression, suggesting proliferation-dependent and apoptosis mechanisms.

In summary, we demonstrate for the first time that under shear stress, ECs treated with PE plasma exhibit elevated phosphorylation of eNOS at residue Ser1177. Also, PE plasma decreases the expression of ICAM-1 and VCAM-1 in a ROS-dependent manner. Additionally, nebivolol intervention influences the NO pathway and total ROS release, while also upregulating adhesion molecules. Together, these findings suggest a deeper investigation into the PE plasma content and how the present molecules shed light on the intricate signaling pathways involved in the endothelial response. Furthermore, the intervention of nebivolol introduces an intriguing dimension to this study. By influencing the NO pathway and total ROS release, the impact of the drug on endothelial function becomes apparent. Moreover, its ability to upregulate adhesion molecules suggests a dynamic interplay between pharmacological intervention and the intricate web of molecular pathways governing endothelial behavior. These discoveries may lay the foundation for future investigations and the development of innovative therapeutic approaches, particularly for conditions marked by endothelial dysfunction, such as PE.

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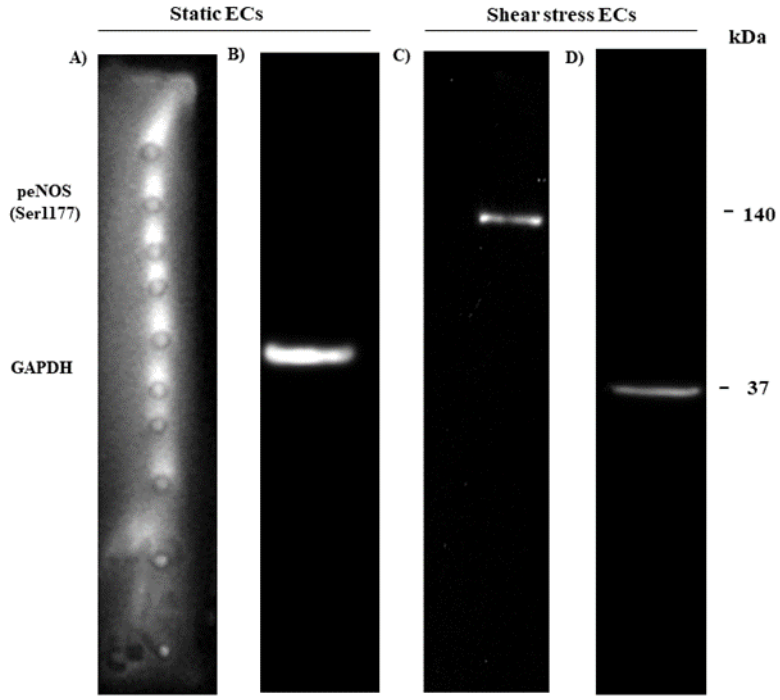
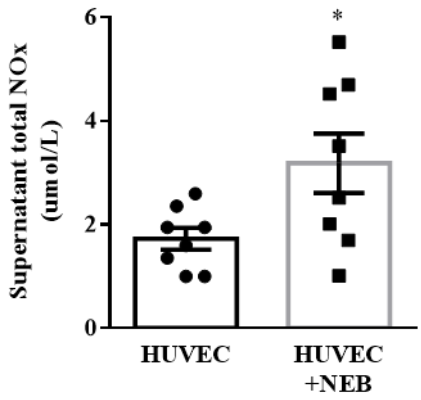
Supplementary Material

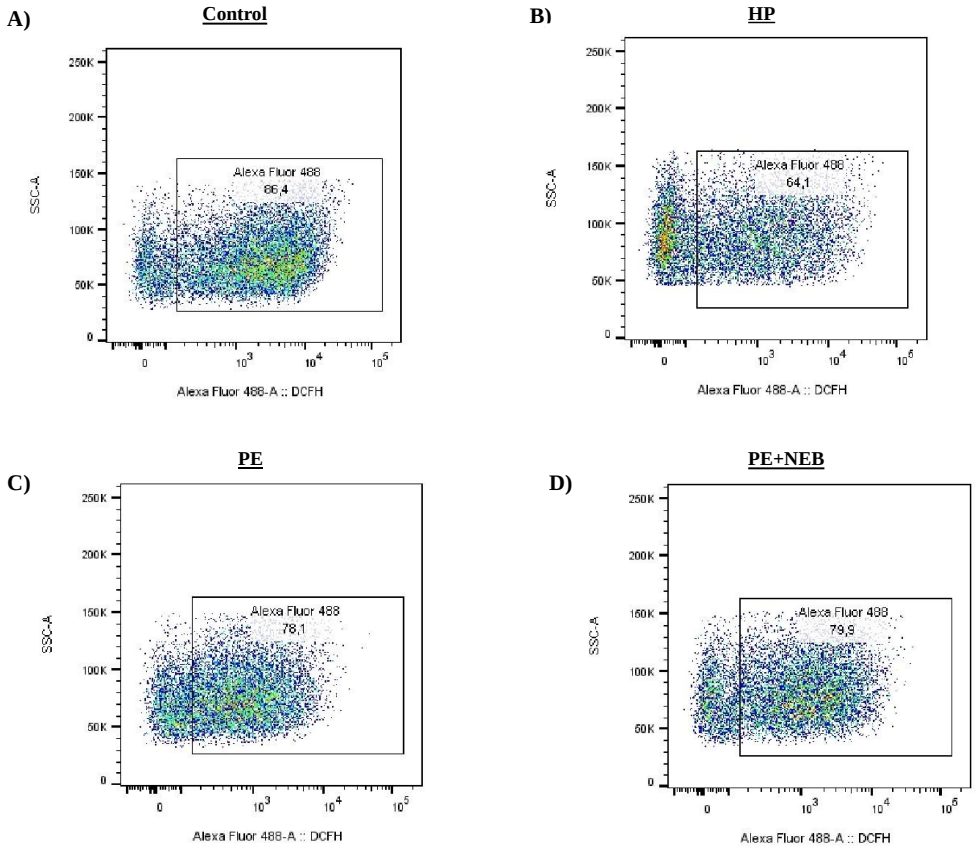
Supplementary Figure S1. Representative western blot showing eNOS phosphorylation at Ser1177 (140kDa) and GAPDH (37 kDa) in static ECs (A, B) and ECs submitted to shear stress (C, D) with no treatment for 4 hours.



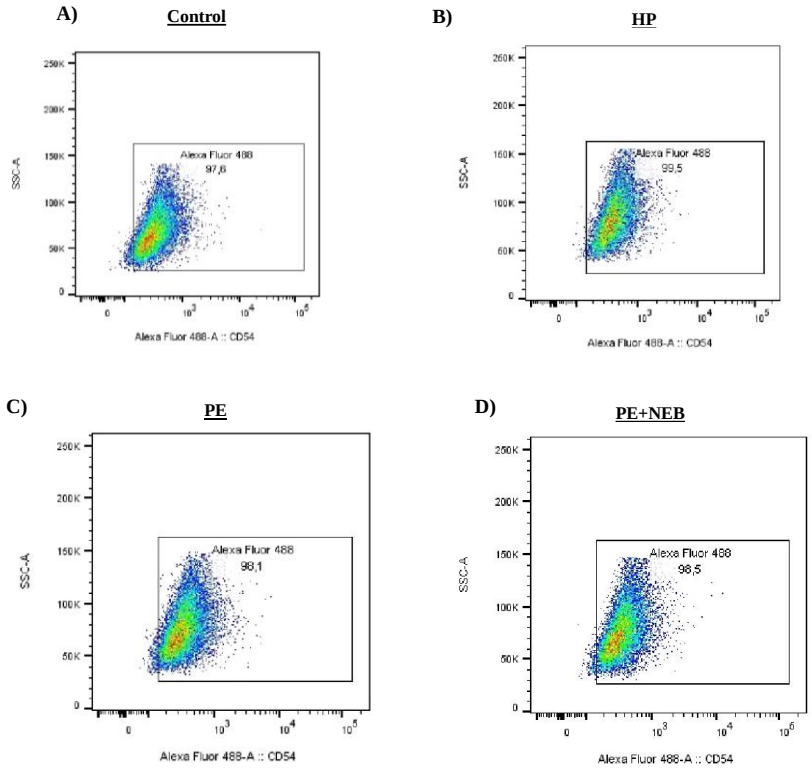
Supplementary Figure S2. The levels of supernatant total nitrite/nitrate oxide (NOx) were measured in ECs with or without a 4-hour incubation of nebivolol. Data were measured as percentages relative to the HUVEC group and the HUVEC+NEB group (control set as 100%). Comparisons between the groups were performed by t test. Data are presented as mean ± SEM. *p<0.05 represents a statistically significant between the NOx levels in HUVEC and nebivolol-treated ECs.



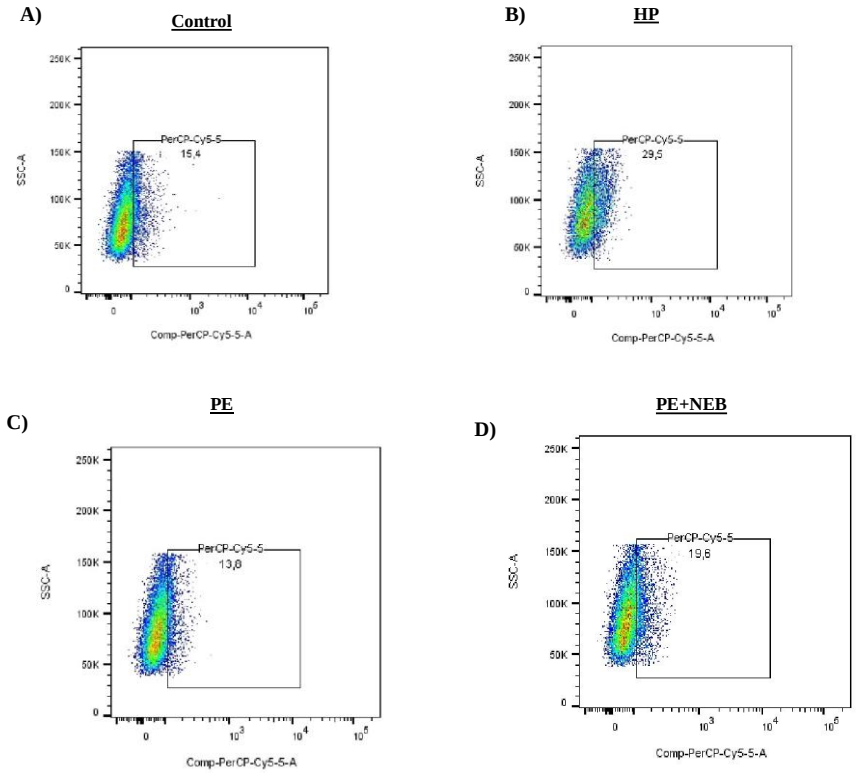
Supplementary Figure S3. The frequency of DCFH-DA fluorescence represented as a percentage in dot plots of Control (86.4%), HP (64.1%), PE (78.1%), and PE+NEB (79.9%) in (A, B, C, and D, respectively).



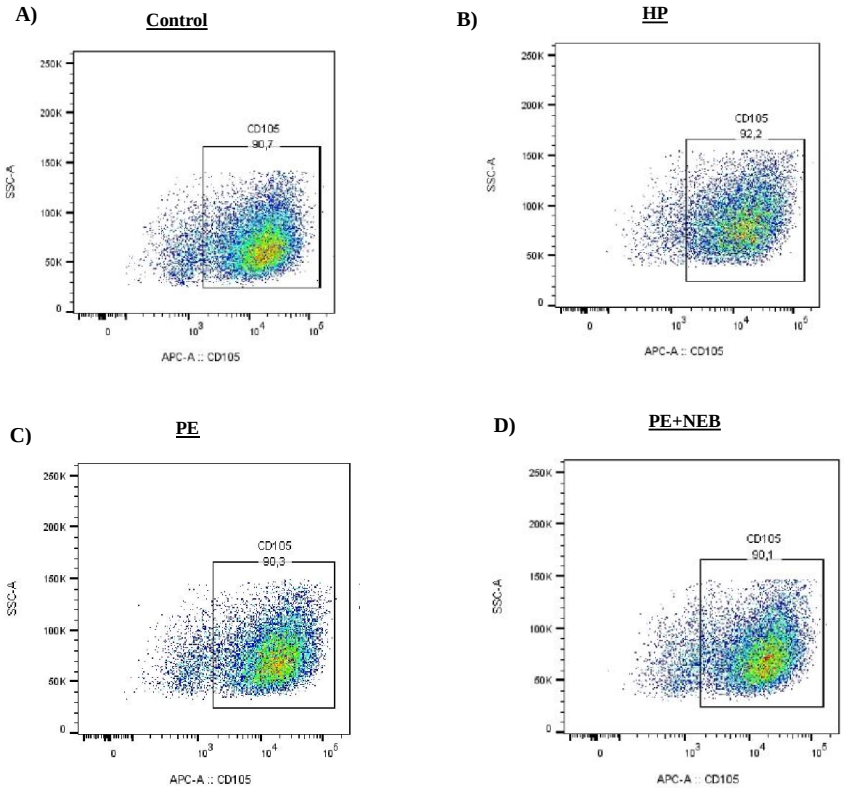
Supplementary Figure S4. The frequency of ICAM-1/CD54 antibody fluorescence represented as a percentage in dot plots of of Control (97.6%), HP (99.5%), PE (98.1%), and PE+NEB (98.5%) in (A, B, C, and D, respectively).



Supplementary Figure S5. The frequency of VCAM-1/CD106 antibody fluorescence represented as a percentage in dot plots Control (15.4%), HP (29.5%), PE (13.8%), and PE+NEB (19.6%) in (A, B, C, and D, respectively).



Supplementary Figure S6. The frequency of ENG/CD105 antibody fluorescence represented as a percentage in dot plots Control (90.7%), HP (92.2%), PE (90.3%), and PE+NEB (90.1%) in (A, B, C, and D, respectively).



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ANEXOS

Disciplinas Cursadas no Programa

Disciplinas Cursadas no Programa	Calendário	Conceito	Frequência	Créditos	Carga Horária
Bases e Atualizações em Farmacologia e Biotecnologia	1º/2021	A	100%	3	45.0
Exploração alternativa de carreiras na ciência	1º/2021	A	100%	1	15.0
Bases e Atualizações em Farmacologia e Biotecnologia	2º/2021	A	100%	3	45.0
Conceitos gerais em Estatística aplicada a modelos biológicos	2º/2021	B	100%	2	30.0
Farmacologia do Endotélio Vascular	1º/2021	A	100%	4	60.0

Disciplinas Aproveitadas

Disciplinas Cursadas no Programa	Calendário	Conceito	Frequência	Créditos	Carga Horária
Pré-eclâmpsia: da bancada ao leito	1/2020	A	100%	4	60.0
Cultura Celular Animal	2/2021	A	97,8%	3	45.0

Artigos Publicados

Bueno-Pereira TO, Nunes PR, Matheus MB, Vieira da Rocha AL, Sandrim VC. Nebivolol Increases Nitric Oxide Synthase via β3 Adrenergic Receptor in Endothelial Cells Following Exposure to Plasma from Preeclamptic Patients. Cells. 2022 Mar 4;11(5):883. doi: 10.3390/cells11050883. PMID: 35269505; PMCID: PMC8909669.

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Participação em eventos científicos

54º Congresso Brasileiro de Farmacologia e Terapêutica Experimental na sessão 14. Pharmacology: Other, o resumo intitulado Nebivolol Mitigates Endothelial Dysfunction in a Model of Preeclampsia.

XI Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC).