UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências de Botucatu

Tese de Doutorado

AVALIAÇÃO DO SISTEMA REDOX PELA VIA DE ATIVAÇÃO DO NRF2 E POTENCIAL EFEITO ANTIOXIDANTE DO RESVERATROL EM MODELO *IN VITRO* DE PRÉ-ECLÂMPSIA

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia e Biotecnologia do Instituto de Biociências de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Doutora em Farmacologia e Biotecnologia.

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Nise da Silveira

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PRÓLOGO

Esta tese será apresentada na forma de uma introdução geral sobre a síndrome gestacional pré-eclâmpsia, o antioxidante resveratrol e seus mecanismos de ação e o potencial da utilização desse antioxidante no tratamento e prevenção da pré-eclâmpsia, seguida por dois capítulos referentes aos manuscritos resultantes do projeto de doutorado.

Sumário

INTRODUÇÃO GERAL

Pré-eclâmpsia

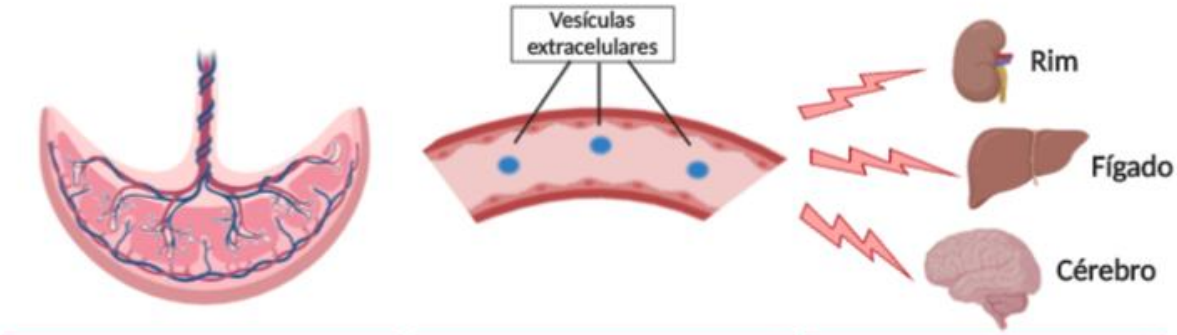
No Brasil e em vários países, a pré-eclâmpsia é uma das principais causas de mortalidade e morbidade entre as gestantes, afetando de 3-8% de todas as gestações (1–3). Ela é definida como um quadro clínico em que, após a 20ª semana de gestação, a gestante apresenta hipertensão, caracterizada por pressão arterial sistólica acima de 140 mmHg e a diastólica acima de 90 mmHg em duas tomadas de pressão, com intervalo de 4 horas, em repouso, acompanhada por proteinúria (0,3 g/L em urina 24 horas) ou presença de outros sintomas como trombocitopenia, disfunção hepática, edema pulmonar e alterações cerebrais/visuais (4). O aumento da pressão sanguínea acompanhado por proteinúria ou outros sintomas pode causar diversas complicações, tanto para a gestante como para o feto. Na gestante, podem ocorrer efeitos deletérios sobre vários sistemas, principalmente o vascular, o hepático, o renal e o cerebral. Já em relação ao feto, este pode ter problemas de crescimento, parto prematuro e morte (1,2).

Atualmente, o tratamento definitivo da pré-eclâmpsia é o parto, que deve ser indicado no momento adequado dependendo de fatores como idade gestacional, gravidade, bem-estar fetal e presença ou não de complicações. Entretanto, a instalação precoce da doença aumenta a chance de prematuridade, e consequentemente, aumento da morbidade e mortalidade perinatal (5). Assim, na tentativa de prevenir tais complicações, várias condutas têm sido propostas quando não é possível ou recomendável interromper a gravidez, como corticoterapia para aceleração da maturidade pulmonar fetal e expansão do volume plasmático; hospitalização com repouso materno; terapia anticonvulsivante com o sulfato de magnésio e tratamento com anti-hipertensivos. Quanto a terapia anti-hipertensiva, os fármacos anti-hipertensivos adotados são metildopa, nifedipino, hidralazina e labetalol (não disponível para uso clínico no Brasil) (5).

A fisiopatologia da pré-eclâmpsia ainda não está completamente elucidada. Fatores maternos relacionados à predisposição genética, má adaptação imunológica à gravidez e doenças vasculares pré-existentes podem estar envolvidos na origem desta doença,

porém atualmente é amplamente aceito que a isquemia da placenta é um fator primordial. Na pré-eclâmpsia, o processo de "pseudovasculogenesis", que consiste na migração dos citotrofoblastos em direção as arteríolas uterinas espiraladas a fim de tornar essas arteríolas mais dilatas, não ocorre devidamente, portanto a invasão das artérias espiraladas do útero é incompleta (6,7). Logo, a média do diâmetro das artérias espiraladas dessas gestantes é metade daquela observada na gravidez normal (8), o que impede uma resposta adequada ao aumento da demanda do fluxo sanguíneo que ocorre durante a gestação, diminuindo a perfusão útero-placentária, provocando isquemia da placenta.

Sugere-se que a isquemia útero-placentária ocasiona a liberação de fatores placentários na circulação materna, levando a uma disfunção endotelial vascular por todo organismo materno (9,10). Diversas evidências suportam este entendimento da doença, onde os sinais clínicos e sintomas da pré-eclâmpsia envolvendo a vasculatura materna tem ligação com essas substâncias circulantes. Por exemplo, a incubação *in vitro* de soro/plasma de mulheres com pré-eclâmpsia induz a injúria de células endoteliais, através de alterações de expressão gênica (11–15), produção alterada de fatores vasoativos (16–18) e danos por estresse oxidativo (19–22). O início da doença se dá pelas alterações placentárias associadas posteriormente a um endotélio materno predisposto. Os mecanismos fisiopatológicos citados acima induzem a formação de fatores que promovem um estado de estresse oxidativo, ou seja, um desequilíbrio entre compostos oxidantes e antioxidantes, em favor da geração excessiva de radicais livres ou em detrimento da velocidade de remoção desses (Figura 1).



Placenta

Hipóxia/Reoxigenação;
↑ estresse oxidativo;
↑ emissão de micropartículas e vesículas contendo fatores vasoativos/pró-inflamatórios e mediadores do estresse oxidativo.

Endotélio

Presença de moléculas vasoativas/pró-inflamatórias e radicais livres;
Ativação da células endoteliais e produção dessas moléculas.

Órgãos alvos

Células endoteliais dos órgãos alvos sofrem efeitos;
Estresse oxidativo local: ↑ apoptose;
Quadro clínico: proteinúria, HELLP, eclâmpsia.

Figura 1. Envolvimento do estresse oxidativo na fisiopatologia da pré-eclâmpsia.

Num estado sadio, mecanismos celulares são ativados no sentido de contrabalancear esse estresse, produzindo, por exemplo, várias enzimas antioxidantes incluindo hemeoxigenase-1 (HO-1), superoxido dismutase (SOD), catalase e glutationa peroxidase (GPx) (23). Assim em uma situação de estresse oxidativo, uma gama de genes codificadores de enzimas antioxidantes e citoprotetoras são ativados, principalmente através da ativação coordenada do Elemento de Resposta aos Antioxidantes (ERA), localizado nas regiões promotoras desses genes (24). Um dos principais fatores de transcrição responsável pela ativação do ERA é o NRF2 (*nuclear factor, erythroid 2-like 2*) (25,26). Esse fator é ativado em resposta ao estado oxidante celular e leva à transcrição desses genes, os quais atuam protegendo a célula contra os danos causados pelo desequilíbrio oxidativo ou contra várias toxinas (25,26).

Na célula endotelial, o NRF2 é considerado um regulador mestre da resposta antioxidante, exercendo papel de proteção do endotélio contra os danos causados por espécies reativas de oxigênio (ERO) (27,28). Em condições normais, o NRF2 está ligado à proteína inibidora Keap-1 (*Kelch-like ECH-associated protein-1*), tornando-se alvo de ubequitinização e degradação proteossomal, mantendo seu nível baixo no citoplasma. Já em condições de estresse, oxidantes endógenos ou pró-oxidantes reagem com o grupo tiol dos resíduos de cisteína presentes na Keap-1, tornando-a oxidada e resultando na dissociação do NRF2 e Keap-1. O NRF2 livre é transportado para o núcleo, onde reconhece e se liga ao ERA no DNA, e, atuando em sinergia com outros fatores de transcrição, promove a transcrição de genes citoprotetores, incluindo a enzima antioxidante HO-1 (29). Essa por sua vez, converte o grupo heme em monóxido de carbono (CO) e biliverdina, que é convertida em bilirubina que apresenta um papel antioxidante (30). Quanto à pré-eclâmpsia, há estudos demonstrando o papel do NRF2 em citotrofoblastos e placenta (31,32). Também foi demonstrado que a administração de HO-1 diminui o perfil antiangiogênico característico da doença, através da inibição da liberação de sFLT-1 (*soluble* VEGFR1) e sEng (*soluble Endoglin*) (33), além de promover o aumento de VEGF em células endoteliais.

Além do mecanismo endógeno de defesa antioxidante, antioxidantes naturais presentes em alimentos atuam como protetores do endotélio (34). Porém em relação à pré-eclâmpsia, os estudos realizados de suplementação com vitaminas antioxidantes

(vitaminas C e E) em gestantes com risco de desenvolvimento da síndrome não tiveram efeito significativo na redução da prevalência da doença comparado ao grupo não suplementado (35,36). Vale ressaltar que as vitaminas C e E atuam como antioxidantes através do sequestro de radicais livres. De maneira interessante, Vanhees e colaboradores (37) demonstraram em camundongos que a exposição do útero a flavonóides, indutores de NRF2, atenua os danos no DNA induzido pelo estresse oxidativo no embrião e protege o adulto através da ativação do sistema antioxidante. Assim intervenções farmacológicas, principalmente com indutores naturais de NRF2, por exemplo o resveratrol (38), os quais promovem sua ativação e consequente aumento de expressão de HO-1, poderiam ter um papel importante na prevenção e terapia da pré-eclâmpsia.

Resveratrol

O resveratrol (3,5,4'-trihidroxiestilbeno) é uma fitoalexina polifenólica da família dos estilbenos produzida por uma variedade de plantas, e é encontrado em maiores concentrações principalmente em uvas vermelhas (39). Existem os isômeros trans e cis-resveratrol, sendo que o trans-resveratrol é um potente antioxidante que também possui outros efeitos biológicos, como atividade anti-inflamatória, cardioprotetoras, antidiabetes, anticancerígeno, quimiopreventivo, neuroprotetor, lipotoxicidade renal, e efeitos protetores renais (40). Por isso o resveratrol tem sido extensivamente estudado como uma possível ferramenta terapêutica e preventiva no câncer (41), diabetes (42), obesidade (43), doenças cardiovasculares (44) e distúrbios na gravidez (45–47).

Além de seu efeito antioxidante através do sequestro de radicais livres, o resveratrol possui uma atividade antioxidante e citoprotetora indireta, através da modulação de genes-alvo (48). É provável que essa ação indireta contribui mais para sua atividade antioxidante do que o sequestro de radicais livres (48). O resveratrol inibe a produção de ERO mediada pela enzima NADPH oxidase, regulando negativamente a expressão e a atividade da enzima (48). Também reduz a geração de superóxido (O_2^-) mitocondrial estimulando a biogênese de mitocôndrias, pois a proliferação mitocondrial reduz o fluxo de elétrons por unidade de mitocôndria (48). Além disso, o resveratrol evita a produção de O_2^- a partir da enzima óxido nítrico sintase endotelial (eNOS) desacoplada, regulando a enzima sintetizadora de tetra-hidrobiopterina (BH_4), a GTP cilohidroxilase 1 (GCH1) (48). O resveratrol também aumenta a expressão de várias enzimas antioxidantes, como

HO-1 e SOD, através da ativação da sirtuína 1 (SIRT1) e do fator de transcrição NRF2 (44,48). Por fim, esse polifenol induz o aumento da expressão de eNOS e consequente aumento da produção de óxido nítrico, causando vasodilatação que é um efeito importante e desejável em doenças cardiovasculares e também na pré-eclâmpsia (49).

Resveratrol e Pré-eclâmpsia

Há poucos estudos na literatura sobre o uso de resveratrol na pré-eclâmpsia (50–57), porém os resultados desses estudos são bastante promissores. Isso justificativa a necessidade de que mais estudos sejam realizados para se entender melhor os efeitos e mecanismos do resveratrol na prevenção e terapêutica da pré-eclâmpsia.

Foi demonstrado que o resveratrol diminuiu a pressão arterial e proteinúria em modelos animais de pré-eclâmpsia (50,51). Quanto aos mecanismos de ação, estudos mostraram que o resveratrol inibe a liberação de sFLT-1 em explantes de placentas oriundas de gestantes com pré-eclâmpsia e em células endoteliais e trofoblásticas mediante estímulo estressor (52–54). Esses resultados evidenciam o benefício do resveratrol de diminuir o perfil antiangiogênico característico da pré-eclâmpsia. O resveratrol também induz a expressão de NRF2 em células endoteliais e de HO-1 em explantes de placentas de gestantes pré-eclâmpticas e em modelos *in vitro* de células endoteliais e trofoblásticas (54,55). Ainda em células trofoblásticas, com estímulo para indução de estresse oxidativo, o resveratrol protegeu essas células dos danos causados por esse estresse através da ativação de SIRT1 (56). Além disso, um estudo mostrou que, em modelo animal de pré-eclâmpsia de ratos tratados com um inibidor da eNOS (N^w-nitro-arginina-metil-ester, L-NAME), o tratamento com resveratrol diminuiu a quantidade de malondialdeído (MDA) e aumentou atividade da SOD no plasma (51). Logo, esses estudos indicam o potencial da ação antioxidante do resveratrol na pré-eclâmpsia. O resveratrol também é capaz de promover a migração e invasão de células trofoblásticas (50), o que é um efeito importante visto que na pré-eclâmpsia essa invasão é incompleta.

O único estudo realizado em humanos testando os efeitos do resveratrol na pré-eclâmpsia mostrou que, ao ser administrado como adjuvante do nifedipino (resveratrol: 50mg e nifedipino:10mg, cada dose, administradas até 5 doses a cada 15min até pressão arterial alcançar ≤150/100mmHg) em gestantes com pré-eclâmpsia (n=174), o resveratrol reduziu o tempo necessário para controlar a pressão arterial em comparação

com o grupo que recebeu somente o nifedipino (n=175) e aumentou o tempo entre crises hipertensivas (57). Além disso, o número de doses necessárias de nifedipino para controlar a hipertensão foi menor com a coadministração de resveratrol comparado com somente o nifedipino. Importante ressaltar que não foram observadas diferenças nos efeitos adversos maternos ou neonatais entre os dois grupos (nifedipino + resveratrol *vs*. nifedipino) (57).

Portanto, em vista das evidências presentes na literatura do potencial do resveratrol na pré-eclâmpsia e da necessidade de maiores estudos, nosso grupo de pesquisa optou por investigar de forma mais profunda os efeitos do resveratrol e seus mecanismos de ação tanto na prevenção quanto no auxílio do tratamento da pré-eclâmpsia.

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CAPÍTULO I

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Resveratrol improves endothelial cell markers impaired by plasma incubation from women who subsequently develop preeclampsia

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TITLE PAGE

Resveratrol improves endothelial cell markers impaired by plasma incubation from women who subsequently develop preeclampsia

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Abstract

In this study, we demonstrated that plasma collected from women who subsequently developed preeclampsia caused increased heme oxygenase-1 (HO-1) production and decreased levels of nitric oxide (NO) markers in endothelial cells (HUVECs). Conversely, no changes in HO-1 or NO markers were found when HUVECs were treated with plasma from women who remained healthy throughout pregnancy. These alterations in HO-1 and NO markers were prevented by cotreatment with the polyphenol resveratrol, which also improved GSH levels. In addition, we evaluated changes induced by plasma incubation in the expression of genes and their related pathways associated with antioxidant defenses, such as Nrf2, ARE activity and GSR. Collectively, our findings suggest that even before the appearance of clinical symptoms of preeclampsia, plasma from affected women is able to induce modifications in endothelial cells with respect to HO-1 production and NO markers. We believe that this *in vitro* strategy may offer an attractive alternative to the exploitation of candidate markers or screening molecules, such as resveratrol, for the prevention and management of preeclampsia.

Keywords: endothelial cells, plasma, preeclampsia, resveratrol.

Abbreviations list

ADMA, asymmetric dimethylarginine; ARE, antioxidant response element; BMI, body mass index; BRISA, Brazilian Ribeirao Preto and São Luis Birth Cohort Studies; DBP, diastolic blood pressure; eNOS, endothelial nitric oxide synthase; GA, gestational age; GSH, glutathione; GSR, glutathione reductase; HMOX1, heme oxygenase-1 gene; HPRT1, hypoxanthine phosphoribosyltransferase 1 gene; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cell; MDA, malondialdehyde; NFE2 L2, nuclear factor-erythroid-derived 2-related factor-2 gene; NO, nitric oxide; NRF2, nuclear factor-erythroid-derived 2-related factor-2; oxLDL, oxidized low-density lipoprotein; qPCR, quantitative PCR; ROS, reactive oxygen species; SBP, systolic blood pressure; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase-1.

INTRODUCTION

Preeclampsia is a pregnancy-related hypertensive disorder characterized by hypertension after 20 weeks of gestation accompanied by proteinuria, thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema or cerebral/visual symptoms.[1] It is a leading cause of maternal and fetal morbidity and mortality worldwide, and the only treatment is delivery of the placenta.[2-4] A common feature in preeclampsia etiology is an oxidative stress imbalance, which contributes to decreased nitric oxide (NO) bioavailability and endothelial dysfunction associated with this syndrome.[5-10] This characteristic justifies the interest in using antioxidants such as vitamins and phytochemicals in the prevention or treatment of preeclampsia.[7, 11] However, clinical trials conducted to verify whether supplementation with antioxidants, such as vitamin C and E, could reduce the risk of preeclampsia development have yielded no convincing evidence.[12, 13] Conversely, polyphenol resveratrol has been studied in preeclampsia[14-18], showing promising results. One study showed that resveratrol is a safe and effective adjuvant of oral nifedipine for attenuating hypertensive symptoms among preeclamptic patients,[14] while other investigations showed that resveratrol can improve trophoblast and endothelial dysfunction in human cells,[15-18] suggesting it may have potential in the treatment and prevention of preeclampsia.

Resveratrol is found in a variety of fruits, and its most common source is the red grape. In addition to being an antioxidant, resveratrol modulates several targets through physical interaction or indirect effects, such as activation of endothelial nitric oxide synthase (eNOS) and the nuclear factor-erythroid-derived 2-related factor-2 (Nrf2).[19] Nrf2 binds to the antioxidant response element (ARE) promoter sequence, regulating the expression of antioxidant proteins, including heme oxygenase-1 (HO-1) and glutathione reductase (GSR), which are able to counter oxidative stress and balance the redox state in cells.[20]

Incubation of endothelial cells with plasma/serum from preeclamptic women is a well-established *in vitro* model of the two stages of preeclampsia that supports a better understanding of the molecular mechanisms of endothelial dysfunction in this syndrome.[6, 8, 21-23] Interestingly,

plasma from pregnant women collected early before the development of preeclampsia has been shown to modulate gene expression in endothelial cells[24] and impair the endothelial function of myometrial vessels.[25]

In this regard, the present study aimed to investigate the effect of resveratrol incubated with plasma collected from women before the development of preeclampsia on modulating key antioxidant defenses and vasodilator factors, such as Nrf2, HO-1, GSR, glutathione (GSH) and NO in endothelial cells. We hypothesized that resveratrol could reduce oxidative stress by improving these pathways in endothelial cells even before the manifestation of clinical symptoms of preeclampsia.

MATERIALS AND METHODS

Study population

This study is part of a broader observational study performed in two Brazilian cities (Brazilian Ribeirao Preto and São Luis Birth Cohort Studies - BRISA).[26-28] The study was approved by the Institutional Review Board at Ribeirao Preto Medical School, Brazil (reference 4116/2008, approval date November 11, 2008), following the principles of the Declaration of Helsinki, and all subjects gave written informed consent. A total of 1,417 pregnant women at 20-25 weeks of gestation were evaluated at Hospital das Clinicas of Ribeirao Preto–University of Sao Paulo. Of these women, 17 did not return, and 460 gave birth in other units. Of the 940 pregnant women remaining, 30 developed preeclampsia (cases), of which 14 were classified as severe cases. For this *in vitro* study, 6 samples of women who developed severe preeclampsia and 6 samples of those who remained healthy throughout pregnancy (controls) were randomly selected. Diagnosis and severity criteria of preeclampsia were defined by the American College of Obstetricians and Gynecologists.[1] Maternal venous blood samples were collected in tubes containing EDTA. The tubes were rapidly centrifuged (1000 *g* for 3 min) at room temperature, and plasma samples were stored at −80°C.

Cell culture, plasma incubation and trans-resveratrol intervention

Human umbilical vein endothelial cells (HUVEC) (CRL 2873, American Type Culture Collection (ATCC), VA, USA) were cultured in DMEM (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 50 μg/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml amphotericin B (Gibco) at 37ºC in an incubator with a 5% CO_2 atmosphere. After reaching 80-90% confluence, HUVECs were incubated in medium containing 10% (v/v) plasma from case and control patients and 1 μM of trans-resveratrol (Cayman Chemical®, MI, USA), the most predominant isoform responsible for many health benefits,[29, 30] or the vehicle DMSO

(Sigma-Aldrich®, Poole, UK) for 24 hours in an incubator**.** The concentration of 1 μM was chosen based on literature research from studies using resveratrol in HUVECs.[31-33] Cells were used until the eighth passage.

Cell viability

After 24 hours of plasma incubation from cases and controls in the presence or absence of trans-resveratrol in HUVECs, cell viability was assessed using a 3-(4,5-dimethylthiazol2-yl)−2,5-diphenyltetrazolium (MTT) assay as described previously.[34] MTT is reduced to blue formazan crystals by metabolically active cells. MTT solution (0.5 mg/ml PBS) (Sigma-Aldrich) was added to a 96-well plate, and the plate was incubated in the incubator for 3 hours. Then, the MTT solution was removed, and DMSO (Sigma-Aldrich®) was added for 10 min. The optical density was measured at 570 nm on a multifunctional plate reader (Synergy 4, BioTek, VT, USA). DMSO alone was used as a blank. Each sample was performed in triplicate. Viability was compared to the control (untreated cells with vehicle DMSO, 100% viability).

Messenger RNA expression by qPCR

Total RNA from HUVECs incubated with plasma from cases and controls in the presence or absence of trans-resveratrol was isolated using a miRNeasy Micro Kit (Qiagen, Leusden, Netherlands) according to the manufacturer's protocol. Isolated RNA from the samples was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, MA, USA) and was consistently found to be pure. RNA was transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, ON, Canada) according to the manufacturer's protocol. qPCR was performed using Luna Universal qPCR Master Mix (New England BioLabs®, MA, USA). Each reaction contained 5 μL Luna Universal qPCR Master Mix, 100 nM of each primer (forward and reverse), 5 μL of cDNA (6.5 ng/μL), and a variable amount of nuclease-free water to yield a final volume of 10 μL. The following genes were analyzed:

NFE2L2, HMOX1, GSR and *HPRT1*. KiCqStart™ SYBR® Green Primers of the mentioned genes were purchased from Sigma-Aldrich. The HPRT1 gene was chosen as an endogenous control because it was the gene most stable in our samples according to a previous study.[20] Thermal cycling was performed under the following conditions: 1 min at 95°C, 40 two-step cycles of 15 s at 95°C and 60 s at 60°C, and a final step for the dissociation curve. Relative expression was calculated using the comparative 2 (-Delta C(T)) method.[35] All PCRs were performed in duplicate for each sample.

Measurement of nuclear Nrf2

HUVECs were seeded into 25 cm^2 bottles at a concentration of $0.7x10^6$ cells. After reaching confluence and receiving the plasma and resveratrol intervention, the nuclear extract was separated and collected using a Nuclear Extraction Kit (Cayman Chemical®), according to the manufacturer's protocol. Nrf2 quantification in the nuclear extract was assessed by *ELISA* using a Nrf2 Transcription Factor Assay Kit (Cayman Chemical®). First, samples and controls were added to a 96-well plate for overnight incubation. The next day, the plate was washed 5 times with wash buffer and incubated with a primary antibody for 1 hour. Washes were repeated, and the second antibody was incubated for 1 hour. After incubation, the washes were repeated, and a developing solution was added for 45 min under gentle agitation and protected from light. Then, stop solution was added, and the plate was read at 450 nm in a spectrophotometer (Synergy 4, BioTek®).

Antioxidant response element (ARE) activation analyzed by a luciferase reporter assay

ARE activation was verified using an ARE Reporter Kit (BPS Bioscience, CA, USA). Briefly, HUVECs were seeded at a concentration of $1x10^4$ cells per well into a white 96-well plate. After reaching confluence, cells were transfected using Lipofectamine® 2000 (Thermo Scientific) with the ARE reporter or negative control reporter for 12 hours. Then, the cells were

incubated with plasma samples from the control and case groups and in the presence or absence of resveratrol. After 24 hours of incubation, luminescence was measured in a multifunctional plate reader (Synergy 4, BioTek®) using the Dual-Glo® Luciferase Assay System (Promega, WI, USA). Trans-resveratrol was added at a concentration of 1 μM for 24 hours (fold=1) as a positive control.

Measurement of HO-1 concentrations

HO-1 concentrations in cell supernatants were measured using the enzyme-linked immunosorbent assay kit Human Total HO-1/HMOX1 *ELISA* (R&D Systems, MN, USA) according to the manufacturer's protocol. Briefly, 50 μL of capture antibody was added to a half area of a 96-well plate and incubated overnight. Then, the plate was washed 3 times with washing buffer, and 50 μL of blocking buffer was added for 1-2 hours. Washes were repeated, and 50 μL of standards and samples were added for 2 hours. After incubation, the washes were repeated, and 50 μL of the detection antibody was added and incubated for 2 hours. After the washes, 50 μL of streptavidin-HRP was added and incubated for 20 min. Then, final washes were performed, and 50 μL of substrate solution was added and incubated for more than 20 min; 25 μL of stop solution was added, and the plate was read at 450 nm in a spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by the incubation of HO-1 solutions (156.25–10000 pg/mL) with the previous reagents. The HO-1 concentration is expressed in pg/mL.

Measurement of nitrite by a Griess assay

Nitrite levels were assessed in the cell supernatants using Griess reagents.[36] Briefly, 50 μL of each sample was added to a 96-well plate with 50 μL of 1% sulfanilamide solution in 5% phosphoric acid for 10 min protected from light. Then, 50 μL of 0.1% N- (1-naphthyl)-ethylenediamine dihydrochloride solution was added and incubated for 10 min. The plate was

read at 540 nm in a spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by incubation of nitrite solutions (0.39–50 μM) with the previous reagents. The nitrite concentration is expressed in μM. Each sample was performed in duplicate.

Measurement of glutathione levels

Total GSH levels were measured in cell lysates using a Glutathione Colorimetric Detection Kit (Invitrogen, MD, USA) according to the manufacturer's protocol. For this assay, plasma samples from each group were pooled and incubated in HUVECs in a 12-well plate. Briefly, 50 μL of each sample was added to a half-area 96-well plate with 25 μL of Colorimetric Detection Reagent and 25 μL of Reaction Mixture. Then, the plate was incubated at room temperature for 20 min. The plate was read at 405 nm in a spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by the incubation of oxidized glutathione standard solutions (0.78–25 μM) with the previous reagents. GSH levels are expressed in μM. Each sample was performed in duplicate.

Measurement of intracellular ROS levels

The levels of intracellular ROS levels were evaluated by measuring the fluorescence of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Cayman Chemical®). DCFH-DA is deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into the highly fluorescent compound 2,7-dichlorofluorescein (DCF). Briefly, after 24 hours of plasma and trans-resveratrol incubation, HUVECs in a 96-well plate were incubated with 25 μM of DCFH-DA in PBS for 45 min in an incubator. Fluorescence was read on a multifunctional plate reader (Synergy 4, BioTek®) using excitation and emission wavelengths of 502 and 523 nm, respectively. Tert-butyl-hydroperoxide solution (Sigma-Aldrich®) at 250 μM for 2 hours was used as a positive control. Each sample was performed in duplicate.

Statistical analysis

For comparison between the case and control groups, t-tests were performed, and for comparison within each group between the presence and the absence of trans-resveratrol, we applied a t-test. These statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA). For all tests, a p value ≤ 0.05 (two-tailed) was considered significant.

RESULTS

The study workflow is shown in Figure 1. The clinical characteristics of the women enrolled in the study are shown in Table 1. At the time of blood collection, all the clinical characteristics were similar between the groups. In the case group, the gestational age (GA) at delivery was significantly lower than that for controls.

First, we verified the cell viability of HUVECs incubated with plasma from the control group (those who remained healthy throughout pregnancy) and the case group (pregnant women who developed preeclampsia), in the absence (-R) and presence (+R) of 1 μM trans-resveratrol. No significant differences in cell viability among groups were found, independently of the addition of resveratrol (all $p > 0.05$).

Next, we verified the gene expression of *NFE2L2*, *HMOX1* and *GSR* in HUVECs (Figure 1). For *NFE2L2* (Figure 2A) and *HMOX1* expression (Figure 2B), there was no significant difference between the groups, and resveratrol did not alter the expression. Regarding *GSR* expression (Figure 2C), there was no difference between the groups), and resveratrol significantly decreased the expression only in the control group. Moreover, *HMOX1* and *GSR* expression was higher in the case group with resveratrol than in controls receiving the same treatment.

To examine the Nrf2 pathway, Nrf2 expression in the nucleus (Figure 3A) and ARE activity (Figure 3B) were assessed. Plasma from the case group did not alter Nrf2 expression or ARE activity compared with that in the controls; however, resveratrol increased the Nrf2 activity by ~ 15% and the ARE activity by ~ 72% only in the control group.

The HO-1 concentration in the cell supernatant (Figure 4A) was increased ~ 2.4-fold in cultures incubated with plasma from the case group compared with that in the control. Furthermore, in the case group, resveratrol decreased the HO-1 concentration by ~ 15%, but this concentration did not change in the control group. In the case group treated with resveratrol, we observed a higher HO-1 concentration than that in the controls with the same treatment.

The nitrite level (Figure 4B) was significantly decreased by ~ 25% in the case group compared with that in the control. However, only in the case group was resveratrol able to increase the nitrite levels.

We also investigated the total GSH concentration in cell lysates (Figure 4C). There was no significant difference between the control and case groups in the absence of resveratrol. Resveratrol increased the GSH concentration in the case group but not in the control group.

Intracellular ROS levels (Figure 4D) were not significantly different between groups, and the addition of resveratrol did not alter ROS levels. The positive control was 4.45±0.23 square root of fluorescence intensity $x10^3$.

DISCUSSION

In this study, we showed for the first time that (1) plasma collected from women who subsequently develop preeclampsia (case group) was able to increase HO-1 production and decrease NO markers in HUVECs, while (2) no changes in HO-1 or NO markers were found when HUVECs were treated with plasma from women who remained healthy throughout pregnancy (control). (3) These alterations in HO-1 and NO markers were prevented by cotreatment with polyphenol resveratrol, which was followed by an improvement in GSH levels. Additionally, we found that resveratrol increased NRF2 expression and ARE activity in cells incubated with plasma from controls but not from cases. Moreover, in the case group, resveratrol increased the gene/protein expression of HO-1 and *GSR* expression compared with that in the control group with the same treatment. Collectively, these findings suggest that even before the appearance of clinical symptoms of preeclampsia, plasma from the women is able to induce modifications in endothelial cells through these mechanisms and that resveratrol is able to prevent this induction.

HO-1 catalyzes heme degradation into carbon monoxide, free iron, and biliverdin and is further degraded to bilirubin, which has antioxidative properties. Recently, the role of HO-1 has been studied in preeclampsia.[37-39] HO-1 inhibition stimulated soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) production in endothelial cells; both of these antiangiogenic markers are implicated in preeclampsia pathophysiology.[39] We showed that plasma from the case group stimulated HO-1 production by HUVECs, and because we used an immortalized healthy cell line, this finding may reflect a protective mechanism of these cells against circulating factors, including oxidative stress inducers, even before development of the syndrome. Indeed, studies have already shown that oxidative stress markers, such as malondialdehyde (MDA) and oxidized low-density lipoprotein (oxLDL), are increased in serum collected in the first trimester from women who develop preeclampsia.[40-42] In our study, we did not observe a difference in ROS production by cells incubated with plasma from the case group compared with that in the control group, which also suggests that a protective mechanism might

occur, probably by HO-1 induction and not by glutathione, as we observed. As mentioned before, we used an immortalized healthy cell line of HUVECs, which is a limitation of the study since it is known that even before preeclampsia development, the maternal endothelium is already impaired.[43, 44]

It is well known that in preeclampsia, NO bioavailability is deficient and contributes to development of the hypertension characteristic of this syndrome.[45-48] More interestingly, in serum/plasma from women in the first trimester of pregnancy, before the manifestation of the clinical symptoms of preeclampsia, the NO concentration was decreased compared with that in healthy controls during the first trimester of pregnancy.[45, 49] Our *in vitro* results also demonstrated that the NO concentration was decreased in endothelial cell supernatants incubated with plasma from the case group compared with the control group. The mechanisms involved in this decrease could be as follows: (1) the presence of ROS in plasma from these women, which could scavenge NO;[7] (2) an antiangiogenic factor, such as sFlt-1 and sEng, which is elevated in serum from women before preeclampsia development[50, 51] and could inhibit NO formation[48] and (3) asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase that is also elevated before preeclampsia development.[52]

As a potent antioxidant with several targets, resveratrol has been extensively studied as a possible therapeutic tool and for the prevention of cancer,[53] diabetes,[54] obesity,[55] cardiovascular diseases[56] and disorders in pregnancy.[57-59] For instance, perinatal resveratrol supplementation is able to prevent the development of hypertension and improve vascular function in adult spontaneously hypertensive rat offspring.[60] Roberts et al.[61] demonstrated that resveratrol use during pregnancy in nonhuman primates improves some maternal factors (maternal weight loss, improved glucose tolerance, increased uterine artery blood flow volume, and decreased placental inflammation and liver triglyceride deposition); however, resveratrol enlarged the fetal pancreatic mass by 42%. This finding raises concerns for resveratrol use by pregnant women; nonetheless, it is difficult to know if this effect would occur in humans, as noted by Hannan et al.[16]

In preeclampsia, although the results might be promising, few studies have investigated the role of resveratrol[14-18], and to the best of our knowledge, no study has focused on prevention. Our findings showed that the addition of resveratrol incubated with plasma from the case group decreased the HO-1 concentration and increased the NO and GSH concentrations. In fact, it was demonstrated in endothelial cells with an oxidative environment that resveratrol is able to increase NO bioavailability[19] and stimulate antioxidant defenses,[62, 63] such as the GSH content. Regarding the decrease in the HO-1 concentration caused by resveratrol, we suggest that since resveratrol can modulate targets directly and activate other protective mechanisms, HO-1 induction was probably not triggered. We also found that resveratrol did not induce Nrf2 activation to the nucleus or ARE activity in the case group, which supports this hypothesis. In the control group, resveratrol was able to activate Nrf2 and ARE; however, these activations did not alter the HO-1 concentration. In the current study, we also observed that resveratrol incubated with plasma from the case group increased the gene and protein expression of HO-1 and *GSR* compared with that in controls with the same treatment. ROS levels were not altered by resveratrol in either group, which was not expected since it is well established that resveratrol scavenges ROS.[64] As a limitation of our study, this lack of a difference may be due to the low concentration of resveratrol that was used and/or the fact that the time of incubation was not enough to evidence this difference.

In conclusion, plasma from women who subsequently develop preeclampsia appears to contain factors that lead to alterations in HO-1 and NO markers in endothelial cells. Moreover, we showed that resveratrol was able to increase NO and GSH levels when incubated with plasma from these women. We believe that the understanding of these early events using our *in vitro* strategy may offer an attractive alternative to exploiting candidate markers for the prevention and management of preeclampsia, as we have demonstrated for resveratrol in the present study.

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Conflict of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Clinical characteristics of pregnant enrolled in the study.

Parameters	**Controls**	**Cases**
GA at sampling (weeks)	23 ± 2	23 ± 2
Maternal Age (years)	28 ± 5	28 ± 4
BMI (kg/m²)	28 ± 4	30 ± 6
SBP at sampling (mmHg)	104 ± 6	118 ± 1
DBP at sampling (mmHg)	64 ± 4	77 ± 15
GA at delivery (weeks)	39 ± 3	36 ± 2*
Newborn weight (g)	3413 ± 252	2665 ± 532

Values are the means ± S.D. GA, gestational age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. * $P<0.05$ vs controls.

Figures

Figure 1.

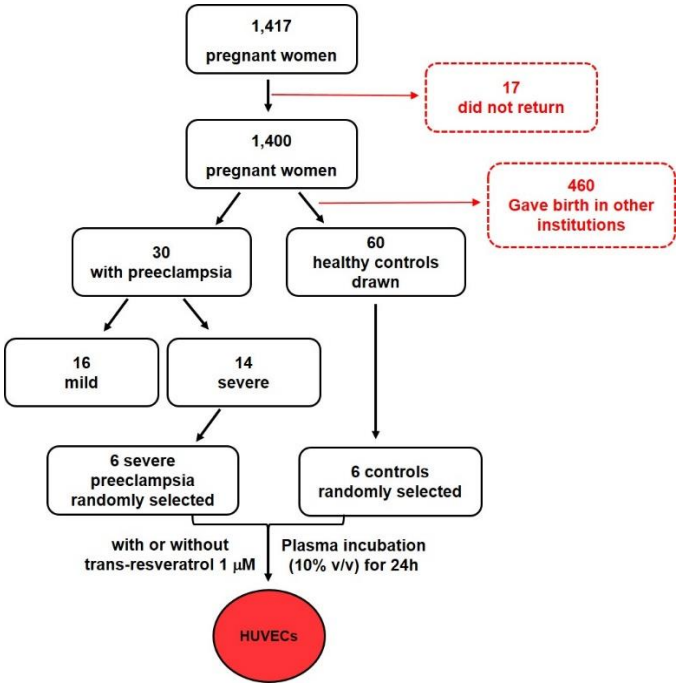

Figure 1. Schematic diagram of the study workflow.

Figure 2.

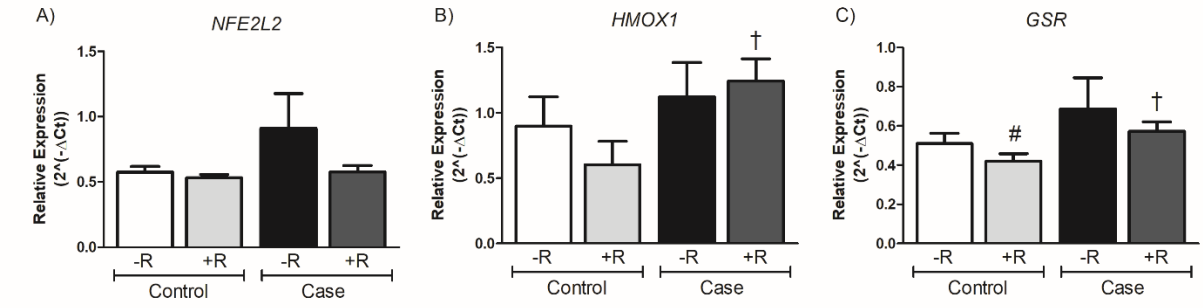

Figure 2. Relative expression of the genes *NFE2L2* (A), *HMOX1* (B) and *GSR* (C) in HUVECs. HUVECs were incubated with 10% (v/v) plasma samples from pregnant women who subsequently developed preeclampsia (case) and who remained healthy during gestation (control) (n=6 per group) for 24 hours in the absence (-R) and presence of trans-resveratrol (+R) at 1 µM. Resveratrol significantly decreased *GSR* expression in the control group. *HMOX1* and *GSR* expression was higher in the case group with resveratrol than in the controls with the same treatment. DMSO was used as a vehicle for trans-resveratrol. Values were normalized using *HPRT1* as an endogenous control. Values are expressed as the mean ± S.E.M. # $p \leq 0.05$ vs. respective group –R; † $p \leq 0.05$ vs. control group +R.

Figure 3.

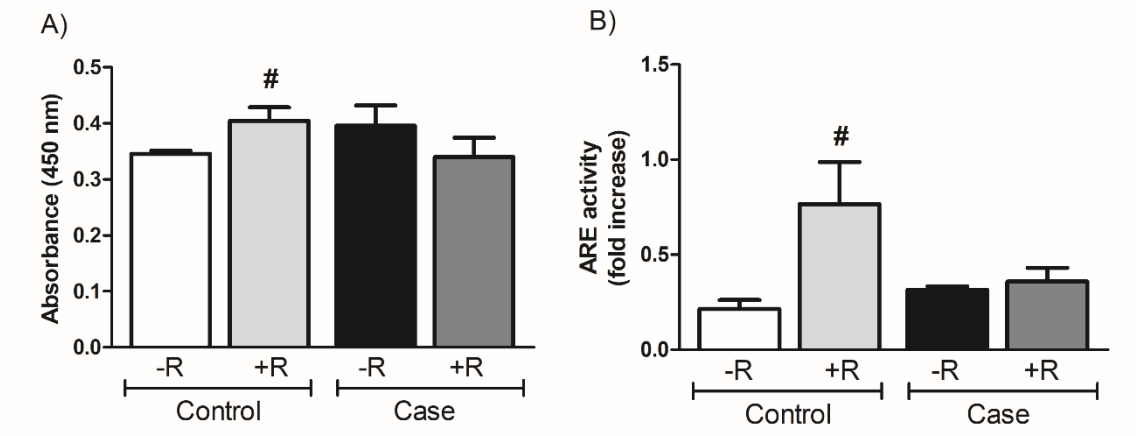

Figure 3. Nrf2 expression in the nucleus (A) and ARE activity (B). HUVECs were incubated with 10% (v/v) plasma samples from pregnant women who subsequently developed preeclampsia (cases) and who remained healthy during gestation (controls) (n=6 per group) for 24 hours in the absence (-R) or presence of trans-resveratrol (+R) at 1 μM. Resveratrol increased Nrf2 expression and ARE activity only in the control group. DMSO was used as a vehicle for trans-resveratrol. Values are expressed as the mean ± S.E.M. # $p \leq 0.05$ vs. respective group –R.

Figure 4.

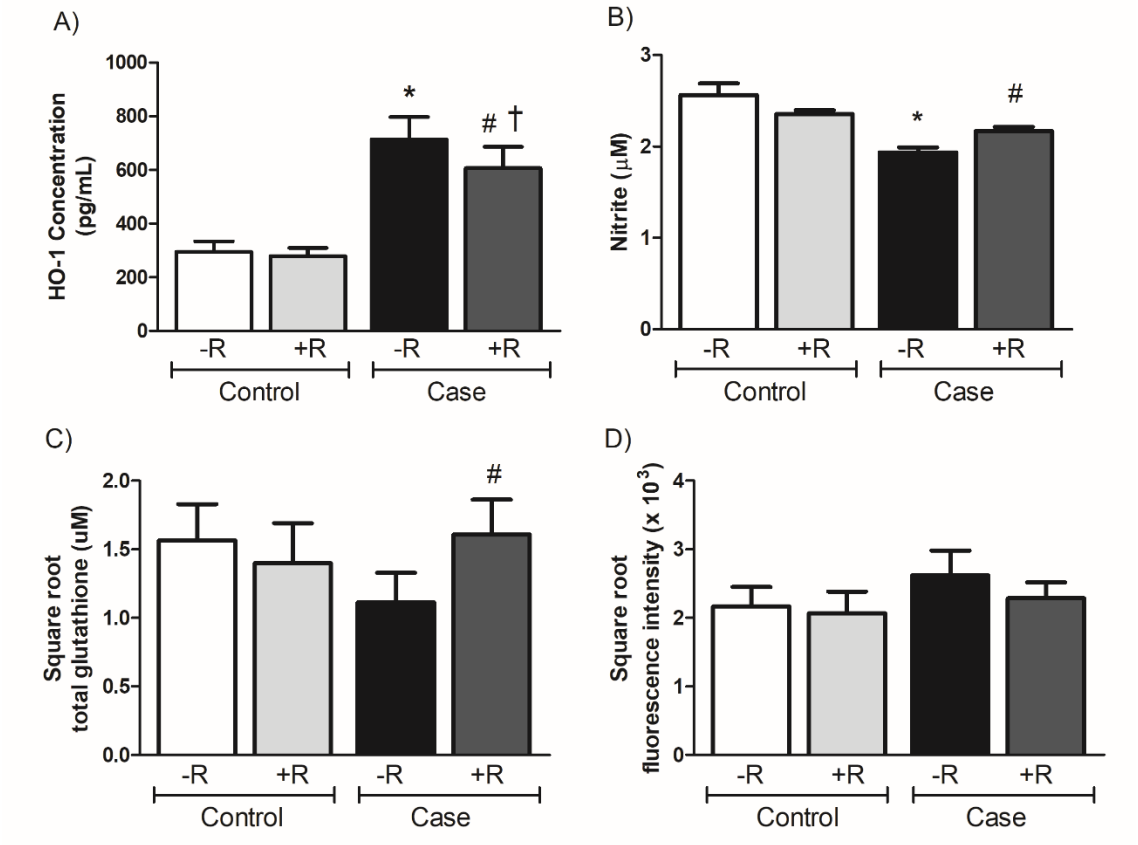

Figure 4. HO-1 levels (A) and nitrite concentration (B) in cell supernatants, total GSH in cell lysates (C) and intracellular ROS levels (D). HUVECs were incubated with 10% (v/v) plasma samples from pregnant women who subsequently developed preeclampsia (cases) and who remained healthy during gestation (controls) (n=6 per group) for 24 hours in the absence (-R) or presence of trans-resveratrol (+R) at 1 μM. For ROS levels, the mean and standard error for the positive control was 4.45±0.23 square root of fluorescence intensity $x10^3$. Plasmas from the case group increased the HO-1 concentration and decreased the nitrite levels compared with those in the controls. Resveratrol decreased the HO-1 concentration and increased the nitrite and glutathione levels in the case group. The HO-1 concentration was higher in the case group with resveratrol than in the controls with the same treatment. DMSO was used as a vehicle for trans-resveratrol. Values are expressed as the mean ± S.E.M. * $p \leq 0.05$ vs. control –R; # $p \leq 0.05$ vs. respective group –R; † $p \leq 0.05$ vs. control group +R.

CAPÍTULO II

Manuscrito para submissão na revista Hypertension

Informações:

Título

Resveratrol and Grape Juice: Effects on Redox Status and Nitric Oxide Production of Endothelial Cells in In Vitro Model of Preeclampsia

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TITLE PAGE

Resveratrol and Grape Juice: Effects on Redox Status and Nitric Oxide Production of Endothelial Cells in *In Vitro* Model of Preeclampsia

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Short title: Resveratrol and grape juice *in vitro* preeclampsia.

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ABSTRACT

Preeclampsia (PE) is a hypertensive disease of pregnancy and is one of the main causes of maternal and fetal morbidity and mortality worldwide. It is known that oxidative stress plays a role in its pathophysiology, therefore the use of antioxidants in the management of PE could be a strategy. In this study we investigated the effects of trans-resveratrol, a potent antioxidant, on Nrf2/ARE pathway, nitric oxide (NO) production and reactive oxygen species (ROS) levels in *in vitro* model of PE. Plasma from PE patients increased ARE activity in endothelial cells compared with plasma from healthy pregnant (HP), and the addition of resveratrol was able to potentiate this increase. Resveratrol also decreased ROS levels in the cells incubated with plasma from PE. Based on these results, we performed a pilot clinical study to compare the effects of serum from PE women before and 1 hour after ingestion of polyphenol-rich whole red grape fruit juice incubated on endothelial cells. Serum from PE patients, obtained one hour after juice intake, decreased antioxidants markers in cells compared with serum before juice intake, besides it increased NO production. In conclusion, resveratrol and polyphenol-rich red grape juice have beneficial effects on endothelial cells incubated with PE plasma/serum, evidencing their potential to assist the management of PE.

Key words: endothelial cells, preeclampsia, plasma, resveratrol, grape juice.

INTRODUCTION

Preeclampsia (PE) affects 3-8% of pregnancies and is one of the main causes of maternal and fetal morbidity and mortality worldwide.[1–3] This multisystemic syndrome is characterized by hypertension after 20 weeks of gestation accompanied by proteinuria or other systemic alterations, such as thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema or cerebral/visual symptoms.[4] There is no treatment, apart from early delivery of the placenta.[3] The pathophysiology of PE is complex and is still not fully elucidated, however it is generally agreed that oxidative stress plays a role, contributing to the endothelial dysfunction and clinical manifestation of this syndrome.[5] Therefore, antioxidants, such as resveratrol, has been extensively studied to verify if they could reduce the risk and aid in the management of PE.[6–9] Studies showed that resveratrol can improve trophoblast and endothelial dysfunction in human cells[10–12] and it is a safe and effective adjuvant of oral nifedipine to attenuate hypertensive symptoms among PE patients,[9] suggesting its potential to assist the treatment of PE.

Resveratrol is a polyphenolic compound present in a variety of fruits, mostly in red grapes. It is an antioxidant that directly scavenges free radicals and also modulates several targets, such as activation of endothelial nitric oxide synthase (eNOS), increasing nitric oxide (NO) production, and the nuclear factor-erythroid-derived 2-related factor-2 (Nrf2).[13] Nrf2 binds to the antioxidant response element (ARE) promoter sequence, upregulating the expression of antioxidant proteins, including heme oxygenase-1 (HO-1) and glutathione reductase (GSR), and hence, countering oxidative stress and balancing the redox state in cells.[14] Interestingly, studies have shown that consumption of dealcoholized red wine and polyphenol-rich juices, such as grape juice, decreased blood pressure levels in humans;[15–17] however, to our knowledge no study has focused in PE.

Progressively studies have shown the role of microRNAs (miRNAs) in the regulation of cellular redox, modulating the expression of genes associated with Nrf2 pathway.[18] MicroRNAs (miRNAs) are small endogenous noncoding RNAs (18-24 nucleotides) that post-

transcriptionally regulate gene expression resulting in the repression of protein expression.[19] Changes in the expression profile of miRNAs has been found in placental tissue and plasma/serum from PE women, suggesting a role of miRNAs in PE.[20–22] In addition, several studies have shown that resveratrol modulates miRNAs in human diseases,[23,24] however apparently there is no study regarding PE.

Incubation of endothelial cells with plasma/serum from PE women is a well-established *in vitro* model that mimics one of the two-stages of PE, allowing the comprehension of molecular mechanisms regarding endothelial dysfunction in this syndrome.[25–29] Indeed, it has been shown that plasma/serum from PE women impaired endothelial cells function, dysregulating the production of vasoactive factors[25,27] and increasing reactive oxygen species (ROS) production.[28,29]

In light of these findings, this study aimed to verify whether resveratrol could activate the Nrf2 pathway, induce the expression of antioxidant molecules and NO production and reduce ROS in *in vitro* model of PE. In addition, we examined whether PE serum collected after grape juice intake incubated in endothelial cells could modulate those factors.

METHODS

More details for Methods in the online-only Data Supplement.

Samples of *in vitro* studies using trans-resveratrol

For *in vitro* studies, eight PE women (PE) and four healthy pregnant (HP) were recruited from the ambulatory clinic at Hospital das Clinicas Faculdade de Medicina de Ribeirao Preto da Universidade de São Paulo (HCFMRP-USP). The study was approved by the Institutional Review Board at Ribeirao Preto Medical School, Brazil (reference 4682/2006, approved date June 20, 2006), following the principles of the Declaration of Helsinki and all subjects gave written informed consent. Diagnosis criteria of PE were defined by the American College of Obstetricians and Gynecologists (ACOG).[4] Exclusion criteria included twin pregnancy, chronic hypertension, hemostatic abnormalities, diabetes mellitus, fetal abnormalities, cancer, and cardiovascular, autoimmune, renal, and hepatic diseases. Maternal venous blood samples were collected in tubes containing heparin, rapidly centrifuged (1000g for 10 min) at room temperature and plasma samples were stored at −70°C.

Pilot clinical study using red grape juice

For this pilot study, four PE patients recruited at HCFMRP-USP were consented to drink 200 mL of a commercial organic whole red grape juice (Carraro, RS, Brazil). The study was approved by the Institutional Review Board at Ribeirao Preto Medical School, Brazil (process number 2.602.100/2018, approved date April 16, 2018). PE was diagnosed according to ACOG guidelines.[4] Exclusion criteria included smokers, chronic alcohol consumption, pre-gestational obesity (BMI > 30), gestational diabetes and diabetes mellitus, dyslipidemia, cardiovascular and inflammatory diseases and cancer. Prior grape juice intake, women were in a fast period of approximately 12 hours. Before grape juice intake (0 h) and after 1 hour (1 h), venous blood

samples were collected in tubes with clot activator gel, which were rapidly centrifuged (1000g for 10 min) at room temperature. Serum was collected after 1 hour of grape juice intake based on a previous study showing that this period was the peak absorption of polyphenols.[30] Serum samples were stored at −70°C for *in vitro* assays.

Cell culture, plasma/serum incubation and trans-resveratrol intervention

Human umbilical vein endothelial cells (HUVEC) (CRL 2873) were cultured until reaching 80-90% of confluence. HUVECs were incubated in supplemented DMEM with 10% (v/v) plasma from PE or HP and 1 μM of trans-resveratrol or the vehicle DMSO in the incubator for 24 hours. The concentration of 1 μM was chosen based on previous studies using trans-resveratrol *in vitro*, including one of our group.[31–34] HUVECs were also incubated with 10% (v/v) serum from PE at 0 h (before grape juice intake) and 1 h (after grape juice intake) for 24 hours. Cells were used until passage 8.

Cell viability by MTT assay

Cell viability was performed using the 3-(4,5-dimethylthiazol2-yl)−2,5-diphenyltetrazolium (MTT) assay as described previously.[35] Viability was compared to control (untreated cells with vehicle DMSO, 100% viability). Each sample was performed in triplicate.

Messenger RNA expression by qPCR

Total RNA from HUVECs incubated with plasma from PE and HP in the presence or absence of trans-resveratrol was isolated according to the manufacturer's protocol. Isolated RNA was consistently found to be pure. RNAs were transcribed into cDNA according to the manufacturer's protocol. qPCR reaction was performed using SYBR Green. The analyzed genes were: *NFE2L2*, *HMOX1* and *HPRT1*. According to previous study,[26] *HPRT1* was selected as an

endogenous control because it was the gene most stable in our samples. Relative quantification was calculated using the comparative 2(-Delta C(T)) method.[36] PCR reactions were performed in duplicate for each sample.

miRNA selection and expression by qPCR

miRNAs from the let-7 family (a, b and c) were selected as they target the mRNA of the *NFE2L2* gene, based on bioinformatics analysis using two databases (Microrna.org[37] and miRWalk 2.0[38]). Those miRNAs are expressed in HUVECs.[39] From the same isolated RNA used for messenger RNA expression, reverse transcription (RT) was performed according to the manufacturer's protocol. qPCR reaction was performed using SYBR Green. Relative quantification was calculated using the comparative 2(-Delta Delta C(T))[36] and normalization was performed to U6 snRNA. PCR reactions were performed in duplicate for each sample.

Measurement of HO-1 concentrations

HO-1 quantification in cell supernatants was accessed using enzyme-linked immunosorbent assay kit Human Total HO-1/HMOX1 ELISA (R&D Systems, MN, USA), according to the manufacturer's protocol. Optical density was determined at 450 nm in the spectrophotometer. A standard curve was generated by incubation of HO-1 solutions (156.25–10000 pg/mL). Each sample was performed in duplicate.

Measurement of intracellular NO

Quantification of intracellular NO was accessed by measuring the fluorescence of 4,5-Diaminofluorescein (DAF-2) diacetate. The fluorescence signal was measured (excitation 495 nm, emission 535 nm) and expressed as arbitrary units. Each sample was performed in duplicate.

Glutathione quantification

Total glutathione (GSH) quantification was performed in cell lysates using the Glutathione Colorimetric Detection Kit according to the manufacturer's protocol. Optical density was determined at 405 nm in the spectrophotometer. A standard curve was generated by incubation of oxidized glutathione standard solutions (0.78–25 μM). Each sample was performed in duplicate.

Measurement of intracellular ROS

Quantification of intracellular ROS was accessed by measuring the fluorescence of 2,7-Dichlorodihydrofluorescein diacetate. Fluorescence was determined using wavelengths of 502 nm of excitation and 523 nm of emission in a multifunctional plate reader. Positive control was addition of Tert-Butyl hydroperoxide solution at 250 μM for 2 hours. Each sample was performed in duplicate.

Antioxidant Response Element (ARE) activation by Luciferase Reporter Assay

ARE activation was accessed using ARE Reporter Kit (BPS Bioscience, CA, USA). HUVECs were seeded into a white 96-well plate at a concentration of $1x10^4$ cells per well in a final media volume of 100 μL. In the next day, cells were transfected using Lipofectamine® 2000 (Thermo Scientific) with ARE reporter or negative control reporter for 12 hours. After transfection, cells were treated with plasmas in the presence or absence of 1 μM trans-resveratrol or serum from the groups for 24 hours. For detecting the luciferase expression after the treatment, the Dual-Glo® Luciferase assay system (Promega, WI, USA) was used and luminescence was measured in a multifunctional plate reader (Synergy 4, BioTek®). Positive control was considered the addition of trans-resveratrol at 1 μM for 24 hours (fold=1).

Statistical analysis

Parametric t-tests were performed for comparisons between HP and PE groups, comparison within each group between the presence and the absence of trans-resveratrol and comparison between 0h and 1h (after grape juice intake) groups. GraphPad Prism 5.0 (GraphPad Software, CA, USA) was used for these analyses. Gene and miRNA expression data were analyzed using the GeneGlobe Data Analysis Center (Qiagen®) online platform. P value < 0.05 (two-tailed) was considered significant.

RESULTS

The study design and workflow are shown in Fig. S1. Clinical characteristics of women whose plasma samples were collected and used to perform the study with resveratrol are shown in Table 1. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly increased in PE group compared with HP group. The gestational age (GA) at delivery was significantly lower in PE compared with HP.

We did not observe a significant difference in cell viability between HP and PE and the addition of resveratrol did not change viability of all cultures (all $p > 0.05$, data not shown).

We observed no significant difference in both gene expressions (*NFE2L2* and *HMOX1*, Figures S2A and 2B, respectively*)* and miRNAs (let-7a, b and c, Figures S2C, D and E, respectively) expressions in HUVECs incubated with plasma from HP and PE, and resveratrol did not alter those expressions in neither the groups. In order to validate the effects of resveratrol alone, resveratrol without plasma was incubated in the cells and we observed increased 1.5 and 7.9-fold the expression of *NFE2L2* and *HMOX1*, respectively, compared with only cells (data not shown).

HO-1 levels in cell supernatant (Figure 1A) was not significantly different between the groups and resveratrol did not alter the concentrations. However, when we analyzed intracellular NO levels (Figure 1B), cells incubated with plasma from PE group had lower levels compared with HP and addition of resveratrol did not alter these levels. We also measured total GSH levels in cell lysates (Figure 1C). There was no difference between PE and HP, however addition of resveratrol increased GSH levels only in HP group. ROS levels were also examined (Figure 1D) and, although there was no difference between PE and HP groups, resveratrol was able to non-statistically decrease ROS levels in PE by ~ 30 %. For ROS levels, positive control was 19.78±2.29 of fluorescence intensity $x10^3$. Finally, ARE activity (Figure 1E) was absent in HP however, in PE it was significantly increased and resveratrol potentiated this increase (~ 78%).

Besides the *in vitro* study of plasma incubation from PE and HP patients and resveratrol, we performed a pilot clinical study to compare the effects of serum incubation in endothelial cells from PE women before and after 1 hour of whole red grape fruit juice ingestion. Serum from the 1 h group significantly decreased ~ 17 % HO-1 concentration (Figure 2A), ~ 91 % GSH levels (Figure 2C) and ~ 69 % ARE activity (Figure 2E) compared with 0 h (basal). However, it increased intracellular NO levels (Figure 2B) and did not alter ROS (Figure 2D). For ROS levels, positive control was 20.38±2.29 of fluorescence intensity $x10^3$.

DISCUSSION

This study was the first to report that plasma from PE patients (PE) increased ARE activity in endothelial cells compared with plasma from healthy pregnant (HP), and the addition of resveratrol was able to potentiate this increase. Most interestingly, serum from PE patients collected after one hour of grape juice intake decreased ARE activity and HO-1 production in endothelial cells compared with serum from PE before grape juice intake, however it increased NO production. These findings show that even after one hour of acute grape juice intake, serum from PE was able to affect endothelial cells, acting on more mechanisms compared with cotreatment of plasma from PE and resveratrol. Collectively, our results suggest that the effects of resveratrol and other components present in grape juice and its metabolites may be beneficial for endothelial cells, and therefore, grape juice intake could aid in the management of PE.

Resveratrol is a polyphenolic compound that has multiple properties and can modulate several cell-signaling molecules. Studies have shown that resveratrol improves the therapeutic outcome of patients suffering from diabetes mellitus, cancer, Alzheimer's disease, kidney diseases, inflammatory diseases, cardiovascular diseases and complicated pregnancies.[40,41] Regarding PE, there are few studies about the effects of resveratrol, however the results are promising.[9–12,34] Our group has recently demonstrated that resveratrol improves endothelial cells markers impaired by plasma incubation from patients before the development of PE symptoms, suggesting its potential in the prevention of this syndrome.[34] In this study, we observed that resveratrol also improves endothelial cells markers impaired by incubation of plasma from women with established symptom of PE, strengthen its potential in PE management.

It is well established that deficient NO bioavailability is involved in PE pathophysiology and it contributes to the development of the symptoms of this syndrome.[42,43] Moreover, it was showed that resveratrol is able to increase NO bioavailability in endothelial cells in situations of oxidative stress.[44] We found that NO production was decreased in HUVECs incubated with plasma from PE compared with HP, however resveratrol cotreatment with plasma from PE was not able to increase NO levels. Probably the used resveratrol

concentration was not sufficient to boost NO production. Another mechanism of action of resveratrol is the scavenging of ROS.[45] In fact, we observed that resveratrol decreased ROS production in HUVECs incubated with plasma from PE. Although it was demonstrated that oxidative stress was elevated in endothelial cells incubated with plasma from PE patients,[29] we did not observe this increase in ROS production in the HUVECs incubated with plasma from PE compared to HP. We speculate that this discrepancy might be because we used an immortalized healthy cell line of HUVECs for this study, as the maternal endothelium has been demonstrated to be impaired in PE.[46] Moreover, the incubation period and/or the quantity of plasma from PE added to these cells maybe was not enough to evidence a difference.

Since resveratrol also activates Nrf2,[45] which binds to ARE sequence upregulating the expression of antioxidant proteins such as HO-1, we investigated the expression of NFE2L2 and HMOX-1 genes and miRNAs from let-7 family in our groups. miRNAs are small noncoding RNAs that post-transcriptionally downregulate gene expression[19] and miRNAs from let-7 family target NFE2L2.[47] We did not find significant difference in NFE2L2, HMOX-1 and miRNAs expression between HUVECs incubated with plasma from PE and HP, and resveratrol did not affect these expressions. Regarding protein expression of HO-1, we also did not observe any significant difference however, ARE activity was increased by plasma from PE compared with HP and resveratrol was able to potentiate this increase. Under oxidative stress conditions, the cellular antioxidant systems are activated to bring back the cell to redox equilibrium,[48] which explains the increased ARE activity in cells incubated with plasma from PE. In addition, we also evaluated GSH levels in the HUVECs incubated with plasma from PE and HP, but there was no significant difference between the groups and resveratrol only increased GSH levels in HP group. Indeed, resveratrol can increase GSH levels via Nrf2/Are pathway activation, which upregulated enzymes involved in GSH biosynthesis.[45] However, it was demonstrated that glutathione system is impaired in PE through decreased glutathione peroxidase (GPX) activity associated with the synthesis of vasoconstrictive eicosanoids such as F2-isoprostanes and thromboxane, which are upregulated in PE.[49] This might explain why we did not find increased GSH levels in PE group.

In light of the interesting results obtained with cotreatment of resveratrol and PE plasma in the endothelial cells, we decided to perform a pilot study with PE patients and acute grape juice intake in order to evaluate the effects of the incubation of serum collected from these patients after one hour of grape juice intake in the endothelial cells, since grape fruit contains large amounts of resveratrol.[50] This one hour period was chosen based on a previous study showing that one hour was the peak absorption of polyphenols.[30] Serum from the 1 h group increased NO production, decreased HO-1, GSH and ARE activity and did not change ROS concentration compared with serum before grape juice intake incubated in the HUVECs. Since grape juices are rich in several antioxidants[51] and they might be present in the serum collected, the antioxidant systems of the cells are probably not being recruited, explaining the decreased content/activity we found in the antioxidant markers evaluated. Regarding NO production, it was showed previously that grape juice is able to induce eNOS expression leading to an increase in NO formation in endothelial cells.[52] Moreover, studies also have showed that grape juice causes endothelium-dependent relaxation in coronary artery and ameliorates cardiovascular autonomic modulation in L-NAME-treated rats.[53,54] Most interestingly, it was demonstrated that consumption of grape-wine extract decreased ambulatory blood pressure in mildly hypertensive subjects.[55] Taking together, our results and these studies evidence the beneficial effects of grape juice in the vascular system, which suggest its potential to assist the management of PE.

Based on our results, it is possible to suggest that resveratrol and grape juice act on endothelial cells in different manners leading to beneficial effects in PE (Figure S3). Although resveratrol is a potent antioxidant and there are several evidences regarding its potential to assist different diseases,[40] the grape juice is a rich source of other antioxidants besides resveratrol[51] that can act synergistically enhancing the effects of resveratrol and activating other mechanisms.[56] In addition, grape juice is more accessible to patients and it is simple to include in the diet. However, it is necessary to be careful with its intake due to the high sugar content.[57]

In conclusion, we found that resveratrol cotreated with plasma from PE patients and serum from women with PE collected after acute grape juice intake ameliorate endothelial cells

markers and antioxidant defense. However, our results are still preliminary and there are limitations in the study. Nevertheless, our study provides more evidence for the use of resveratrol treatment and introduce the potential of grape juice supplementation in the management of PE.

Perspectives

This study shows the beneficial effects of resveratrol and grape juice in endothelial cell function using an *in vitro* model of preeclampsia. Resveratrol alone and the grape juice that has several antioxidants can respectively improve redox status and NO production, which are essential to attenuate preeclampsia symptoms. Based on our results and the literature available, it is possible to suggest that more efforts should be made to deeper explore preclinical and clinical effects of resveratrol and grape juice in preeclampsia. We strongly believe in their potential to assist and improve the preeclampsia management.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Plasma from PE patients activates ARE sequence in endothelial cells and resveratrol is able to potentiate this activation.
- Serum collected from PE patients after one hour of grape juice intake induces NO production in endothelial cells and decreases ARE activity, HO-1 expression and GSH levels.

What Is Relevant?

- Resveratrol and grape juice have beneficial effects on endothelial cells suggesting their potential in attenuating the hypertension symptom in PE.

Summary

Resveratrol cotreated with plasma from PE patients and serum from women with PE collected after acute grape juice intake ameliorate endothelial cells markers and antioxidant defense, in different manners. Our results evidence the potential of resveratrol and grape juice to assist the management of PE.

Figure Legends

Figure 1. HO-1 concentrations (A), NO (B), total glutathione (C) and ROS (D) levels and ARE activity (E) in HUVECs. Cells were incubated with 10% (v/v) plasma samples from preeclamptic patients (PE, n=8) and healthy pregnant (HP, n=4) in the absence (-R) or presence of trans-resveratrol (+R) at 1 μM for 24 hours. For ROS levels, mean and standard error for positive control was 19.78±2.29 of fluorescence intensity $x10^3$. Plasmas from PE decreased NO levels and increased ARE activity compared with HP. Addition of resveratrol decreased ROS levels and ARE activity only in PE group. Resveratrol was able to increase total glutathione levels only in HP group. DMSO was used as vehicle for trans-resveratrol. Values are means ± S.E.M. Comparisons between HP and PE groups were by t-test and paired t-test when comparing each group with respective trans-resveratrol treatment. * $p < 0.05$ vs. HP –R, # $p < 0.05$ vs. respective group –R.

Figure 2. HO-1 concentrations (A), NO (B), total glutathione (C) and ROS (D) levels and ARE activity (E) in HUVECs. Cells were incubated with 10% (v/v) serum from preeclamptic (PE) patients before (0 h) and after (1 h) 200 mL of whole red grape juice ingestion for 24 hours. For ROS levels, mean and standard error for positive control was 20.38±2.29 of fluorescence intensity $x10^3$. Serum from PE patients after 1 hour of grape juice ingestion decreased HO-1 concentration, total glutathione levels and ARE activity and increased NO levels compared with before. Values are means ± S.E.M. Comparisons between groups were by t-test. * $p < 0.05$ vs. 0 h.

Tables

Table 1. Clinical characteristics of pregnant enrolled in the study.

Parameters	**HP (n=4)**	**PE (n=8)**
GA at sampling (weeks)	36 ± 1	35 ± 2
Maternal Age (years)	24 ± 3	26 ± 3
BMI (kg/m²)	28 ± 3	30 ± 3
SBP at sampling (mmHg)	110 ± 7	133 ± 4*
DBP at sampling (mmHg)	73 ± 5	86 ± 3*
GA at delivery (weeks)	40 ± 1	35 ± 2*
Newborn weight (g)	2945 ± 135	2513 ± 419
Antihypertensive drugs at blood collection [1]		
α-Methyldopa (%)	NA	75
Nifedipine (%)	NA	12.5
Hydralazine (%)	NA	12.5

Values are the means ± S.D. or percentage. GA, gestational age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NA, not applicable. * P<0.05 vs controls.

[1] All PE patients were under antihypertensive drugs treatment.

ONLINE SUPPLEMENT

Resveratrol and Grape Juice: Effects on Redox Status and Nitric Oxide Production of Endothelial Cells in In Vitro Model of Preeclampsia

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DETAILED METHODS

Cell culture, plasma/serum incubation and trans-resveratrol intervention

Human umbilical vein endothelial cells (HUVEC) (CRL 2873, American Type Culture Collection (ATCC), VA, USA) were cultured in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 50 μg/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml amphotericin B (Gibco) in an incubator with a 5% CO_2 atmosphere at 37ºC. When reaching 80-90% of confluence, HUVECs were incubated in supplemented DMEM with 10% (v/v) plasma from PE or HP and 1 μM of trans-resveratrol (Cayman Chemical®, MI, USA) or the vehicle DMSO (Sigma-Aldrich®, Poole, UK) in the incubator for 24 hours. The concentration of 1 μM was chosen based on previous studies using trans-resveratrol *in vitro*, including one of our group.[1–4] HUVECs were also incubated with 10% (v/v) serum from PE at 0 h (before grape juice intake) and 1 h (after grape juice intake) for 24 hours. Cells were used until passage 8.

Cell viability by MTT assay

Cell viability was performed using the 3-(4,5-dimethylthiazol2-yl)−2,5-diphenyltetrazolium (MTT) assay as described previously.[5] HUVECs were seeded in a 96-well plate and, after 24 hours of incubation, MTT solution (0.5 mg/ml PBS) (Sigma-Aldrich®) was added and the plate was incubated for 3 hours. MTT is reduced to blue formazan crystals by metabolically active cells. After 3 hours, MTT solution was removed and DMSO (Sigma-Aldrich®) was added for 10 min incubation. Optical density was measured at 570 nm on the spectrophotometer (Synergy 4, BioTek, VT, USA). Blank well was DMSO alone. Viability was compared to control (untreated cells with vehicle DMSO, 100% viability). Each sample was performed in triplicate.

Messenger RNA expression by qPCR

Total RNA from HUVECs incubated with plasma from PE and HP in the presence or absence of trans-resveratrol was isolated using the miRNeasy Micro Kit (Qiagen, Leusden, Netherlands), according to the manufacturer's protocol. Isolated RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, MA, USA), and it was consistently found to be pure. RNAs were transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, ON, Canada), according to the manufacturer's protocol. Each qPCR reaction contained 10 μL of Power SYBR™ Green PCR Master Mix (Thermo Scientific), 100 nM of each primer (forward and reverse), 5 μL of cDNA (6.5 ng/μL), and a variable amount of nuclease-free water to a final volume of 20 μL. The analyzed genes were: *NFE2L2*, *HMOX1* and *HPRT1*. According to previous study,[6] *HPRT1* was selected as an endogenous control because it was the gene most stable in our samples. Primers of these genes (KiCqStart™ SYBR® Green Primers) were purchased from Sigma-Aldrich®. Thermal cycling conditions were: 10 min at 95 °C, 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C, and a final step for the dissociation curve. Relative quantification was calculated using the comparative 2(-Delta C(T)) method.[7] PCR reactions were performed in duplicate for each sample.

miRNA selection and expression by qPCR

miRNAs from the let-7 family (a, b and c) were selected as they target the mRNA of the *NFE2L2* gene, based on bioinformatics analysis using two databases (Microrna.org[8] and miRWalk 2.0[9]). Those miRNAs are expressed in HUVECs.[10] From the same isolated RNA used for messenger RNA expression, reverse transcription (RT) was performed using the miScript II RT Kit (Qiagen®), according to the manufacturer's protocol. The reaction was performed using miScript HiSpec Buffer, which selectively converts mature miRNAs and certain small nucleolar RNAs and small nuclear RNAs into cDNA. For qPCR, miScript SYBR® Green PCR Kit (Qiagen®) was used and each reaction contained 10 μL of QuantiTect SYBR Green PCR Master Mix, 2 μL of universal primer, 300 nM of each primer, 4 μL of nuclease-free water and 2 μL of cDNA (1.5 ng/μL), in a final volume of 20 μL. Primers of the selected miRNAs were purchased from Qiagen®. Thermal cycling conditions were: 15 min at 95 °C, 40 three step cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 70 °C, and a final step for the dissociation curve. Relative quantification was calculated using the comparative 2(-Delta Delta C(T))[7] and normalization was performed to U6 snRNA. PCR reactions were performed in duplicate for each sample.

Measurement of HO-1 concentrations

HO-1 quantification in cell supernatants was accessed using enzyme-linked immunosorbent assay kit Human Total HO-1/HMOX1 ELISA (R&D Systems, MN, USA), according to the manufacturer's protocol. Briefly, in a half area 96-well, 50 μL of capture antibody was added and incubated overnight. Then, the plate was washed 3 times with wash buffer and 50 μL of block buffer was added for 1 h. Washes were repeated and 50 μL of standards and samples was added for 2 h. After 2 h, washes were performed again and 50 μL of the detection antibody was added and incubated for other 2 h. Washes were repeated and 50 μL of streptavidin-HRP was added and incubated for 20 min. Final washes were performed and 50 μL of substrate solution was added and incubated for more 20 min. 25 μL of stop solution was added and optical density was determined at 450 nm in the spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by incubation of HO-1 solutions (156.25–10000 pg/mL) with the previous reagents. Each sample was performed in duplicate.

Measurement of intracellular NO

Quantification of intracellular NO was accessed by measuring the fluorescence of 4,5-Diaminofluorescein (DAF-2) diacetate (Cayman Chemical®). A total of $5x10^4$ HUVEC per well were plated and treated into a black 96-well plate. After 24h of incubation, the cells were washed with 95 μL of saline solution (PBS) and incubated for 30 minutes at 37ºC. Then, cells were loaded with 5 μL of DAF-2 and read for 2 hours in 37ºC in a microplate reader (Synergy 4, BioTek®). The fluorescence signal was measured (excitation 495 nm, emission 535 nm) and expressed as arbitrary units. Each sample was performed in duplicate.

Glutathione quantification

Total glutathione (GSH) quantification was performed in cell lysates using the Glutathione Colorimetric Detection Kit (Invitrogen, MD, USA), according to the manufacturer's protocol. HUVECs were seeded in a 6-well plate and after reaching

confluency, cells were treated with plasmas in the presence or absence of 1 μM trans-resveratrol or serum from the groups for 24 hours. Then, cells were lysate and 50 μL of each sample was added in a half area 96-well plate with 25 μL of Colorimetric Detection Reagent and 25 μL of Reaction Mixture. The plate was incubated in room temperature for 20 min. Optical density was determined at 405 nm in the spectrophotometer (Synergy 4, BioTek®). A standard curve was generated by incubation of oxidized glutathione standard solutions (0.78–25 μM) with the previous reagents. Each sample was performed in duplicate.

Measurement of intracellular ROS

Quantification of intracellular ROS was accessed by measuring the fluorescence of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Cayman Chemical®). DCFH-DA is deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into the highly fluorescent compound 2,7-dichlorofluorescein (DCF). After plasma and resveratrol incubation, HUVECs were incubated with 25 μM of DCFH-DA in PBS for 45 min in the incubator. Fluorescence was determined using wavelengths of 502 nm of excitation and 523 nm of emission in a multifunctional plate reader (Synergy 4, BioTek®). Positive control was addition of Tert-Butyl hydroperoxide solution (Sigma-Aldrich®) at 250 μM for 2 hours. Each sample was performed in duplicate.

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FIGURES

Figure S1.

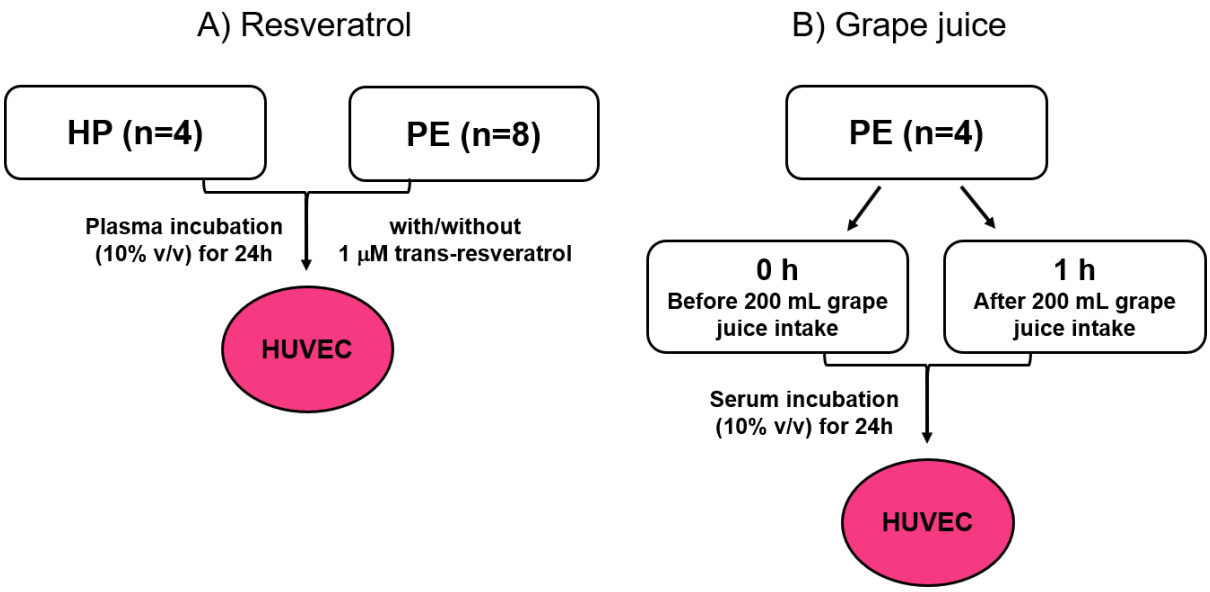

Figure S1. Schematic diagram of the study workflow.

Figure S2.

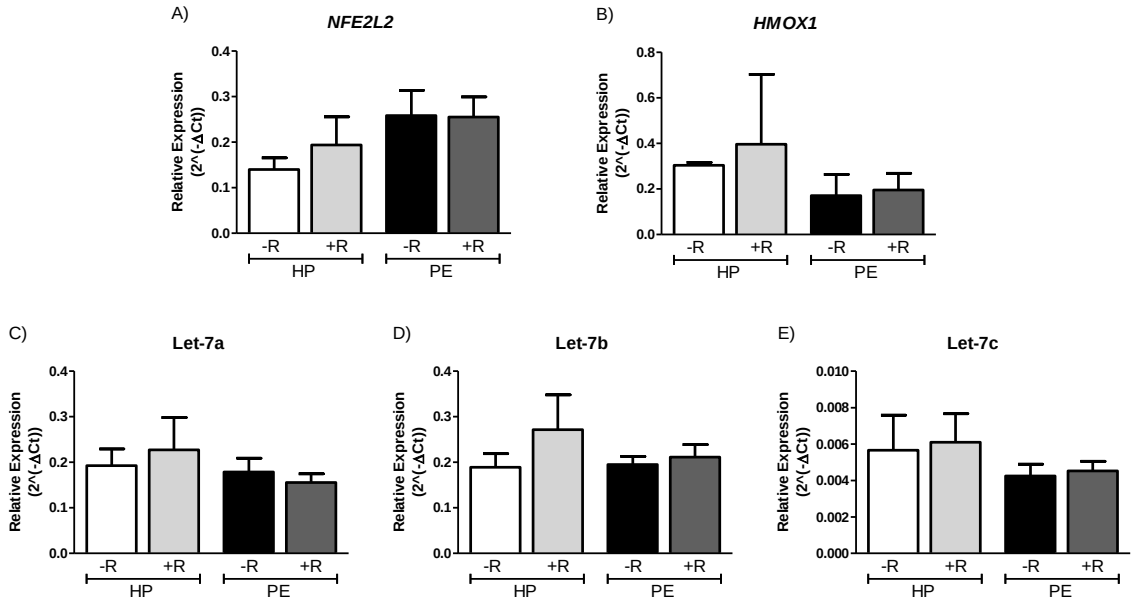

Figure S2. Relative expression of the genes *NFE2L2* (A) and *HMOX1* (B) and miRNAs let-7a (C), let-7b (D) and let-7c (E) in HUVECs. Cells were incubated with 10% (v/v) plasma samples from PE patients (PE, n=8) and healthy pregnant (HP, n=4) for 24h in the absence (-R) or presence of trans-resveratrol (+R) at 1 μM. Gene and miRNAs expression was not significantly different between HP and PE, and resveratrol did not alter the expression in none of the groups. DMSO was used as vehicle for trans-resveratrol. Values were normalized using *HPRT1* as endogenous control for gene expression and RNU6-2 for miRNAs. Values are means ± S.E.M.

Figure S3.

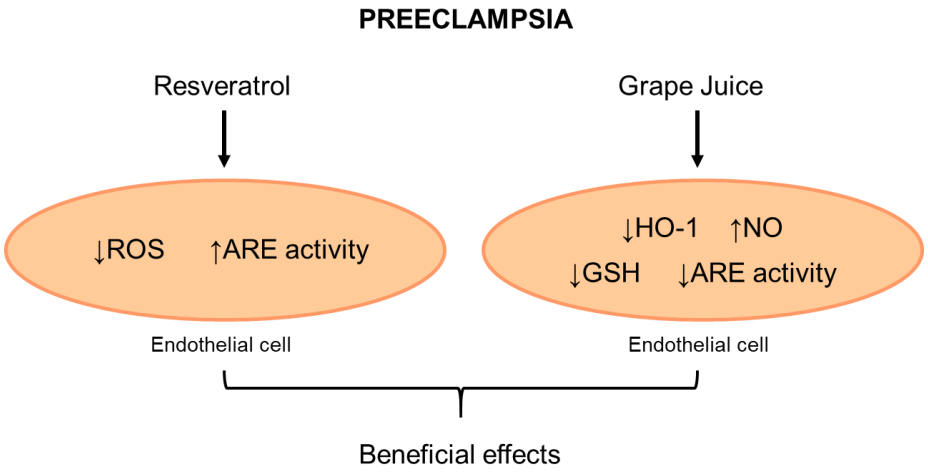

Figure S3. Mechanisms of action of resveratrol and grape juice in the endothelial cells in preeclampsia.

CONCLUSÃO

Com base nos estudos presentes na literatura e nos resultados dos manuscritos expostos, concluímos que há um potencial de uso do resveratrol na prevenção e tratamento da pré-eclâmpsia. O resveratrol é um composto natural presente em alimentos, como a uva, que podemos ingerir rotineiramente, é seguro na gravidez e não interfere na conduta clínica realizada atualmente nessa síndrome. Considerando ainda o uso do suco de uva ao invés do resveratrol isolado, por ser rico em diversos antioxidantes, esses podem ter uma ação conjunta potenciando ainda mais os efeitos benéficos na pré-eclâmpsia. Os estudos ainda são escassos e há muito que se investigar e aprofundar, logo futuros estudos devem ser realizados, principalmente estudos clínicos, para que haja embasamento científico suficiente para se sugerir como prática clínica o uso do resveratrol/suco de uva como auxílio na prevenção e manejo da pré-eclâmpsia.