JONAS TADEU CAU SERTORIO

“IMPLICAÇÕES FISIOPATOLÓGICAS DA DESCOMPARTIMENTALIZAÇÃO DA HEMOGLOBINA PARA A BIOLOGIA DO ÓXIDO NÍTRICO EM PACIENTES COM PRÉ-ECLÂMPSIA”

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ORIENTAÇÃO: Prof. Dr. JOSÉ EDUARDO TANUS DOS SANTOS

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para a obtenção do título de Doutor em Farmacologia

ESTE EXEMPLAR CORRESPONDE À
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JONAS TADEU CAU SERTORIO, E ORIENTADO PELO
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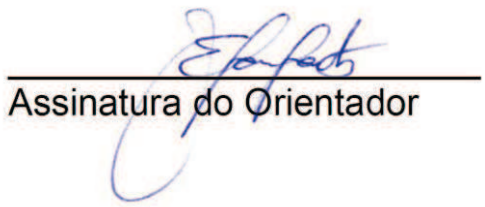

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DEDICATÓRIA

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Wilson e Isabel,
minha irmã Pâmela e
minha amada Daiane.

Por fazerem a palavra amor ter sentido.

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“O temor do Senhor é o princípio da sabedoria, e a ciência do Santo, a prudência”.

(Provérbios 9.10)

RESUMO

O óxido nítrico (NO) exerce um importante papel durante as alterações hemodinâmicas que normalmente acompanham uma gravidez sadia (GS). Entretanto, em mulheres com pré-eclâmpsia (PE), uma síndrome caracterizada por hipertensão e proteinúria, a vasodilatação da circulação sistêmica materna está comprometida. Isso possivelmente ocorre devido a uma menor biodisponibilidade de NO, a qual resulta de uma menor produção e uma maior degradação do NO. Como a hemoglobina livre no plasma sequestra o NO pela rápida reação de dioxigenação, o que pode comprometer a eficiência do processo de vasodilatação mediado pela via do NO/guanilato ciclase, formulamos a hipótese de que pacientes com PE, quando comparadas com grávidas sadias, apresentariam concentrações aumentadas de hemoglobina livre no plasma, o que resultaria em maior degradação (consumo) do NO e menor biodisponibilidade de NO. Além disso, também formulamos a hipótese de que os diferentes fenótipos da haptoglobina (Hp) podem modular a biodisponibilidade de NO ao influenciar o consumo de NO na PE. A haptoglobina é uma proteína polimórfica (Hp1-1, Hp2-1 e Hp2-2) que se liga à hemoglobina para formar um complexo (Hb-Hp) que é removido da circulação, prevenindo o consumo de NO e o estresse oxidativo induzido pela hemoglobina. Os produtos do gene da haptoglobina apresentam diferentes propriedades bioquímicas e biofísicas, o que pode influenciar a taxa de eliminação (clearance) do complexo Hb-Hp. Para avaliar a biodisponibilidade de NO, foram analisadas as concentrações de nitrito no plasma e no sangue total em um analisador de NO. O consumo de NO foi determinado no plasma através do método de quimioluminescência. As concentrações de hemoglobina plasmática e haptoglobina foram analisadas com kits de imunoensaio enzimático (ELISA). Os genótipos da haptoglobina foram determinados pela Reação em cadeia da polimerase em tempo real (RT-PCR). Encontramos concentrações reduzidas de nitrito no plasma e no sangue total de grávidas com PE comparado com grávidas sadias. As amostras de plasma de grávidas com PE consumiram mais NO e apresentaram maiores concentrações de hemoglobina livre do que as amostras de grávidas sadias. Além disso, verificamos que há correlação positiva entre as concentrações de hemoglobina plasmática e o consumo de NO, correlação negativa entre o consumo de NO e as concentrações de nitrito plasmático e no sangue total e, por fim, correlação negativa entre as concentrações de hemoglobina livre plasmática e as concentrações de nitrito no

plasma e no sangue total. Esses dados sugerem que níveis aumentados de Hb livre resultam em consumo de NO aumentado e baixa biodisponibilidade de NO em pacientes com PE, quando comparados com grávidas sadias. Não encontramos diferenças nas frequências dos genótipos da haptoglobina entre grávidas com PE e GS. Em grávidas sadias, os genótipos da haptoglobina não apresentaram efeitos nos níveis de hemoglobina livre, no consumo de NO e nas concentrações de nitrito plasmático. Entretanto, pacientes com PE e genótipos Hp2-1 e Hp2-2 apresentaram níveis aumentados de hemoglobina livre (heme), consumo de NO elevado e concentrações reduzidas de nitrito quando comparados com pacientes com PE e genótipo Hp1-1. Esses achados indicam que apesar de o genótipo da haptoglobina não afetar o risco de se desenvolver PE, o genótipo Hp1-1 possivelmente exerce um papel de proteção na PE ao reduzir o sequestro de NO, enquanto que os genótipos Hp2-1 e Hp2-2 podem agravar a PE ao reduzir a biodisponibilidade de NO.

Palavra-chave: Haptoglobina, hemoglobina, hemólise, oxido nítrico, polimorfismo, pré-eclâmpsia.

ABSTRACT

Nitric oxide (NO) plays an important role in the hemodynamic changes found during normal pregnancy (NP). However, preeclampsia (PE), a syndrome characterized by hypertension and proteinuria, is associated with impaired vasodilation of the maternal systemic circulation, possibly due to decreased NO bioavailability resulting of lower NO production and increased NO degradation. Since cell-free hemoglobin scavenges NO through a high speed dioxygenation reaction, thus compromising the efficiency of the NO/soluble guanylyl cyclase pathway to elicit vasodilatory activity, we hypothesized that higher plasma hemoglobin concentrations exist in patients with preeclampsia compared with normal pregnant women, and the higher plasma hemoglobin concentrations could lead to increased NO consumption and lower NO bioavailability in preeclamptic patients. Moreover, we hypothesized that haptoglobin (Hp) phenotypes might modulate NO bioavailability by influencing NO consumption in preeclampsia. Haptoglobin is a polymorphic protein (Hp1-1, Hp2-1 and Hp2-2) that binds hemoglobin to form a complex that is removed from circulation, thus preventing Hb-driven oxidative stress and NO scavenging. Hp protein products differ in biochemical and biophysical properties, which reflects on the Hb-Hp complex clearance rate. To assess NO bioavailability, we measured plasma and whole blood nitrite concentrations using an ozone-based chemiluminescence assay. A NO consumption assay was used to measure NO consumption. Plasma hemoglobin and haptoglobin concentrations were assessed with a commercial immunoassay (ELISA). Haptoglobin genotypes were determined using Real Time Polymerase Chain Reaction. We found lower whole blood and plasma nitrite concentrations in preeclamptic patients compared with normal pregnant women. Plasma samples from preeclamptic women consumed more NO and had higher plasma Hb levels than those found in normal pregnant women. We found significant positive correlations between plasma Hb and plasma NO consumption, negative correlations between plasma NO consumption and whole blood and plasma nitrite concentrations, and negative correlations between plasma Hb and whole blood and plasma nitrite concentrations. These findings suggest that increased plasma Hb levels lead to

increased NO consumption and lower NO bioavailability in preeclamptic patients compared with healthy pregnant women. Furthermore, we found no differences in Hp genotype frequencies between preeclamptic and NP groups. Haptoglobin genotypes had no effects on plasma heme levels, NO consumption and plasma nitrite in normal pregnant women. However, in preeclampsia, Hp2-1 and Hp2-2 were associated with higher plasma heme levels, increased NO consumption, and lower plasma nitrite compared with Hp1-1. These findings indicate that although haptoglobin genotype does not affect the risk of preeclampsia, Hp1-1 genotype may exert a protective role in preeclampsia by reducing NO scavenging, whereas Hp2-1 and Hp2-2 may further aggravate preeclampsia by reducing NO bioavailability.

Keywords: Haptoglobin, hemoglobin, hemolysis, nitric oxide, polymorphism, preeclampsia.

BH_4	Tetrahidrobiopterina
ELISA	Ensaio imunoenzimático ligado à enzima
eNOS	Sintase Endotelial do Óxido Nítrico
FAD	Flavina-adenina dinucleotídeo
FMN	Flavina mononucleotídeo
GMPc	Monofosfato Cíclico de Guanosina
GS	Gestantes saudáveis
Hb	Hemoglobina
HELLP	Hemólise, aumento das enzimas hepáticas e plaquetopenia
mmHg	Milímetros de mercúrio
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NO	Óxido Nítrico
NO_2^-	Nitrito
NO_3^-	Nitrato
NOS	Sintase de Óxido Nítrico
PA	Pressão arterial
PLGF	Fator de crescimento placentário
PE	Pré-eclâmpsia
RTPCR	Reação da polimerase em cadeia em tempo real

sENG Endoglina solúvel

sFlt-1 Tirosina quinase 1 semelhante fms solúvel

sVEGFR1 Receptor solúvel para o fator de crescimento endotelial vascular-1

TGFβ Fator transformador de crescimento beta

VEGF Fator de crescimento endotelial vascular

SUMÁRIO

1 – INTRODUÇÃO

1.1 – Fisiopatologia da Pré-eclâmpsia

A pré-eclâmpsia (PE) é uma síndrome sistêmica clinicamente caracterizada por hipertensão e proteinúria, após a 20° semana de gestação. A PE afeta de 3 a 5% das mulheres grávidas, sendo a principal causa de mortalidade materna e fetal no mundo [1]. No Brasil, a PE é responsável por 37% das causas de morte obstétricas diretas [2]. Diversos fatores tais como doença autoimune crônica, obesidade, diabetes e hipertensão pré-existentes, doença renal, etnia, primigravidez, PE em uma gestação prévia e história familiar de PE estão associados com maior risco de se desenvolver PE [3].

A pré-eclâmpsia parece progredir em dois estágios: pré-clínico (1° e 2° trimestres) e clínico (3° trimestre). O primeiro estágio é marcado por um processo de placentação anormal, seguido pela produção de fatores antiangiogênicos pela placenta, os quais entram na circulação materna e causam disfunção endotelial generalizada. O segundo estágio é caracterizado por hipertensão e proteinúria, os sinais clínicos da pré-eclâmpsia [4].

Em uma gestação normal, os citotrofoblastos invadem a decídua e as arteríolas espiraladas maternas, promovendo o remodelamento do endotélio e a destruição do tecido elástico-muscular das arteríolas, transformando-as em vasos de alta capacitância e baixa resistência [4]. Esse processo, referido como “pseudovasculogênese”, tem início no final do primeiro trimestre e termina entre a 18° e a 20° semana de gestação, o que permite um maior suprimento sanguíneo para o feto em crescimento [5]. Entretanto, na pré-eclâmpsia, parte das artérias uterinas espiraladas não sofre invasão trofoblástica, impedindo o remodelamento vascular adequado à crescente demanda de fluxo sanguíneo durante o progresso da gestação [6]. Com isso, devido à redução na perfusão uteroplacentária, a placenta torna-se isquêmica com o progresso da gestação, liberando fatores antiangiogênicos para a circulação materna, os quais podem causar disfunção endotelial generalizada [7-9].

A expressão aumentada de fatores antiangiogênicos produzidos pela placenta está associada a níveis plasmáticos aumentados desses fatores em

mulheres com pré-eclâmpsia [10, 11]. Um desses fatores, a forma solúvel do receptor para o fator de crescimento endotelial vascular-1 (sVEGFR1), também conhecido como sFLT-1 (do inglês soluble fms-like tyrosine kinase-1), liga-se ao fator de crescimento endotelial vascular (VEGF) e ao fator de crescimento placentário (PLGF), bloqueando efeitos como a angiogênese [12], a síntese de óxido nítrico pelas células endoteliais [13] e os efeitos vasodilatadores promovidos pelo VEGF e pelo PLGF [14].

Um segundo fator antiangiogênico que está aumentado na PE, a endoglina solúvel (sENG), é o co-receptor para os ligantes TGFβ-1 e TGFβ-3 [15]. Ao se ligar ao TGβ-1 e ao TGβ-3, a sENG impede que esses fatores liguem-se à endoglina, o que pode reduzir a produção de NO e, consequentemente, a vasodilatação arterial induzida pelo NO [11]. Além disso, a endoglina pode modular a expressão e a atividade da eNOS, afetando, portanto, o tônus vascular [16, 17].

1.2 – Papel do óxido nítrico na gestação

O óxido nítrico (NO) desempenha papéis fisiológicos fundamentais no sistema cardiovascular. Neste sistema, é produzido principalmente pela enzima sintase endotelial do óxido nítrico (eNOS), a qual é expressa constitutivamente em células endoteliais [18]. O NO produzido pela eNOS exerce importante controle no fluxo sanguíneo ao promover o relaxamento do músculo liso vascular [19]. A eNOS utiliza L-arginina, oxigênio molecular e diferentes substratos (NADPH, FAD, FMN e BH_4) para a produção de NO e L-citrulina. Após sua produção e liberação, o NO ativa a enzima guanilato ciclase ao se ligar à porção heme desta enzima, levando à produção de GMP cíclico. Este último relaxa o músculo liso vascular por interferir na concentração citoplasmática de cálcio [20]. Além de contribuir para a vasodilatação, o NO é um regulador crítico da homeostase vascular, pois inibe a agregação plaquetária [21], a adesão de leucócitos [22], a proliferação de células do músculo liso vascular [23] e estimula a angiogênese [24], além de ter atividade antioxidante e antiinflamatória [25].

Durante uma gestação normal, o organismo materno está sujeito a uma série de alterações cardiovasculares e hemodinâmicas [26, 27]. A partir do primeiro trimestre de gestação, há aumento da frequência cardíaca, do volume plasmático e do débito cardíaco, os quais são acompanhados por uma queda na resistência vascular periférica e na pressão sanguínea [28]. Entretanto, em mulheres com pré-eclâmpsia, a vasodilatação da circulação sistêmica materna está comprometida [29], o que pode ser devido a uma reduzida biodisponibilidade de NO [8], a qual resulta de uma menor produção [30] e de uma maior degradação de NO [31]. De fato, a quantidade de NO biodisponível resulta do balanço entre a sua síntese e a sua degradação. Esse balanço é controlado dinamicamente por diversos processos, vários dos quais podem estar alterados na pré-eclâmpsia. Por exemplo, níveis alterados de fatores angiogênicos [10], aumento na expressão da enzima arginase [32], maior estresse oxidativo [33], concentrações alteradas de tetrahidrobiopterina (cofator para a eNOS) [34], inibição da eNOS devido a altas concentrações de dimetilarginina assimétrica (ADMA) [35] e variações genéticas [36, 37] parecem comprometer a biodisponibilidade de NO na pré-eclâmpsia.

1.3 – Hemólise como causa da baixa biodisponibilidade do NO

Além dos diversos fatores brevemente discutidos acima, há evidências apontando a relevância de outro mecanismo capaz de aumentar a degradação do NO. Este mecanismo fisiopatológico envolve o aumento das concentrações de hemoglobina livre no plasma, comprometendo a biodisponibilidade do NO produzido pelo endotélio, o que pode contribuir para a disfunção endotelial, aumento da expressão de moléculas de adesão, ativação plaquetária e produção de espécies reativas de oxigênio [38-44].

Usualmente encontrada em altas concentrações em pacientes com anemias hemolíticas, a hemoglobina livre no plasma reage rapidamente com o NO através da clássica reação de dioxigenação, produzindo metahemoglobina e nitrato, o que destrói efetivamente as atividades protetoras cardiovasculares do NO [45]. Em condições fisiológicas, a taxa de reação entre o NO e a hemoglobina é muito lenta, uma vez que a hemoglobina está

compartimentalizada dentro dos eritrócitos [46, 47]. Além da barreira física criada pela membrana dos eritrócitos, o fluxo sanguíneo intravascular tende a manter os eritrócitos fluindo nas regiões mais centrais da luz dos vasos, distantes do endotélio, onde o NO é produzido, o que contribui para criação de uma barreira que evita a degradação (consumo) do NO pela hemoglobina [48].

Entretanto, quando há hemólise intravascular, nenhum desses mecanismos protege o NO contra interações imediatas com a hemoglobina, levando a consumo aumentado de NO quando a hemoglobina sai do compartimento eritrocitário [47, 49]. Deste modo, a rápida dioxigenação do NO reduz sua biodisponibilidade, comprometendo a ativação fisiológica da via NO/cGMP e o processo de vasodilatação [50].

Outro fator agravante após a descompartimentalização da hemoglobina é a liberação da enzima arginase-1, a qual compete com a eNOS pela L-arginina (substrato para a produção de NO), reduzindo as concentrações deste aminoácido, o que contribui para menor produção de NO [40]. A baixa biodisponibilidade de L-arginina também leva ao desacoplamento da eNOS, a qual passa a produzir radicais livres que reduzirão ainda mais as concentrações de NO [20]. Deste modo, a hemoglobina livre causa diferentes efeitos pró-oxidantes, proliferativos e pró-inflamatórios no sistema cardiovascular [44, 51].

As alterações bioquímicas induzidas pela hemoglobina livre e pelo consumo de NO podem agravar diversas doenças e condições em que a disfunção endotelial possui um papel central, tais como anemia falciforme [52], talassemias [42], malária [53], “bypass cardiopulmonar” [42], anemia induzida por drogas [54] e, mais recentemente, transfusão de bolsas de sangue armazenadas por longos períodos [43]. Ainda que pacientes com estas diferentes condições hemolíticas apresentem sintomas únicos, eles frequentemente compartilham sequelas relacionadas ao aumento das concentrações de hemoglobina livre tais como hemoglobinúria, dor abdominal, espasmo esofágico, disfagia, aumento na pressão arterial, ativação plaquetária, etc, com aumento da taxa de mortalidade [42]. Ainda, observações realizadas após administração de carreadores de oxigênio à base de hemoglobina

(HbOCs) em humanos, apoiam as evidências de que há uma relação entre o excesso de hemoglobina livre na corrente sanguínea e alterações cardiovasculares como hipertensão e aumento da resistência vascular sistêmica nestes pacientes [55-57].

Recentemente, as complicações resultantes do consumo de NO pela hemoglobina livre em doenças hemolíticas passaram a receber maior atenção. Isto se deve em parte, ao fato de se acreditar que os níveis de hemoglobina plasmática eram considerados muito baixos para produzirem alguma alteração fisiopatológica relevante. Entretanto, evidências encontradas em experimentos com animais transgênicos, animais de grande porte e estudos epidemiológicos em humanos tem contribuído para o entendimento do papel da hemoglobina na patofisiologia de doenças hemolíticas [41, 50, 58, 59]. Em pacientes com anemia falciforme, mostrou-se que concentrações de hemoglobina livre tão baixas quanto 6 μM (em heme) são suficientes para inibir a vasodilatação mediada pela acetilcolina [60]. Outro experimento mostrou respostas reduzidas ao NO em tecido de coelhos que foram infundidos com baixas concentrações de hemoglobina (6μM em heme) [61]. Também foi demonstrado que a hemólise intravascular resulta em vasoconstrição e comprometimento renal em um modelo canino de hemólise intravascular [50].

Na pré-eclâmpsia, Sarrel PM *et al* propôs que níveis aumentados de hemoglobina livre poderiam limitar a vasodilatação mediada pelo NO [62]. Além disso, mostrou-se que as subunidades da hemoglobina alfa2 e gama estão expressas em grande quantidade na placenta de grávidas com PE, o que poderia levar ao acúmulo de hemoglobina no lúmen dos vasos da placenta, resultando em extravasamento de hemoglobina fetal (HbF) e adulta (HbA) para a circulação materna [63].

1.4 – Clearance da hemoglobina na hemólise intravascular

A haptoglobina (Hp) é uma proteína plasmática produzida pelos hepatócitos, a qual apresenta alta afinidade pela hemoglobina [64]. Sua síntese é rapidamente aumentada em resposta a diferentes estímulos inflamatórios e sua concentração normal varia de 0,3 à 3 mg/ml [65]. Quando os eritrócitos são destruídos dentro do compartimento vascular, o tetrâmero que constitui a

hemoglobina se divide, formando dímeros que rapidamente se ligam à haptoglobina [66]. O complexo hemoglobina-haptoglobina (Hb-Hp) é reconhecido pelo receptor de macrófagos/monócitos CD163 [67], ao qual se liga com grande afinidade, sendo então internalizado e degradado por enzimas lisossomais após sua endocitose. Após a degradação da proteína ligante, o grupo heme é convertido pela enzima heme oxigenase-1 em monóxido de carbono, biliverdina e ferro [68]. O ferro é estocado pela ferritina, evitando seus efeitos oxidativos, enquanto que a biliverdina é convertida em bilirrubina pela enzima biverdina redutase [69]. Diversos trabalhos mostram que o monóxido de carbono e a biliverdina apresentam propriedades anti-inflamatórias e antioxidantes [70, 71].

Outro mecanismo de proteção contra a hemoglobina livre atua em paralelo com a haptoglobina [72, 73]. Após a descompartimentalização da hemoglobina, o grupo heme contendo o átomo de ferro no estado ferroso (Fe^{2+}) é oxidado para o estado férrico (Fe^{3+}), sendo então liberado da molécula de hemoglobina. Este grupo heme no estado férrico se liga com alta afinidade à glicoproteína plasmática hemopexina, a qual é internalizada após ser reconhecida pelo receptor LRP/CD91, presente em monócitos, hepatócitos e em outras células. Por fim, o complexo hemopexina-heme é degradado por enzimas lisossomais e pela heme oxigenase [72].

Além desses mecanismos diretos de proteção contra os efeitos da hemoglobina livre, ao se ligar ao receptor CD163, o complexo Hb-Hp sinaliza para a produção de heme oxigenase-1 nos monócitos circulantes e para a produção da citocina antiinflamatória IL-10 [74]. Deste modo, os efeitos antioxidantes, anticoagulantes e antiproliferativos dos sistemas CD163/heme oxigenase-1/biliverdina redutase parecem representar uma forma compensatória para o consumo do NO e para os efeitos pró-oxidantes e vasoconstritores promovidos pela hemoglobina livre e pelo grupo heme.

1.5 – Polimorfismos da Haptoglobina

O gene para a haptoglobina está localizado no cromossomo 16q22. Existem no homem duas classes de alelos para a Hp (1 e 2), que geram três

diferentes genótipos (1-1, 2-1 e 2-2) [66]. O alelo humano Hp1 é composto por 5 exons, sendo encontrado em todos os mamíferos. O alelo Hp2 está presente somente em humanos, é composto por 7 exons e parece ter evoluído a partir do alelo Hp1 pela duplicação dos exons 3 e 4 [75]. O produto protéico do gene da haptoglobina é um monômero, encontrado no plasma como um polímero constituído de 2-10 monômeros covalentemente ligados. A estequiometria do polímero da haptoglobina é Hp-genótipo-dependente devido à diferença nas valências da Hp1 (monovalente) e Hp2 (bivalente). Isso ocorre, pois o domínio de multimerização do Hp está localizado no exon 3 do gene da Hp, o qual está duplicado no alelo Hp2 [76]. Como resultado dessas diferenças, em indivíduos Hp1-1, a haptoglobina apresenta-se na forma de dímeros (2 monomêros de Hp), nos indivíduos Hp2-1 como um polímero linear (2-8 monômeros) e nos Hp2-2 como um polímero cíclico (3-10 monômeros) [77].

Alguns trabalhos mostram que, mesmo após a ligação entre a hemoglobina e a haptoglobina, o complexo Hb-Hp é capaz de inibir o relaxamento dependente do endotélio [78]. Isso pode ser explicado pela capacidade da hemoglobina em consumir o NO produzido pelo endotélio, mesmo estando ligada à haptoglobina [39, 46]. Desse modo, é possível que, devido às diferenças nas estruturas moleculares, os polimorfismos da haptoglobina possam influenciar os mecanismos de proteção contra o estresse oxidativo dirigido pela hemoglobina e contra o consumo do NO na pré-eclâmpsia [46, 79].

2 – HIPÓTESE

Formulamos a seguinte hipótese: mulheres com pré-eclâmpsia apresentam níveis aumentados de hemoglobina livre no plasma, os quais estão associados com maior consumo de NO e menor biodisponibilidade de NO quando comparados com os níveis desses marcadores em grávidas sadias. Também propusemos que os diferentes genótipos da haptoglobina podem modular as concentrações de hemoglobina livre, os níveis de consumo de NO e a biodisponibilidade de NO na pré-eclâmpsia.

3 – OBJETIVOS

1- Determinar os níveis de hemoglobina livre no plasma, consumo de NO pelo plasma e as concentrações dos produtos de oxidação do NO, nitrito e nitrato em grávidas sadias e mulheres com pré-eclâmpsia.
2- Correlacionar o consumo de NO com os níveis de hemoglobina livre.
3- Correlacionar o consumo de NO e os níveis de hemoglobina livre com as concentrações de nitrito no plasma e no sangue total.
4- Determinar os genótipos e as concentrações de haptoglobina.
5- Avaliar a influência dos genótipos da haptoglobina sobre os níveis de hemoglobina livre, consumo de NO e nitrito plasmático em pacientes com pré-eclâmpsia e em grávidas sadias.

4 - CAPÍTULOS

CAPÍTULO 1

INCREASED CIRCULATING CELL-FREE HEMOGLOBIN LEVELS REDUCE NITRIC OXIDE BIOAVAILABILITY IN PREECLAMPSIA

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Original Contribution

Increased circulating cell-free hemoglobin levels reduce nitric oxide bioavailability in preeclampsia

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ABSTRACT

Contrasting with increased nitric oxide (NO) formation during healthy pregnancy, reduced NO bioavailability plays a role in preeclampsia. However, no study has examined whether increased NO consumption by enhanced circulating levels of cell-free hemoglobin plays a role in preeclampsia. We studied 82 pregnant women (38 healthy pregnant and 44 with preeclampsia). To assess NO bioavailability, we measured plasma and whole blood nitrite concentrations using an ozone-based chemiluminescence assay. Plasma ceruloplasmin concentrations and plasma NO consumption (pNOc) were assessed and plasma hemoglobin (pHb) concentrations were measured with a commercial immunoassay. We found lower whole blood and plasma nitrite concentrations in preeclamptic patients (-48 and -39%, respectively; both $P<0.05$) compared with healthy pregnant women. Plasma samples from preeclamptic women consumed 63% more NO ($P=0.003$) and had 53% higher pHb and 10% higher ceruloplasmin levels than those found in healthy pregnant women ($P<0.01$). We found significant positive correlations between pHb and pNOc ($r=0.61$; $P<0.0001$), negative correlations between pNOc and whole blood or plasma nitrite concentrations ($P=0.02$; $r=-0.32$ and $P=0.01$; $r=-0.34$, respectively), and negative correlations between pHb and whole blood or plasma nitrite concentrations ($P=0.03$; $r=-0.36$ and $P=0.01$; $r=-0.38$, respectively). These findings suggest that increased pHb levels lead to increased NO consumption and lower NO bioavailability in preeclamptic compared with healthy pregnant women.

Introduction

A healthy pregnancy is supported by important changes in maternal cardiovascular hemodynamics including increased blood volume and cardiac output without increases in arterial blood pressure. A major reason for this lack of increases in arterial blood pressure during normal pregnancy is enhanced nitric oxide (NO) formation and systemic vascular dilation [1]. Indeed, NO is a critical regulator of vascular homeostasis [1,2], and it maintains the basal vasodilator tone, inhibits platelet aggregation and leukocytes adhesion, and exerts antioxidant and anti-inflammatory effects [3]. It should be noted that the bioactive products of nitric oxide synthase (NOS) do not only include NO radicals, but also include nitrosonium stabilized by other molecules including thionitrites, for example [4,5].

In contrast with the increases of NO formation during healthy pregnancy [6,7], growing evidence indicates that reduced NO bioavailability plays a role in the pathogenesis of hypertensive disorders of pregnancy [8–10]. Recently, we have reported lower circulating nitrite levels in patients with preeclampsia, as compared with those found in healthy pregnant women, thus suggesting that impaired NO formation may be involved in the pathogenesis of preeclampsia [10]. In fact, NO bioavailability results of a balance between NO synthesis and NO degradation and this balance are dynamically regulated by several processes [3], which may be altered in preeclampsia. For example, altered concentrations of endogenous inhibitors of endothelial NOS (eNOS) [11], deficient tetrahydrobiopterin (a cofactor for eNOS) levels [12], increased concentrations of antiangiogenic factors [10], genetic variations [13,14], and oxidative stress [15] have been suggested to impair NO bioavailability in preeclampsia. However, no previous study has examined whether increased NO consumption by enhanced circulating levels of cell-free hemoglobin plays a role in preeclampsia. This is important because increased intravascular cell-free hemoglobin levels could reduce NO bioavailability and its diffusion to vascular smooth muscle cells, thereby decreasing vasodilation [16–19].

In the present study we hypothesized that higher plasma hemoglobin concentrations exist in patients with preeclampsia compared with

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healthy pregnant women, and these higher plasma hemoglobin concentrations could lead to increased NO consumption and lower NO bioavailability in preeclampsia compared with healthy pregnancy. Moreover, we hypothesized that a direct relationship exists between plasma cell-free hemoglobin levels and NO consumption.

Material and methods

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. A total of 82 pregnant women were enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto. Of these, 38 were healthy pregnant women with uncomplicated pregnancies and 44 had preeclampsia. Hypertensive disorders were defined in accordance with the guidelines of the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy) [20]. Preeclampsia was defined as increased blood pressure (≥140 mm Hg systolic or ≥90 mm Hg diastolic on 2 or more measurements at least 6 h apart) with significant proteinuria (≥0.3 g/24 h) in a woman after 20 weeks of gestation. No women with HELLP syndrome or preexisting hypertension, with or without superimposed preeclampsia, were included in the present study. Exclusion criteria included twin or multiple pregnancies or any evidence of previous medical illness.

At the time of clinic attendance, maternal venous blood samples were collected into standard EDTA or into heparin Vacutainer tubes (Becton-Dickinson, Sao Paulo, Brazil) with a 21 gauge needle. The tubes were immediately centrifuged at 1000 *g* for 3 min at room temperature, and plasma samples were stored at -70 °C until used to measure plasma nitrite, plasma hemoglobin levels, and NO consumption. An aliquot of whole blood was mixed with a nitrite preservation solution in a 4:1 dilution, as previously described [21–23], and stored at -70 °C until analyzed to measure whole blood nitrite concentrations. Briefly, this solution contains 0.8 M ferricyanide and 1% NP-40 [21–23].

To avoid sample contamination with nitrites, especial care was taken with regard to the reagents and tubes used in the experiments, which were prewashed with Milli-Q (Synthesis; Millipore) water to make sure that we would have no nitrite contamination.

Measurement of plasma and whole blood nitrite concentrations

Plasma aliquots were analyzed in triplicate for their nitrite content using two different ozone-based chemiluminescence assays, as previously described [17,24–28]: tri-iodine and ascorbic acid reduction. Briefly, when using the tri-iodine assay, 200 µl of plasma samples was injected into a solution of acidified tri-iodide and purged with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, Boulder, CO, USA). Approximately 8 ml of tri-iodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was placed in the purge vessel into which plasma samples were injected in a total volume of 8 ml. However, when using the ascorbic acid reduction assay, 100 µl of plasma was injected into a solution containing glacial acetic acid (8 ml) and ascorbic acid 60 mM, and purged with nitrogen in-line with a gas-phase chemiluminescence in the NO analyzer as described above. Both the tri-iodide solution and the ascorbic acid solution reduce nitrites to NO gas, which is detected by the NO analyzer. Some samples were pretreated without acidified sulfanilamide (AS, 5 wt%; 9:1, v/v) for 3 min, and then analyzed, as previously described [29]. To measure whole blood nitrite concentrations in triplicate, whole blood samples were deproteinated with methanol (1:1) and centrifuged at 14,000 *g* for 3 min. Then, 300 µl supernatant was injected into the solution of acidified tri-iodide and purged with nitrogen in-line with a gas-phase chemiluminescence NO analyzer, as described above.

Measurement of plasma nitrate concentrations

Plasma NO_x (nitrate + nitrite) concentrations were determined in duplicate by using the Griess reaction as previously described [30]. Briefly, 40 µl of plasma was incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM beta nicotinamide adenine dinucleotide phosphate, and 2 units of nitrate reductase/ml) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37 °C in the dark. Eighty microliters of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) was added to each well and the plate was incubated for an additional 15 min at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 µM) with the same reductase buffer. The total amount of nitrite recovery was >98% and absorbances were measured at 540 nm using a microplate reader.

NO consumption assay

To measure NO consumption by plasma samples, we used a NO consumption assay previously described [17,31]. Briefly, a solution of 40 µM DETA NONOate (Cayman Chemical, Ann Arbor, MI) in PBS (pH 7.4) was prepared in a glass vessel purged with nitrogen in-line with an NO chemiluminescence analyzer (Sievers Model 280) to produce a steady-state NO signal of about 50–70 mV. This signal is generated by the decay of DETA-NONOate and the release of NO from DETA NONOate, thus producing a stable baseline signal. Thereafter, we injected 100 µl of plasma samples in triplicate, which produced decreases in baseline NO signal (NO consumption, mV).

Additional experiments were carried out to evaluate whether NO consumption by plasma from preeclamptic women are mediated by thiol- or iron-dependent mechanisms. Therefore, in some experiments, plasma samples from preeclamptic women were mixed with *N*-ethylmaleimide (a thiol blocker; 50 mM) for 10 min at room temperature, and the amounts of NO consumed by these plasma samples or by 300 µl of glutathione solution (GSH 500 nmol/ml) were determined as described above. In addition, we have also compared NO consumption plasma samples from preeclamptic women which were incubated (or not) with potassium ferricyanide plus potassium cyanide (0.5 mM final concentrations of both reagents) or PBS (control) for 45 min at room temperature.

Data were analyzed with the software program ORIGIN Version 6.1 (OriginLab, Northampton, MA) and the area under the curve of decreasing NO signal over time was measured [17,31] for each plasma sample. The intraassay and interassay coefficients of variation were less than 7 and 9%, respectively.

To examine whether adding exogenous nitrite to plasma could affect NO consumption by plasma samples, we added exogenous nitrite to plasma samples from preeclamptic patients in amounts that would be enough to achieve nitrite levels found in the plasma of healthy pregnant women. Nitrite (or vehicle) was added to these plasma samples and incubated for 3 h at 37 °C, as described previously [32]. Nitrite concentrations were measured in nitrite (or vehicle)-added samples by the tri-iodine technique described above. NO consumption by these samples was also measured as described above.

Enzyme immunoassays of plasma hemoglobin and whole blood hemoglobin measurement

The plasma concentrations of hemoglobin were measured with a commercial immunoassay (ELISA; Bethyl Laboratories, Montgomery, TX) according to manufacturer's instructions.

The total blood hemoglobin was spectrophotometrically determined at 550 nm, in an ABX Pentra 80 hematology system (Horiba Medical, Sao Paulo, Brazil).

Assessment of serum ceruloplasmin

The serum concentrations of ceruloplasmin were measured with a commercial kit using antisera to ceruloplasmin (LabPack, Sao Paulo, Brazil) and a Siemens nephelometer (BN II System) following the manufacturer's instructions.

Reagents and solutions

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and solutions were prepared immediately before use.

Statistical analysis

Data were reported as the mean ± SEM. The clinical and biochemical characteristics of study groups were compared by the unpaired Student *t* test or chi-squared tests. The Pearson's correlation (*r*, *P*) was calculated (Statview for Windows, Cary, NC) for associations between parameters studied here. A probability value <0.05 was considered the minimum level of statistical significance.

Results

Table 1 summarizes the clinical characteristics of the 82 subjects enrolled in the present study. We found no significant differences in age, race, body mass index, hemoglobin, hematocrit, gestational age at sampling, and percentage primigravida when patients with preeclampsia were compared with healthy pregnant women (Table 1; all $P>0.05$). However, we found higher systolic and diastolic blood pressure, lower birth weight, and gestational age at delivery when the group of preeclamptic pregnancies was compared with the groups of healthy pregnant women (Table 1; both $P>0.05$).

We found lower whole blood and plasma nitrite concentrations in preeclamptic patients (-48 and -39%, respectively; both $P<0.05$; Fig. 1B) compared with those found in healthy pregnant women. We have also validated these results by measuring nitrite concentrations by another method and we confirmed significant reductions in plasma nitrite concentrations in preeclamptic women compared with healthy pregnant women (-42% $P<0.05$; Fig. 1C). To test whether we were really measuring nitrite with these assays, some samples were pretreated with acid sulfanilamide, which converts nitrite into a diazonium cation that is undetectable by the chemiluminescence assays used here. Pretreatment of plasma samples with acid sulfanilamide abolished the chemiluminescence signal (Fig. 2), thus indicating that the chemiluminescence signal reflects mostly nitrite. In contrast to the changes in the nitrite levels found, plasma nitrate levels were similar in preeclamptic women and healthy pregnant women ($P>0.05$; Fig. 1D).

We found that plasma samples from preeclamptic women consumes 63% more NO ($P=0.003$; Figs. 3A and B) and has 53% higher cell-free plasma hemoglobin concentrations that those found in healthy pregnant women ($P<0.01$; 5.51 ± 0.56 mg/dl versus 3.62 ± 0.37 mg/dl, corresponding to 3.18 ± 0.35 μM versus 1.63 ± 0.22 μM heme, respectively; Fig. 3C).

To examine whether direct relationships exist between the plasma cell-free hemoglobin concentrations and the plasma NO consumption, we carried out correlation analysis between these parameters. We found significant positive correlations between plasma hemoglobin concentrations and NO consumption by respective plasma samples (Fig. 4; $r=0.61$; $P<0.0001$). In addition, to examine whether these two parameters are associated with lower NO bioavailability, we carried out correlation analysis between these two parameters and whole blood or plasma nitrite concentrations (Table 2). Interestingly, we found significant negative correlations between NO consumption and whole blood or plasma nitrite concentrations ($P=0.02$; $r=-0.32$ and $P=0.01$; $r=-0.34$, respectively). In addition, we found significant negative correlations between plasma hemoglobin concentrations and whole blood or plasma nitrite concentrations ($P=0.03$; $r=-0.36$ and $P=0.01$; $r=-0.38$, respectively).

Finally, we found that adding the thiol blocker *N*-ethylmaleimide produced no effects on NO consumption by plasma samples from preeclamptic women ($P>0.05$; Figs. 5A and B). Moreover, glutathione in amounts comparable those found in plasma from preeclamptic women [33] produced no significant NO consumption (Fig. 4A). Adding exogenous nitrite to plasma samples from preeclamptic patients in amounts that would be enough to achieve nitrite levels found in the plasma of healthy pregnant women produced no significant changes in NO consumption by these plasma samples (Fig. 6). Conversely, NO consumption was completely abolished when plasma samples from preeclamptic women were mixed with potassium ferricyanide and potassium cyanide, which are hemoglobin oxidants (Fig. 7).

Since ceruloplasmin has recently been shown to be a nitrite synthase, we also measured serum ceruloplasmin levels. We found significantly higher serum ceruloplasmin levels in preeclamptic women compared with healthy pregnant women (Fig. 8; $P<0.05$).

Table 1
Demographic characteristics of study participants

	Healthy pregnant	Preeclampsia	*P* value
N	38	44	
Age (years)	23.5 ± 0.8	23.5 ± 0.8	NS
Race (white, %)	68	69	NS
Primigravida (%)	42	45	NS
BMI (kg/m^2)	26.8 ± 0.6	27.1 ± 0.2	NS
SBP (mm Hg)	101.2 ± 1.0	146.6 ± 0.3	<0.05
DBP (mm Hg)	68.8 ± 1.0	94.2 ± 0.4	<0.05
24-h-Pr (mg/24 h)	ND	1194.5 ± 38.5	
Hb (g/dl)	12.2 ± 0.2	11.9 ± 0.3	NS
Hct (%)	35.8 ± 1.0	35.5 ± 0.9	NS
GA at sampling	35.9 ± 0.4	34.4 ± 0.5	NS
GAD (weeks)	40.1 ± 0.6	34.4 ± 0.8	<0.05
Newborn weight (g)	3266 ± 112	2352 ± 189	<0.05

BMI, body mass index; GA, gestational age; SBP, systolic blood pressure; DBP, diastolic blood pressure; 24-h-Pr, 24-h proteinuria; Hb, hemoglobin concentration; Hct, hematocrit; GA, gestational age; GAD, gestational age at delivery; ND: not determined. Values are the mean ± SEM.

Discussion

This study was the first to measure cell-free hemoglobin and NO consumption in preeclamptic and in healthy pregnant women. Interestingly, we found increased plasma cell-free hemoglobin levels and NO consumption in preeclamptic compared with healthy pregnant women, and significant correlation between these parameters. In addition, we found inverse relationships between these parameters and markers of NO bioavailability. Collectively, these findings suggest that reduced NO bioavailability in preeclampsia is probably due, at least in part, to increased NO consumption. These findings are consistent with a previous hypothesis by Sarrel et al. suggesting that vasoconstriction in preeclampsia may be attributed to increased concentrations of free hemoglobin found in preeclampsia compared with normal pregnancy [34].

To evaluate NO bioavailability in the present study, we measured nitrite concentrations in both plasma and whole blood samples. This approach was based on recent studies showing that plasma nitrite levels may reflect endogenous NO formation, with approximately 70% of plasma nitrite deriving from endothelial NO synthase activity [35], and that inhibition of NO synthase activity decreased the circulating

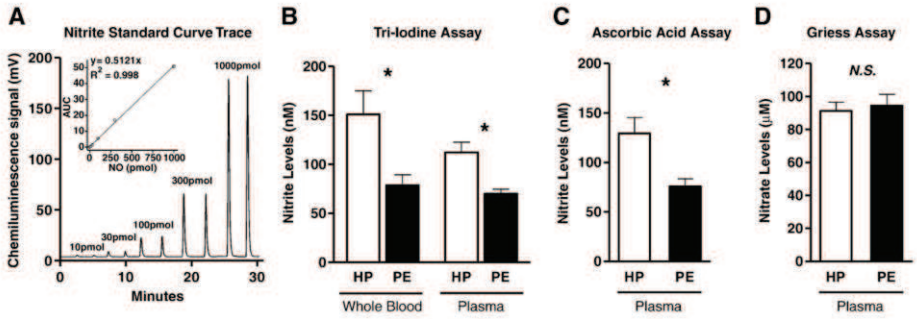

Fig. 1. Whole blood and plasma nitrite levels in healthy pregnant (HP) women and in patients with preeclampsia (PE). Panel A shows chemiluminescence signal obtained with standard samples (the inset shows standard curve obtained by integrating the area under the curve). Panel B shows whole blood and plasma nitrite levels in healthy pregnant (HP) women and in patients with preeclampsia (PE) assessed by the tri-iodine method. Panel C shows plasma nitrite levels in healthy pregnant (HP) women and in patients with preeclampsia (PE) validated by the ascorbic acid assay. Panel D shows plasma nitrate levels in healthy pregnant (HP) women and in patients with preeclampsia (PE), assessed by Griess reagent. The bars indicate the means ± SEM. *N.S.*, not significant. $^{*}P<0.05$ vs HP group.

nitrite concentrations [35,36]. However, other factors may modify nitrite concentrations [37], including the reduction of nitrate to nitrite by commensal bacteria in the oral cavity and gastrointestinal tract, nitrite ingested from the diet [38], and nitrite synthase activity associated with ceruloplasmin [39]. In the present study, we assessed the nitrate levels in both groups of pregnant women and found no significant differences between groups. Conversely, we found lower nitrite levels in preeclamptic patients than in healthy pregnancy women, thus suggesting that the nitrite levels may better reflect the reduced nitric oxide bioavailability in preeclamptic women.

Shiva et al. showed that ceruloplasmin acts as a nitrite synthase [39]. This is especially relevant to the present study because we found higher ceruloplasmin levels in preeclamptic women compared to healthy pregnant women, thus confirming previous findings [40,41]. Although increased ceruloplasmin levels would lead to corresponding increases in nitrite levels in preeclamptic women, we found lower nitrite concentrations in these patients. These findings are consistent with a major reduction in NO formation in these patients compared with healthy pregnant women.

Although NO consumption by hemoglobin has already been well established, no previous study has addressed the possible association between this phenomenon and a reduction of bioavailability of NO in preeclampsia. Here, we found a significant reduction in plasma nitrite levels, assessed by two independent methods, as well as reduced whole blood nitrite levels. Our results provide evidence indicating that cell-free hemoglobin can reduce NO bioavailability in preeclamptic women and explain, at least in part, previous findings [10,42,43] showing lower plasma nitrite levels in preeclampsia compared with those found in healthy pregnancies.

Under normal conditions, hemoglobin is compartmentalized within red blood cell, thus allowing NO produced by endothelial cells to reach smooth muscle cells and produce vasodilation [17]. However, limited NO bioavailability may result when hemoglobin is released from erythrocytes into plasma during pathological hemolysis, leading to hemoglobin-mediated NO dioxygenation and nitrosylation, as previously shown [17]. Our findings support the idea that this is also the case of patients with preeclampsia.

In this study, the increased NO consumption by plasma samples from preeclamptic patients could be due the thiol groups than to iron reactivity from free hemoglobin. We found evidence against a thiol-mediated NO consumption because similar NO consumption was found with plasma samples with or without *N*-ethylmaleimide, a thiol blocker. In agreement with these findings, we found that a thiol compound (glutathione) in amounts comparable to those found in plasma from preeclamptic women [33] produced no NO consumption. These findings are consistent with the idea that thiol groups are not

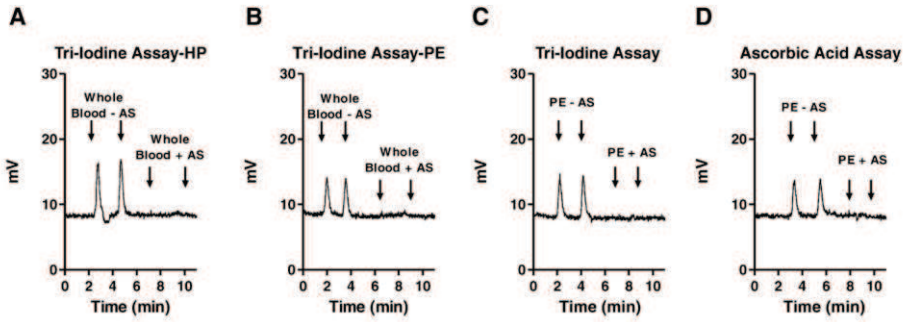

Fig. 2. Lack of significant contribution of other nitroso species to the chemiluminescence signal obtained. Samples were pretreated with (+AS) or without (−AS) acidified sulfanilamide (AS, 5 wt%; 9:1, v/v) for 3 min, and then analyzed. Sample injections are indicated by the arrows. Panel A and B shows signals obtained with whole blood from healthy pregnant (HP) women and patients with preeclampsia (PE), respectively, assessed by tri-iodine assay. Panels C and D shows signals obtained with plasma from patients with preeclampsia (PE) assessed by tri-iodine and ascorbic acid assays, respectively.

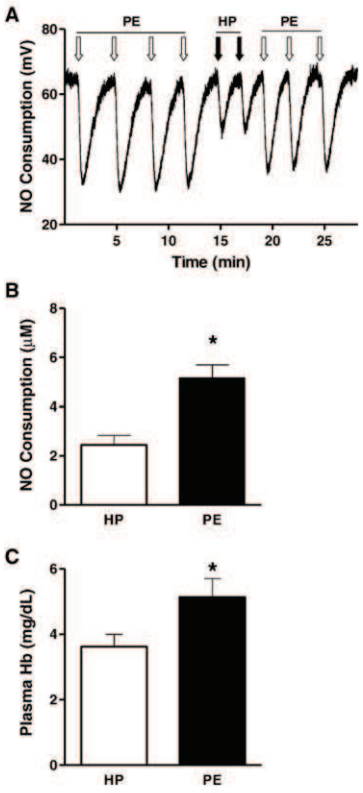

Fig. 3. NO consumption assay and plasma hemoglobin concentrations in healthy pregnant women and in patients with preeclampsia. Panel A shows transient decreases in the baseline NO signal (NO consumption, mV) generated by a solution of DETA-NONOate in PBS, which was purged with a nitrogen stream in-line with an NO analyzer. NO consumption by plasma samples from healthy pregnant (HP) and preeclaptic (PE) women is indicated by arrows. Panel B shows NO consumption and panel C shows plasma hemoglobin levels in healthy pregnant (HP) women and in patients with preeclampsia (PE). The bars indicate the means ± SEM. *$P<0.05$ vs HP group.

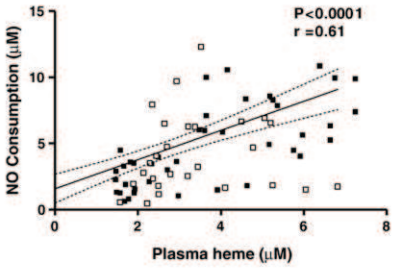

Fig. 4. Correlation between plasma hemoglobin (Hb) and NO consumption in healthy pregnant (□) and preeclamptic (■) women. The regression line and the 95% CI are plotted. *P* and Pearson correlation (*r*) values are reported.

Table 2
Correlation analysis between NO consumption or plasma hemoglobin concentrations and whole blood nitrite or plasma nitrite levels in pregnant women (both healthy pregnant and preeclamptic women)

		P	Pearson *r*
NO consumption	Whole blood nitrite	0.02	-0.32
	Plasma nitrite	0.01	-0.34
Plasma hemoglobin	Whole blood nitrite	0.03	-0.36
	Plasma nitrite	0.01	-0.38

involved in NO consumption. However, NO consumption by plasma samples was abolished when these samples were mixed with potassium ferricyanide and potassium cyanide, which are hemoglobin oxidants. Ferricyanide oxidizes Fe (II) hemoglobin to methemoglobin (Fe (III) hemoglobin) and cyanide converts methemoglobin to cyanomethemoglobin, which has low affinity for NO [17].

We examined whether reduced nitrite levels in plasma from preeclamptic patients could modify NO consumption. Adding exogenous nitrite in amounts that were enough to achieve nitrite levels found in the plasma of healthy pregnant women did not affect NO consumption. This finding is consistent with the idea that near physiological levels of nitrite (nanomolar range) probably do not oxidize all free heme content in plasma from preeclamptic women, which is in the micromolar range. Our findings suggest that the iron reactivity of cell-free hemoglobin in plasma samples is involved in NO consumption and not thiol groups.

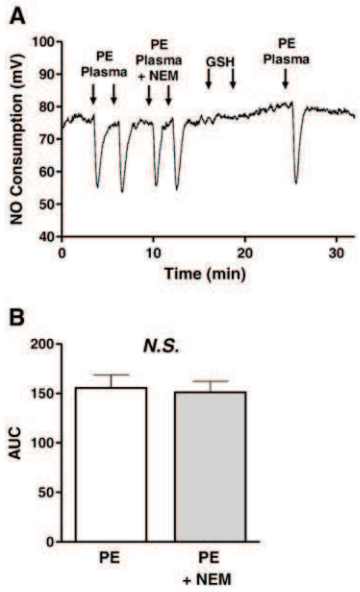

Fig. 5. Lack of significant contribution of thiol groups to NO consumption by plasma from patients with preeclampsia. Panel A shows NO consumption by plasma from preeclaptic (PE) women with or without pretreatment with 50 mM of NEM and by GSH (500 nmol/ml). Panel B shows NO consumption as the area under curve after the injection of plasma samples from preeclamptic patients with or without NEM pretreatment. NEM, *N*-ethylamaleimide; GSH, glutathione; *N.S.*, not significant. The bars indicate the means ± SEM.

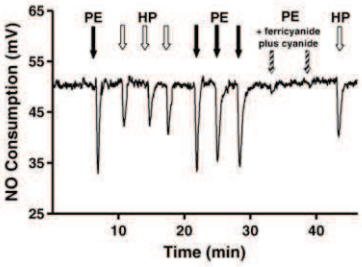

Fig. 6. NO consumption induced by plasma samples from patients with preeclampsia (PE) or healthy pregnancy (HP) after iron oxidation of hemoglobin. Plasma samples from patients with PE were assayed for NO consumption either after treatment with PBS or with potassium ferricyanide plus potassium cyanide, which abolished NO consumption by plasma samples.

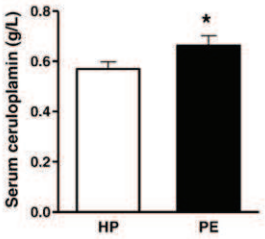

Fig. 8. Serum ceruloplasmin levels in healthy pregnant (HP) women and in patients with preeclampsia (PE). The bars indicate the means ± SEM. *$P<0.05$ vs HP group.

While other circulating substances may also consume NO [31], the significant correlation that we found between NO consumption and plasma hemoglobin concentrations is consistent with the idea that cell-free hemoglobin is a major player leading to reduced NO bioavailability in preeclampsia. Indeed, cell-free hemoglobin completely inhibited NO-mediated dilation in coronary arteries [44] and produced potent dose-dependent vasoconstrictor effects that were not found with encapsulate hemoglobin [45]. Moreover, many conditions associated with increased plasma hemoglobin concentrations including paroxysmal nocturnal hemoglobinuria, cardiopulmonary bypass, mechanical heart valve-induced anemia, and sickle cell disease disrupt normal cardiovascular function [17,19,46,47]. Curiously, several studies report associations between hypertensive disorders of pregnancy and sickle cell disease in pregnancy [48,49], thus giving additional support to our findings.

Other mechanisms may be relevant and further impair NO activity in preeclamptic patients. For example, increased plasma hemoglobin concentrations lead to an excess of free heme during protein degradation, thus causing endothelial cell damage [50] and generating reactive oxygen species, which in turn scavenge NO [51] and stimulate the release of inflammatory mediators [52,53]. Moreover, the activity of the enzyme that degrades heme (heme oxygenase) is reduced in preeclampsia, thus further increasing heme plasma levels [54]. It is also possible that lower haptoglobin concentrations in preeclampsia [55] contribute to the elevated plasma hemoglobin concentrations. This is because haptoglobin forms a complex with hemoglobin, which

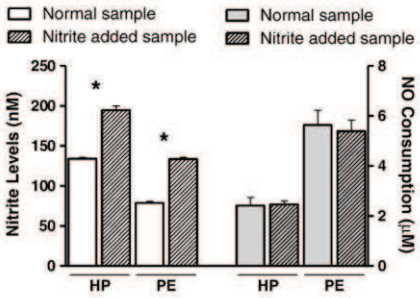

Fig. 7. Influence of nitrite levels on NO consumption by plasma from healthy pregnant (HP) women or patients with preeclampsia (PE). The left axis shows nitrite levels in each group of pregnant women, before and after adding exogenous nitrite. Adding exogenous nitrite to plasma samples produced no effects on NO consumption by plasma samples (right axis). The bars indicate the means ± SEM. *$P<0.05$ vs HP group.

is recognized by scavenger receptor CD163 on macrophages/monocytes [56,57], thus promoting hemoglobin clearance. While we have not examined these mechanisms in the present study, it is highly probable that they may explain the negative correlations that we found between plasma hemoglobin concentrations/NO consumption and nitrite levels (both in plasma and whole blood).

The chemical biology of NO is very complex and involves many processes that may interact with the pathophysiological alterations of preeclampsia [58]. These may include alterations in ADMA (an endogenous inhibitor of eNOS activity), antiangiogenic factors [10], oxidative stress [15], and genetic variations [13,14]. The findings reported here may help to understand the pathophysiology of preeclampsia, especially when severe hemolysis is present, as is the case of HELLP syndrome [59]. We believe that a study should be carried out to compare NO bioavailability and consumption in patients with more severe forms of preeclampsia, including the HELLP syndrome. If our findings are confirmed, we would have strong evidence supporting the use of plasma hemoglobin concentrations as a biomarker of disease.

We must emphasize that our nitrite results should be interpreted very carefully due to possible technical limitations. Although the tri-iodine assay has been widely used to assess plasma and whole blood nitrite concentrations, concerns have been raised about these measurements [60]. The reducing solution used with the tri-iodine method can reduce other nitroso species that may be present in plasma/blood samples, such as *S*-nitrosothiols, iron-nitrosyls, and *N*-nitrosamines. These species would generate NO and falsely contribute to the nitrite levels being measured [60]. However, treating plasma/blood samples with acid sulfanilamide allows a subtractive measurement of nitrites because this reagent converts nitrite, a diazonium cation that is undetectable by chemiluminescence [29,61,62]. In the present study, we confirmed out nitrite results by using another chemiluminescence method based on ascorbic acid, which detects nitrites without interference of other *S*-, *N*-nitroso and nitrated species [28]. Both methods used in the present study produced almost identical results, and consistently showed lower nitrite levels in preeclamptic women. Finally, we showed that pretreatment with acid sulfanilamide was associated with no NO signal, thus suggesting that we were really measuring nitrites in our samples. However, we cannot completely rule out the possibility that other species may have some minor contribution to the peaks detected with our NO analyzer. In addition, the pregnant women enrolled in the present study were not fasting for more than 8 h, thus possibly affecting our measurements.

In conclusion, we found increased cell-free hemoglobin levels and NO consumption in preeclamptic compared with healthy pregnant women, which is associated with lower NO bioavailability. Moreover, a positive correlation found between these two parameters may suggest that cell-free hemoglobin is an important molecule that regulates NO consumption by the plasma, thus altering its bioavailability. It is possible that improving NO activity in these patients produces beneficial therapeutic effects.

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References

[1] Sladek, S. M.; Magness, R. R.; Conrad, K. P. Nitric oxide and pregnancy. *Am. J. Physiol.* **272:**R441–R463; 1997.
[2] Moncada, S.; Higgs, A. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329:** 2002–2012; 1993.
[3] Forstermann, U.; Munzel, T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* **113:**1708–1714; 2006.
[4] Mayer, B.; Pfeiffer, S.; Schrammel, A.; Koesling, D.; Schmidt, K.; Brunner, F. A new pathway of nitric oxide/cyclic GMP signaling involving *S*-nitrosoglutathione. *J. Biol. Chem.* **273:**3264–3270; 1998.
[5] Schmidt, H. H.; Hofmann, H.; Schindler, U.; Shutenko, Z. S.; Cunningham, D. D.; Feelisch, M. No.NO from NO synthase. *Proc. Natl. Acad. Sci. U. S. A.* **93:**14492–14497; 1996.
[6] Williams, D. J.; Vallance, P. J.; Neild, G. H.; Spencer, J. A.; Imms, F. J. Nitric oxide-mediated vasodilation in human pregnancy. *Am. J. Physiol.* **272:**H748–H752; 1997.
[7] Weiner, C. P.; Knowles, R. G.; Moncada, S. Induction of nitric oxide synthases early in pregnancy. *Am. J. Obstet. Gynecol.* **171:**838–843; 1994.
[8] Seligman, S. P.; Buyon, J. P.; Clancy, R. M.; Young, B. K.; Abramson, S. B. The role of nitric oxide in the pathogenesis of preeclampsia. *Am. J. Obstet. Gynecol.* **171:** 944–948; 1994.
[9] Choi, J. W.; Im, M. W.; Pai, S. H. Nitric oxide production increases during normal pregnancy and decreases in preeclampsia. *Ann. Clin. Lab. Sci.* **32:**257–263; 2002.
[10] Sandrim, V. C.; Palei, A. C.; Metzger, I. F.; Gomes, V. A.; Cavalli, R. C.; Tanus-Santos, J. E. Nitric oxide formation is inversely related to serum levels of antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endogline in preeclampsia. *Hypertension* **52:**402–407; 2008.
[11] Savvidou, M. D.; Hingorani, A. D.; Tsikas, D.; Frolich, J. C.; Vallance, P.; Nicolaides, K. H. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. *Lancet* **361:**1511–1517; 2003.
[12] Kukor, Z.; Meszaros, G.; Hertelendy, F.; Toth, M. Calcium-dependent nitric oxide synthesis is potently stimulated by tetrahydrobiopterin in human primordial placenta. *Placenta* **17:**69–73; 1996.
[13] Sandrim, V. C.; de Syllos, R. W.; Lisboa, H. R.; Tres, G. S.; Tanus-Santos, J. E. Influence of eNOS haplotypes on the plasma nitric oxide products concentrations in hypertensive and type 2 diabetes mellitus patients. *Nitric Oxide* **16:**348–355; 2007.
[14] Sandrim, V. C.; Palei, A. C.; Cavalli, R. C.; Araujo, F. M.; Ramos, E. S.; Duarte, G.; Tanus-Santos, J. E. eNOS haplotypes associated with gestational hypertension or preeclampsia. *Pharmacogenomics* **9:**1467–1473; 2008.
[15] Li, H.; Gu, B.; Zhang, Y.; Lewis, D. F.; Wang, Y. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. *Placenta* **26:**210–217; 2005.
[16] Gladwin, M. T.; Crawford, J. H.; Patel, R. P. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radic. Biol. Med.* **36:**707–717; 2004.
[17] Reiter, C. D.; Wang, X.; Tanus-Santos, J. E.; Hogg, N.; Cannon III, R. O.; Schechter, A. N.; Gladwin, M. T. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat. Med.* **8:**1383–1389; 2002.
[18] Kato, G. J.; Gladwin, M. T. Evolution of novel small-molecule therapeutics targeting sickle cell vasculopathy. *JAMA* **300:**2638–2646; 2008.
[19] Rother, R. P.; Bell, L.; Hillmen, P.; Gladwin, M. T. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA* **293:**1653–1662; 2005.
[20] Anonymous Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am. J. Obstet. Gynecol.* **183:** S1–S22; 2000.
[21] Pelletier, M. M.; Kleinbongard, P.; Ringwood, L.; Hito, R.; Hunter, C. J.; Schechter, A. N.; Gladwin, M. T.; Dejam, A. The measurement of blood and plasma nitrite by chemiluminescence: pitfalls and solutions. *Free Radic. Biol. Med.* **41:**541–548; 2006.
[22] Nagassaki, S.; Sertorio, J. T. C.; Metzger, I. F.; Bem, A. F.; Rocha, J. B. T.; Tanus-Santos, J. E. eNOS gene T-786C polymorphism modulates atorvastatin-induced increase in blood nitrite. *Free Radic. Biol. Med.* **41:**1044–1049; 2006.
[23] Dejam, A.; Hunter, C. J.; Pelletier, M. M.; Hsu, L. L.; Machado, R. F.; Shiva, S.; Power, G. G.; Kelm, M.; Gladwin, M. T.; Schechter, A. N. Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* **106:**734–739; 2005.
[24] Metzger, I. F.; Souza-Costa, D. C.; Marroni, A. S.; Nagassaki, S.; Desta, Z.; Flockhart, D. A.; Tanus-Santos, J. E. Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men. *Pharmacogenet. Genomics* **15:**565–570; 2005.
[25] Nagassaki, S.; Metzger, I. F.; Souza-Costa, D. C.; Marroni, A. S.; Uzuelli, J. A.; Tanus-Santos, J. E. eNOS genotype is without effect on circulating nitrite/nitrate level in healthy male population. *Thromb. Res.* **115:**375–379; 2005.
[26] Yang, B. K.; Vivas, E. X.; Reiter, C. D.; Gladwin, M. T. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Radic. Res.* **37:**1–10; 2003.
[27] Dias-Junior, C. A.; Gladwin, M. T.; Tanus-Santos, J. E. Low-dose intravenous nitrite improves hemodynamics in a canine model of acute pulmonary thromboembolism. *Free Radic. Biol. Med.* **41:**1764–1770; 2006.
[28] Nagababu, E.; Rifkind, J. M. Measurement of plasma nitrite by chemiluminescence without interference of *S*-, *N*-nitroso and nitrated species. *Free Radic. Biol. Med.* **42:** 1146–1154; 2007.
[29] MacArthur, P. H.; Shiva, S.; Gladwin, M. T. Measurement of circulating nitrite and *S*-nitrosothiols by reductive chemiluminescence. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **851:**93–105; 2007.
[30] Montenegro, M. F.; Pessa, L. R.; Gomes, V. A.; Desta, Z.; Flockhart, D. A.; Tanus-Santos, J. E. Assessment of vascular effects of tamoxifen and its metabolites on the rat perfused hindquarter vascular bed. *Basic Clin. Pharmacol. Toxicol.* **104:**400–407; 2009.
[31] Wang, X.; Tanus-Santos, J. E.; Reiter, C. D.; Dejam, A.; Shiva, S.; Smith, R. D.; Hogg, N.; Gladwin, M. T. Biological activity of nitric oxide in the plasmatic compartment. *Proc. Natl. Acad. Sci. U. S. A.* **101:**11477–11482; 2004.
[32] Greenberg, L. A.; Lester, D.; Haggard, H. W. The reaction of hemoglobin with nitrite. *J. Biol. Chem.* **280:**665–673; 1943.
[33] Llurba, E.; Gratacos, E.; Martin-Gallan, P.; Cabero, L.; Dominguez, C. A comprehensive study of oxidative stress and antioxidant status in preeclampsia and normal pregnancy. *Free Radic. Biol. Med.* **37:**557–570; 2004.
[34] Sarrel, P. M.; Lindsay, D. C.; Poole-Wilson, P. A.; Collins, P. Hypothesis: inhibition of endothelium-derived relaxing factor by haemoglobin in the pathogenesis of pre-eclampsia. *Lancet* **336:**1030–1032; 1990.
[35] Kleinbongard, P.; Dejam, A.; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Godecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G. A.; Bryan, N. S.; Feelisch, M.; Kelm, M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic. Biol. Med.* **35:**790–796; 2003.
[36] Kelm, M.; Preik-Steinhoff, H.; Preik, M.; Strauer, B. E. Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. *Cardiovasc. Res.* **41:**765–772; 1999.
[37] Gladwin, M. T.; Schechter, A. N.; Kim-Shapiro, D. B.; Patel, R. P.; Hogg, N.; Shiva, S.; Cannon III, R. O.; Kelm, M.; Wink, D. A.; Espey, M. G.; Oldfield, E. H.; Pluta, R. M.; Freeman, B. A.; Lancaster Jr., J. R.; Feelisch, M.; Lundberg, J. O. The emerging biology of the nitrite anion. *Nat. Chem. Biol.* **1:**308–314; 2005.
[38] Lundberg, J. O.; Weitzberg, E.; Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* **7:**156–167; 2008.
[39] Shiva, S.; Wang, X.; Ringwood, L. A.; Xu, X.; Yuditskaya, S.; Annavajjhala, V.; Miyajima, H.; Hogg, N.; Harris, Z. L.; Gladwin, M. T. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat. Chem. Biol.* **2:**486–493; 2006.
[40] Kristensen, K.; Wide-Swensson, D.; Lindstrom, V.; Schmidt, C.; Grubb, A.; Strevens, H. Serum amyloid a protein and C-reactive protein in normal pregnancy and preeclampsia. *Gynecol. Obstet. Invest.* **67:**275–280; 2009.
[41] Orhan, H. G.; Ozgunes, H.; Beksac, M. S. Correlation between plasma malondialdehyde and ceruloplasmin activity values in preeclamptic pregnancies. *Clin. Biochem.* **34:**505–506; 2001.
[42] Bernardi, F.; Constantino, L.; Machado, R.; Petronilho, F.; Dal-Pizzol, F. Plasma nitric oxide, endothelin-1, arginase and superoxide dismutase in pre-eclamptic women. *J. Obstet. Gynaecol. Res.* **34:**957–963; 2008.
[43] Mayret-Mesquiti, M.; Perez-Mendez, O.; Rodriguez, M. E.; Fortoul, T. I.; Gorocica, P.; Bernal-Alcantara, D.; Montano, L. F.; Alvarado-Vasquez, N. Hypertriglyceridemia is linked to reduced nitric oxide synthesis in women with hypertensive disorders of pregnancy. *Hypertens. Pregnancy* **26:**423–431; 2007.
[44] Liao, J. C.; Hein, T. W.; Vaughn, M. W.; Huang, K. T.; Kuo, L. Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc. Natl. Acad. Sci. U. S. A.* **96:** 8757–8761; 1999.
[45] Nakai, K.; Ohta, T.; Sakuma, I.; Akama, K.; Kobayashi, Y.; Tokuyama, S.; Kitabatake, A.; Nakazato, Y.; Takahashi, T. A.; Sadayoshi, S. Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J. Cardiovasc. Pharmacol.* **28:** 115–123; 1996.
[46] Gladwin, M. T.; Sachdev, V.; Jison, M. L.; Shizukuda, Y.; Plehn, J. F.; Minter, K.; Brown, B.; Coles, W. A.; Nichols, J. S.; Ernst, I.; Hunter, L. A.; Blackwelder, W. C.; Schechter, A. N.; Rodgers, G. P.; Castro, O.; Ognibene, F. P. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *N. Engl. J. Med.* **350:** 886–895; 2004.
[47] Lin, E. E.; Rodgers, G. P.; Gladwin, M. T. Hemolytic anemia-associated pulmonary hypertension in sickle cell disease. *Curr. Hematol. Rep.* **4:**117–125; 2005.
[48] Villers, M. S.; Jamison, M. G.; De Castro, L. M.; James, A. H. Morbidity associated with sickle cell disease in pregnancy. *Am. J. Obstet. Gynecol.* **199:**125; 2008.
[49] Tsaras, G.; Owusu-Ansah, A.; Boateng, F. O.; Amoateng-Adjepong, Y. Complications associated with sickle cell trait: a brief narrative review. *Am. J. Med.* **24:**24; 2009.
[50] Balla, G.; Vercellotti, G. M.; Eaton, J. W.; Jacob, H. S. Iron loading of endothelial cells augments oxidant damage. *J. Lab. Clin. Med.* **116:**546–554; 1990.
[51] Scalera, F. Intracellular glutathione and lipid peroxide availability and the secretion of vasoactive substances by human umbilical vein endothelial cells after incubation with TNF-alpha. *Eur. J. Clin. Invest.* **33:**176–182; 2003.
[52] Lavrovsky, Y.; Schwartzman, M. L.; Levere, R. D.; Kappas, A.; Abraham, N. G. Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc. Natl. Acad. Sci. U. S. A.* **91:**5987–5991; 1994.
[53] Porto, B. N.; Alves, L. S.; Fernandez, P. L.; Dutra, T. P.; Figueiredo, R. T.; Graca-Souza, A. V.; Bozza, M. T. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. *J. Biol. Chem.* **282:**24430–24436; 2007.

[54] Appleton, S. D.; Marks, G. S.; Nakatsu, K.; Brien, J. F.; Smith, G. N.; Graham, C. H.; Lash, G. E. Effects of hypoxia on heme oxygenase expression in human chorionic villi explants and immortalized trophoblast cells. *Am. J. Physiol. Heart Circ. Physiol.* **284:**H853–H858; 2003.

[55] Knapen, M. F.; Peters, W. H.; Mulder, T. P.; Merkus, H. M.; Jansen, J. B.; Steegers, E. A. Plasma glutathione *S*-transferase Pi 1-1 measurements in the study of hemolysis in hypertensive disorders of pregnancy. *Hypertens. Pregnancy* **18:** 147–156; 1999.

[56] Nagel, R. L.; Gibson, Q. H. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J. Biol. Chem.* **246:**69–73; 1971.

[57] Kristiansen, M.; Graversen, J. H.; Jacobsen, C.; Sonne, O.; Hoffman, H. J.; Law, S. K.; Moestrup, S. K. Identification of the haemoglobin scavenger receptor. *Nature* **409:** 198–201; 2001.

[58] Gilbert, J. S.; Ryan, M. J.; LaMarca, B. B.; Sedeek, M.; Murphy, S. R.; Granger, J. P. Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction. *Am. J. Physiol. Heart Circ. Physiol.* **294:**H541–H550; 2008.

[59] Hladunewich, M.; Karumanchi, S. A.; Lafayette, R. Pathophysiology of the clinical manifestations of preeclampsia. *Clin. J. Am. Soc. Nephrol.* **2:**543–549 (Electronic publication 2007 Apr 2004); 2007.

[60] Hausladen, A.; Rafikov, R.; Angelo, M.; Singel, D. J.; Nudler, E.; Stamler, J. S. Assessment of nitric oxide signals by triiodide chemiluminescence. *Proc. Natl. Acad. Sci. U. S. A.* **104:**2157–2162; 2007.

[61] Samouilov, A.; Zweier, J. L. Development of chemiluminescence-based methods for specific quantitation of nitrosylated thiols. *Anal. Biochem.* **258:**322–330; 1998.

[62] Marley, R.; Feelisch, M.; Holt, S.; Moore, K. A chemiluminescense-based assay for *S*-nitrosoalbumin and other plasma *S*-nitrosothiols. *Free Radic. Res.* **32:**1–9; 2000.

CAPÍTULO 2

HAPTOGLOBIN POLYMORPHISM AFFECTS NITRIC OXIDE BIOAVAILABILTY IN PREECLAMPSIA

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ORIGINAL ARTICLE

Haptoglobin polymorphism affects nitric oxide bioavailability in preeclampsia

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Studies showed elevated cell-free hemoglobin (Hb) in preeclampsia (PE), and Hb reacts with nitric oxide (NO), decreasing its bioavailability. Haptoglobin (Hp) is a polymorphic protein (Hp1-1, Hp2-1 and Hp2-2) that binds Hb to form a complex that is removed from circulation, thus preventing Hb-driven oxidative stress and NO scavenging. Hp protein products differ in biochemical and biophysical properties, which reflects on the Hb–Hp complex clearance rate. We hypothesized that Hp phenotypes modulate NO bioavailability by influencing NO consumption in PE. We studied 92 PE subjects and 105 normal pregnant women (NP). Hp genotypes were determined using real-time PCR. To assess NO bioavailability, we measured plasma nitrite using an ozone-based chemiluminescence assay. Plasma Hb and Hp were assessed with commercial immunoassays. A NO consumption assay was used to measure NO consumption. We found no differences in Hp genotype frequencies between PE and NP groups. Hp genotypes had no effects on plasma heme levels, NO consumption and plasma nitrite in NP. However, in PE, Hp2-1 and Hp2-2 were associated with higher plasma heme levels (48 and 55% higher, respectively; $P<0.05$), increased NO consumption (42 and 44% more, respectively; $P<0.05$) and lower plasma nitrite (39% less for Hp2-2; $P<0.05$) compared with Hp1-1. These findings indicate that although Hp genotype does not affect the risk of PE, Hp1-1 genotype may exert a protective role in PE by reducing NO scavenging, whereas Hp2-1 and Hp2-2 further may aggravate PE by reducing NO bioavailability.

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Keywords: haptoglobin; hemoglobin; hemolysis; nitric oxide; polymorphism; preeclampsia

INTRODUCTION

Nitric oxide (NO) has an important role in the hemodynamic changes found during healthy pregnancy.[1] Increased heart rate, plasma volume and cardiac output over the course of a normal pregnancy are accompanied by decreased total peripheral vascular resistance and arterial blood pressure.[2] However, preeclampsia (PE) is associated with impaired vasodilation of the maternal systemic circulation,[3] possibly due to decreased NO bioavailability resulting due to lower NO production[4] and increased NO degradation.[5] In fact, altered circulating levels of angiogenic factors,[6] increased arginase expression,[7] oxidative stress,[8] eNOS inhibition by high concentrations of asymmetric dimethylarginine[9] and genetic variations[10] have all been suggested to impair NO bioavailability in PE.

We have recently reported increased circulating cell-free hemoglobin (Hb) levels in PE,[5] and this alteration may contribute to impaired NO bioavailability, because cell-free Hb scavenges NO through a high speed dioxygenation reaction in which NO reacts with oxyhemoglobin to form methemoglobin and nitrate,[11] thus compromising the efficiency of the NO/soluble guanylyl cyclase pathway to elicit vasodilatory activity.

In other disease conditions associated with Hb decompartmentalization into plasma such as sickle-cell disease, NO consumption by cell-free plasma Hb has been shown to cause hypertension, increased systemic and pulmonary vascular resistance and endothelial dysfunction.[12] Moreover, free heme catalyzes the formation of reactive oxygen species , such as superoxide anion, which in turn reacts with NO further impairing NO bioavailability and producing a much more powerful oxidant, peroxynitrite,[13] which is an important oxidant leading to endothelial cell injury and vascular inflammatory disorders.[14]

In order to avoid iron loss and oxidative damage mediated by cell-free heme after intravascular hemolysis, the serum protein haptoglobin (Hp) binds to Hb to form a complex Hb–Hp that is rapidly removed from the circulation by the macrophage CD163 receptor expressed on Kupffer cells in the liver.[15] The human Hp gene is polymorphic with two common alleles (Hp^1 and Hp^2) encoding 3 different proteins (Hp1-1, Hp2-1 and Hp2-2). The allele Hp^1 is composed of 5 exons and the allele Hp^2 of 7 exons. The allele Hp^2, present only in humans, apparently resulted from duplicated exons 3 and 4 of the Hp^1 gene.[16] As the multimerization domain is located in the exon 3 of the *Hp* gene, the duplication generates a bivalent Hp2 protein, unlike Hp1, which is monovalent. Therefore, Hp protein is a dimer in Hp1-1, a linear polymer in Hp2-1 and a cyclic polymer in Hp2-2 individuals.[17] These differences in Hp structure appear to have clinical importance due to functional differences in protecting against Hb-driven oxidative stress and NO consumption.[11]

Indeed, it has been reported that Hb–Hp complex holds Hb dimer in a ferrous oxidation state that scavenges and inactivates NO through dioxygenation reaction, inhibiting endothelium-dependent relaxation.[18,19] This observation provides a possible mechanism to explain the associations of Hp phenotypes recently

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described in PE[20] with increased cell-free Hb and decreased NO levels found in women with this syndrome.[5]

Considering the effects of reduced NO bioavailability in PE, and that increased levels of cell-free Hb and Hb–Hp complexes rapidly scavenge NO, we hypothesized that different Hp phenotypes might differ in their protective effects against NO consumption by plasma of normal pregnant (np) and PE women. We tested this possibility in the present study.

MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. A total of 197 pregnant women were enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto. Of these, 105 were healthy pregnant women with uncomplicated pregnancies and 92 had PE. Hypertensive disorders were defined in accordance with the guidelines of the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy).[21] Preeclampsia was defined as increased blood pressure ($\geqslant$140 mm Hg systolic or $\geqslant$90 mm Hg diastolic on two or more measurements at least 6 h apart) with significant proteinuria ($\geqslant$0.3 g/24 h) in a woman after 20 weeks of gestation. No women with HELLP (hemolysis, elevated liver enzymes and low platelet count) syndrome or preexisting hypertension, with or without superimposed PE, were included in the present study. Exclusion criteria included twin or multiple pregnancies or any evidence of previous medical illness. At the time of clinic consultation, maternal venous blood samples were collected into standard EDTA or into heparin Vacutainer tubes (Becton-Dickinson, Sao Paulo, Brazil) with a 21 gauge needle. The tubes were immediately centrifuged at 1000 g for 3 min at room temperature, and plasma samples were stored at $-70\,^{\circ}$C until they were used to measure plasma nitrite, NO consumption, plasma Hb and Hb levels.[19,22] To avoid sample contamination with nitrites, especial care was taken with regard to the reagents and tubes used in the experiments, which were prewashed with Milli-Q (Synthesis; Millipore, Billerica, MA, USA) water to make sure that we would have no nitrite contamination. Genomic DNA was extracted from the cellular component of 1 ml of whole blood and stored at $-20\,^{\circ}$C to determine Hp genotypes.

Measurement of Hb and Hp concentrations

ELISA kits were used to measure plasma Hb (Cat.No.E88-135, Bethyl Laboratories Inc., Montgomery, TX, USA) and Hp (ab108856, Abcam Inc., Cambridge, MA, USA) concentrations, according to the manufacturers' instructions.

Measurement of plasma nitrite concentrations

Plasma aliquots were analyzed in triplicate for their nitrite content using an ozone-based chemiluminescence assay, as previously described.[22] In brief, 200 µl of plasma samples were injected into a solution of acidified tri-iodide, purging with nitrogen inline with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, Boulder, CO, USA). Approximately 8 ml of tri-iodide solution (2.0 g of potassium iodide and 1.3 g of iodine dissolved in 40 ml of water and 140 ml of acetic acid) were placed in the purge vessel into which plasma samples were injected. The tri-iodide solution reduced nitrites to NO gas, which was detected by the NO analyzer. For data acquisition we used the Liquid Program (version 3.21, Sievers, Boulder, CO, USA) and for data analysis we integrated the tracings peak by peak to obtain the area under the curve (measured in mV) with ORIGIN Version 8.5 (OriginLab, Northampton, MA, USA).

NO Consumption Assay

In order to assess NO consumption by plasma samples, we used a previously described assay.[19] Briefly, a solution of 40 µM DETA-NONOate (Cayman Chemical, Ann Arbor, MI, USA) in PBS (pH 7.4) was prepared in a glass vessel purged with nitrogen inline with an NO chemiluminescence analyzer (Sievers Model 280 NO Analyzer, Boulder, CO, USA) to produce a steadystate NO signal of about 30–50 mV. This signal is generated by the decay of DETA-NONOate and the release of NO from DETA-NONOate, thus producing a stable baseline signal. Thereafter, we injected 50 µl of plasma samples in triplicate, which produced decreases in baseline NO signal (NO consumption, mV), which was acquired with the Liquid Program (version 3.21).

The data were transferred and analyzed with the software program ORIGIN Version 8.5 (OriginLab, Northampton, MA, USA) and the area under the curve of decreasing NO signal over time was measured for each plasma sample. The intraassay and interassay coefficients of variation were $<6\%$.

To demonstrate that NO scavenging was sensitive to iron oxidation, we incubated plasma samples from PE women with potassium ferricyanide III (Sigma Aldrich, St Louis, MO, USA; 0.5 mM final concentration) for 45 min at room temperature before injecting the plasma into the NO analyzer.

Haptoglobin genotyping

Genotypes for the duplication of exons 3 and 4 of Hp gene (alleles Hp^1 and Hp^2) were determined by a previously described method using real-time PCR[23] with slight modifications. Briefly, a Taqman probe (Hp2; Sigma Aldrich; sequence in Table 1) was designed to anneal at the junction of the end of exon 4 and the beginning of the duplicated allele (sequence that does not exist in Hp^1 homozygotes, only in heterozygous Hp^1/Hp^2—1 copy, and in homozygous Hp^2—2 copies). Another probe (Hp5′, Sigma Aldrich; sequence in Table 1) was designed to anneal at the promoter of *Hp* gene acting as an internal control. Copy number of the Hp2 sequence was assessed relatively to the amplification of the internal control using the $2^{-\Delta\Delta C_T}$ method (Table 2). Real-time PCR reactions were performed with Taqman Core Reagents Kit (Applied Biosystems, Carlsbad, CA, USA) as multiplex assays at the following conditions: 1 × reaction buffer, dNTPs at 200 µM, $MgCl_2$ at 3.5 mM, primers Hp5′ forward and reverse at 60 nM, Hp2 forward and reverse at 105 nM, probe HP5′ at 42 nM, probe Hp2 at 100 nM, 0.25 units of AmpliTaq Gold, 0.1 units of AmpErase and between 3–8 ng of template DNA in a total reaction volume of 10 µl. Standard cycling programs for Taqman probes were used and reactions were performed at a StepOne Plus Real Time PCR machine (Applied Biosystems). Samples in which genotypes were previously assessed by direct sequencing were used as positive controls for each genotype and all samples were genotyped at least twice to ensure data quality.

Statistical analysis

The clinical and biochemical characteristics of study groups were compared by unpaired Student *t*-test or χ^2-tests. The distribution of genotypes was assessed for deviation from the Hardy–Weinberg equilibrium and differences in genotype frequencies were assessed using χ^2-tests. Two-way (polymorphisms *vs.* disease) analysis of variance followed by Bonferroni's post-test and one-way analysis of variance followed by Dunn's multiple comparison post-test were used to compare polymorphisms and biochemical parameters between group. The Spearman's correlation (rs, *P*) was calculated for associations between plasma heme concentrations and NO consumption. The data are shown as mean ± s.e.m. and a probability $P<0.05$ was considered statistically significant. Statistical analysis was performed with Prism 5 Software (Graphpad Prism, San Diego, CA, USA).

RESULTS

Patients' characteristics are detailed in Table 3. No significant differences were found in race, age, Hb and hematocrit. However, subjects in the PE group had higher systolic and diastolic blood pressure, proteinuria, higher body mass index, lower gestational age at delivery and lower birth weight when compared with NP women (Table 3; $P<0.05$).

When comparing NP and PE groups, we found that plasma from patients with PE had lower nitrite levels (Figure 1a; $P<0.05$) associated with increased plasma cell-free Hb concentrations (Figure 1b; $P<0.05$) and reduced Hp levels (Figure 1c; $P<0.05$).

Interestingly, we found higher NO consumption by plasma samples from patients with PE compared with NP (Figure 2c; $P<0.05$) and this variable correlated significantly with Hb concentrations, expressed as heme (Figure 2d; rs = 0.641; $P<0.001$). This is consistent with the dioxygenation reaction, where ferrous oxyhemoglobin reacts with NO to form nitrate and methemoglobin in a 1:1 stoichiometry reaction (1 mole of heme consumes 1 mole of NO). Known concentrations of Hb solutions were used to calculate a standard curve for NO consumption

Table 1. Primers and probes used for Hp^1/Hp^2 genotyping

Sequence name	*Sequence*	*Fluorescent dye*	*Quencher*
HP5′—Forward	5′-CACATTTACTGATTTCAGGCTGGA-3′		
HP5′—Reverse	5′-CCTTTTCACAGTAATTTTCTCCACCT-3′		
HP5′—Probe	5′-AGCTTTTAAGCAATAGGGAGATGGCCACA-3′	FAM	BHQ1
HP2—Forward	5′-GGAGCTGCTCTGCACATCAA-3′		
HP2—Reverse	5′-CCCTTTCAATGAATTTCAGGGA-3′		
HP2—Probe	5′-ACCCCGAATAGAAGCTCGCGAACTGTA-3′	JOE	BHQ1

Table 2. Hp genotying by copy number variation relative quantification

Genotype	*Number of individuals*	$2^{-\Delta\Delta C_T}$ *for each genotype (s.d.)*	*Range*
HP^1/HP^1	48	0 (0)	0–0
HP^1/HP^2	97	0.57 (0.04)	0.44–0.68
HP^2/HP^2	52	1.04 (0.07)	0.91–1.29

Abbreviation: s.d., standard deviation. The copy number variation was assessed by relative quantification of the junction between exon 4 and the duplicated allele and the promoter of the gene by the well-established $2^{-\Delta\Delta C_T}$ method. As the internal control always has two copies, the relative quantity of the homozygous HP^2/HP^2, which carries two copies of the HP2 sequence is around one. Therefore, as the heterozygous HP^1/HP^2 will have only one copy of HP2 sequence for each two copies of the internal control, the relative quantity is around 0.5. Finally, we did not detect the amplification of the HP2 probe in HP^1/HP^1 homozygous samples, therefore, the ratio with the two copies of the internal control is zero.

Table 3. Demographic characteristics of study participants

	Normal pregnancy	*Preeclampsia*	*P-value*
N	105	92	
Race (white, %)	68	65	NS
Age (years)	25.1 ± 0.6	26.8 ± 0.6	NS
Primigravida (%)	40	46	NS
BMI (kg m^{-2})	23.3 ± 0.4	27.7 ± 0.6	<0.05
SBP (mm Hg)	111 ± 1.1	155 ± 1.7	<0.05
DBP (mm Hg)	72 ± 0.8	97 ± 1.1	<0.05
24-h-Pr (mg/24 h)	ND	780.6 ± 97.6	—
Hb (g dl^{-1})	11.9 ± 0.1	11.9 ± 0.1	NS
Hct (%)	35.6 ± 0.6	35.9 ± 0.5	NS
Newborn weight (g)	3420 ± 50	2689 ± 85	<0.05
GA at sampling	36.4 ± 0.3	34.1 ± 0.4	<0.05
GAD (weeks)	39.8 ± 0.1	36.4 ± 0.3	<0.05

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; GAD, gestational age at delivery; Hb, hemoglobin concentration; Hct, hematocrit; ND, not determined; NS, not significant; SBP, systolic blood pressure; 24-h-Pr, 24-h proteinuria. Values are the mean ± s.e.m.

(Figure 2a). To further illustrate NO scavenging by plasma samples in PE and NP, we show decreases in the baseline NO signal (NO signal, mV) after plasma samples injections (Figure 2b).

Therefore, as Hb scavenges NO and Hp binds Hb with high affinity to accelerate its clearance,[11] we evaluated if Hp polymorphisms (which are known to alter Hp half-life) are associated with variations in plasmatic NO levels, cell-free Hb, and NO consumption by plasma from PE and NP groups.

Table 4. Frequency of the different Hb phenotypes

Phenotype	*Normal pregnancy, n (%)*	*Preeclampsia, n (%)*	*P-value*
Hp 1-1	26 (25)	22 (24)	NS
Hp 2-1	56 (53)	41 (45)	NS
Hp 2-2	23 (22)	29 (31)	NS

The distribution of genotypes for the three polymorphisms studied here showed nodeviation from Hardy–Weinberg equilibrium.

Although Hp genotype distribution (Table 4) did not show differences in frequency between PE patients and NP women, we found significantly lower plasma nitrite in samples from PE patients carrying the allele Hp^2 (Hp2-1 and Hp2-2), compared with NP women carrying the same allele (33 and 54% less, respectively; Figure 3a, $P<0.05$). Interestingly, Hp2-1 and Hp2-2 phenotypes in PE resulted in significantly higher plasma levels of heme (41 and 45% more, respectively; Figure 3b, $P<0.05$) and also higher NO consumption (40 and 33% more, respectively; Figure 3c, $P<0.05$) compared with NP women.

Moreover, among patients with PE, Hp2-2 genotype showed lower nitrite levels when compared with Hp1-1 (80.22 ± 7.42 nM versus 131.60 ± 13.73 nM, respectively; Figure 2a, $P<0.05$). Though not statistically significant, nitrite concentrations in Hp2-1 were also reduced in relation to Hp1-1 (Figure 2a).

In parallel with the reduction in nitrite levels found among patients with PE, we found higher plasma levels of heme in Hp2-1 and Hp2-2 in comparison with Hp1-1, (5.60 ± 0.49 μM and 6.39 ± 0.63 μM versus 2.86 ± 0.35 μM respectively; Figure 3b, $P<0.05$) and increased NO consumption (4.96 ± 0.44 μM and 5.14 ± 0.52 μM versus 2.87 ± 0.20 μM respectively; Figure 3c, $P<0.05$).

Finally, we found lower Hp concentrations associated with genotypes Hp2-1 and Hp2-2 in PE, comparing them to NP women (Figure 3d; $P<0.05$) and to PE women with Hp1-1 genotype (Figure 3d). The reduction in Hp levels is consistent with the increased plasma cell-free Hb concentration in PE.

DISCUSSION

This is the first study to show that Hp polymorphisms modulate NO bioavailability in women with PE. Interestingly, we found increased NO consumption in association with increased cell-free Hb in plasma from PE patients carrying the allele Hp^2 of the Hp gene. Moreover, we show for the first time that Hp1-1 phenotype may protect PE women against NO scavenging by cell-free Hb.

Recently, different groups have reported increased circulating cell-free Hb levels in patients with PE, as compared with those found in normal pregnancy (NP),[5,24] thus suggesting that Hb decompartmentalization may be involved in the pathogenesis of PE. Additionally, Hb subunits alpha2 and gamma are overexpressed in the PE placenta leading to accumulation of Hb in the vascular lumen of the placenta and consequently to fetal and adult Hb leakage into the maternal circulation.[24,25]

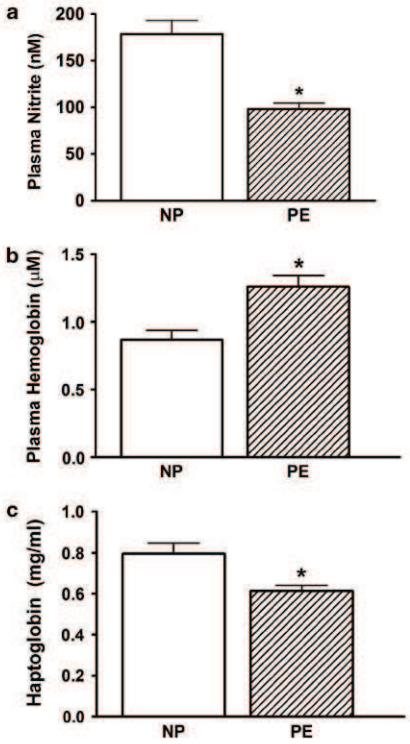

Figure 1. Plasma nitrite, cell-free Hb and Hb levels in NP women and in patients with PE. (**a**) Shows plasma nitrite levels in NP women and in patients with PE. (**b**, **c**) Show Hb and Hb levels, respectively, in plasma from NP women and patients with PE. Values are the mean ± s.e.m. *$P<0.05$ versus the NP group.

Red blood cell lysis of maternal systemic or intervillous circulation, or from fetal red blood cells in a leaky placental barrier, scavenges NO in a fast and irreversible dioxygenation reaction that produces nitrate (NO_3^-) and methemoglobin.[19] Under normal physiological conditions, this reaction rate is highly limited as NO reacts much more slowly with compartmentalized Hb than with cell-free Hb. This is explained by diffusional barriers to NO around the red blood cell and intravascular blood flow. Red blood cellss occupy a central position in flowing blood, and are separated from the endothelium by laminar plasma, and therefore are relatively far from where NO is synthesized.[26] However, Hb release during hemolysis blocks these mechanisms that protect NO from being scavenged by Hb.

Our present results expand previous findings[5,12] and show that micromolar concentrations of plasma cell-free Hb are capable of scavenging NO from plasma (Figure 2d), possibly contributing to the cardiovascular derangements commonly found in PE. Interestingly, we found lower nitrite levels in PE (Figure 1a), and this finding may partly reflect increased NO consumption. In another clinical study performed in patients with sickle-cell anemia, it was reported that NO consumption by cell-free plasma Hb impaired the vascular function in subjects with heme concentrations as low as 6 $\mu mol\, l^{-1}$.[12]

Kim-Shapiro *et al.*[27] have shown in a theoretical model for NO availability within blood vessels that, at low hematocrits, small concentrations of cell-free Hb have more important effects on NO bioavailability due to increased NO consumption. However this factor cannot be invocated in PE, which presents with increased hematocrit when compared with normal pregnancies due to decreased volume expansion. Owing to this, another NO scavenging factor that may participate in PE is the complex formed by Hb and Hp (Hb–Hp). This complex holds Hb dimer in a ferrous oxidation state, which consumes NO through a dioxygenation reaction, thus inhibiting endothelium-dependent relaxation.[18,19] These observations may help to explain the differences in iron-driven oxidative stress and antioxidant properties associated with the different Hp protein structures found in humans.[15]

In our experiments, we found increased cell-free Hb associated with higher NO consumption and reduced Hp concentrations in Hp2-1 and Hp2-2 genotypes in PE compared with normal pregnancy (Figures 3b, c and d). Interestingly, Hp1-1 in PE did not show significant increases in plasma heme or NO consumption compared with normal pregnancy. These findings are consistent with other studies in PE where Hp1-1 was considered a protective factor against Hb-driven oxidative stress,[20] whereas Hp2-2 was shown to be a risk factor for cardiovascular disease.[28] However, we found no significant differences in blood pressure or proteinuria when the different Hp genotype groups were compared, thus suggesting that PE subjects with Hp1-1 genotype did not have milder forms of PE.

In Hp1-1 subjects, the Hp protein is found as a dimer, and as it has a smaller size than the linear (Hp2-1) and the cyclic (Hp2-2) polymers found in other genotypes, it can bind to and clear more molecules of Hb via monocyte/macrophage scavenger receptor CD163, thus conferring protection against oxidative stress.[11]

In our study, we show that PE patients with phenotypes Hp2-1 and Hp2-2 had increased concentrations of plasma heme associated with increased NO consumption when compared with Hp 1-1 (Figures 3b and c). This is consistent with other findings where Hb–Hp1-1 complexes were demonstrated to be cleared more rapidly than Hb–Hp2-2 complexes.[28] As NO consumption via dioxygenation reaction occurs at the same rate for cell-free Hb and for Hb bound to either Hp1-1 or Hp2-2, it has been proposed that different uptake rates of the Hb–Hp1-1 and Hb–Hp2-2 complexes by macrophages could have a profound effect on NO bioavailability.[11] Furthermore, the CD163 macrophage/monocyte receptor, which binds Hb–Hp complexes, has been shown to be down-regulated[29] and upregulated[30] in different clinical conditions. Thus, besides Hp phenotype, CD163 expression might also have a clinical importance in regulating the rate in which Hb–Hp complexes are scavenged in PE, but this possibility remains to be tested.

The present study has some limitations. Firstly, we have not proven that cell-free Hb directly contributes to hypertension in PE. However, there are many previous studies showing that hemolysis may contribute to hypertension and other clinical conditions.[26] Secondly, we have not shown differences in Hp genotype frequencies, which might be due to our small sample size. However, the two largest studies comparing Hp phenotypes frequencies in PE have shown that Hp1-1 have a higher prevalence in normal pregnancy[20] and that Hp2-1 is associated with higher PE risk in Caucasian women,[31] thus supporting our results. Moreover, Hp2-2 was shown to have a higher incidence in pregnancy-associated hypertension.[32] Further prospective studies to examine whether plasma Hb concentrations and NO consumption vary over the course of pregnancy and correlate with hemodynamic parameters in PE are necessary to refine our findings.

This study may have relevant clinical implications. It is possible that pharmacological therapies that restore NO-cGMP activity or protect against hemolysis-driven NO scavenging in PE may attenuate the hemodynamic changes associated with this syndrome. In fact, small-molecule therapies like inhaled NO and carbon monoxide, nitrite infusion and endothelin receptor antagonists have been shown to ameliorate clinical signs of patients with sickle-cell disease, which is an important hemolytic condition.[33] Furthermore, treatment with eNOS substrate

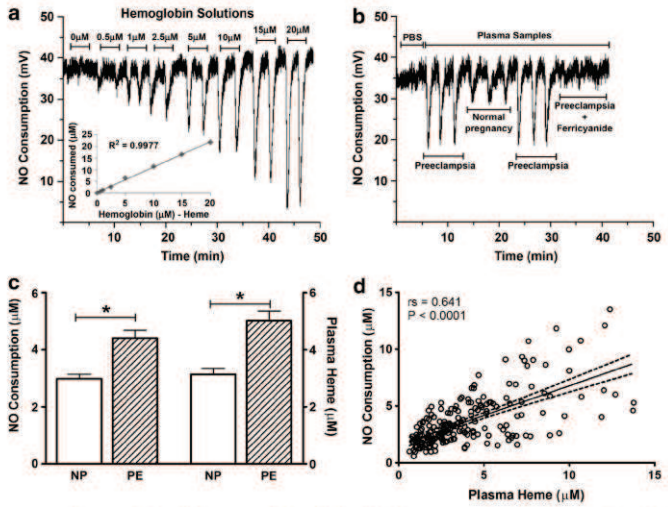

Figure 2. NO consumption assay and correlation between cell-free Hb and NO consumption. (**a**) Shows transient decreases in baseline NO signal generated by a solution of DETANONOate in PBS, which was purged with a nitrogen stream inline with an NO analyzer. The decreases in NO signal correspond to NO consumption (mV) by Hb solutions (standard curve). The inset shows significant correlation between the amounts of NO consumed and the Hb concentrations in the solutions. (**b**) Shows NO consumption by plasma samples from healthy pregnant women and PE patients. (**c**) Shows levels of NO consumption and Hb concentrations, expressed as heme, in plasma from NP women and patients with PE. (**d**) Shows significant positive correlation between NO consumption by plasma samples and plasma heme concentrations. The regression line and the 95% confidence interval are plotted. *P* and Spearman's correlation (rs) are reported. Values are the mean ± s.e.m. $^{*}P<0.05$ versus the NP group.

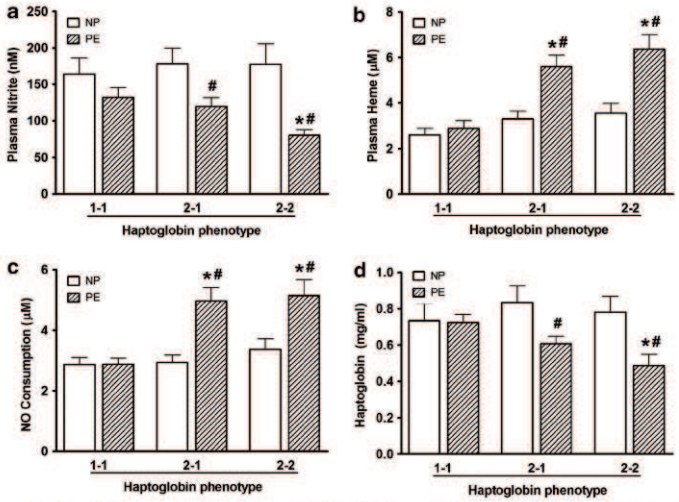

Figure 3. Hb polymorphism effects on NP womenand patients with PE. (**a**) Shows Hb genotype effects on plasma nitrite levels in NP women and PE patients. (**b**) Shows Hb genotype effects on cell-free Hb levels measured by ELISA. (**c**) Shows Hb genotype effects on NO consumption levels by plasma from NP women and PE patients. (**d**) Shows Hb levels according to each Hb genotype. Values are the mean ± s.e.m. $^{*}P<0.05$ compared with Hb phenotype 1-1 in PE (striped bars). $^{\#}P<0.05$ compared with respective phenotype in NP women (open bars).

L-arginine,[34] inhibition of phosphodiesterase type 5 with sildenafil[35] and enhancing heme oxygenase activity (which degrades heme) by administering statins[36] have been shown to ameliorate PE symptoms in animal models of PE through pathways that might increase NO bioavailability. However, these therapies remain to be tested in clinical trials.

In conclusion, our results may have clinical importance because they suggest a functional association between Hp polymorphisms,

increased Hb decompartmentalization and augmented NO consumption, which possibly contribute to the hemodynamic derangements of PE.

What is known about this topic

- Nitric oxide has a key role in the hemodynamic changes seen in healthy pregnancy.
- Increased circulating cell-free Hb levels reduce NO bioavailability by scavenging it, thus impairing vasodilation in the maternal systemic circulation in PE.
- Haptoglobin forms a complex with Hb, increasing its clearance rate. However, no previous study has examined if Hb polymorphisms contribute to NO scavenging and NO bioavailability in PE.

What this study adds

- Haptoglobin genotypes Hp2-1 and Hp2-2 increase NO scavenging by cell-free Hb and decrease NO bioavailability in PE.
- Haptoglobin phenotype Hp1-1 may be associated with cardiovascular protection by reducing Hb-driven NO consumption.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. *Am J Physiol* 1997; **272**(2 Part 2): R441–R463.
2 Thornburg KL, Jacobson S-L, Giraud GD, Morton MJ. Hemodynamic changes in pregnancy. *Semin Perinatol* 2000; **24**: 11–14.
3 Jim B, Sharma S, Kebede T, Acharya A. Hypertension in pregnancy: a comprehensive update. *Cardiol Rev* 2010; **18**(4): 178–189.
4 Palei AC, Sandrim VC, Cavalli RC, Tanus-Santos JE. Comparative assessment of matrix metalloproteinase (MMP)-2 and MMP-9, and their inhibitors, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in preeclampsia and gestational hypertension. *Clin Biochem* 2008; **41**(10-11): 875–880.
5 Sandrim VC, Montenegro MF, Palei AC, Metzger IF, Sertorio JT, Cavalli RC *et al.* Increased circulating cell-free hemoglobin levels reduce nitric oxide bioavailability in preeclampsia. *Free Radic Biol Med* 2010; **49**(3): 493–500.
6 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF *et al.* Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004; **350**(7): 672–683.
7 Sankaralingam S, Xu H, Davidge ST. Arginase contributes to endothelial cell oxidative stress in response to plasma from women with preeclampsia. *Cardiovasc Res* 2010; **85**(1): 194–203.
8 Wikstrom AK, Nash P, Eriksson UJ, Olovsson MH. Evidence of increased oxidative stress and a change in the plasminogen activator inhibitor (PAI)-1 to PAI-2 ratio in early-onset but not late-onset preeclampsia. *Am J Obstet Gynecol* 2009; **201**(6): 597 e591–597 e598.
9 Sandrim VC, Palei AC, Metzger IF, Cavalli RC, Duarte G, Tanus-Santos JE. Interethnic differences in ADMA concentrations and negative association with nitric oxide formation in preeclampsia. *Clin Chim Acta* 2010; **411**(19–20): 1457–1460.
10 Sandrim VC, Palei AC, Cavalli RC, Araujo FM, Ramos ES, Duarte G *et al.* eNOS haplotypes associated with gestational hypertension or preeclampsia. *Pharmacogenomics* 2008; **9**(10): 1467–1473.
11 Azarov I, He X, Jeffers A, Basu S, Ucer B, Hantgan RR *et al.* Rate of nitric oxide scavenging by hemoglobin bound to haptoglobin. *Nitric Oxide* 2008; **18**(4): 296–302.
12 Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon III RO, Schechter AN *et al.* Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 2002; **8**(12): 1383–1389.
13 Pacher P, Beckman JS, Liaudet L. Nitric Oxide and Peroxynitrite in Health and Disease. *Physiol Rev* 2007; **87**(1): 315–424.
14 Wagener FA, Eggert A, Boerman OC, Oyen WJ, Verhofstad A, Abraham NG *et al.* Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood* 2001; **98**(6): 1802–1811.
15 Levy AP, Asleh R, Blum S, Levy NS, Miller-Lotan R, Kalet-Litman S *et al.* Haptoglobin: basic and clinical aspects. *Antioxid Redox Signal* 2010; **12**(2): 293–304.
16 Bowman BH, Kurosky A. Haptoglobin: the evolutionary product of duplication, unequal crossing over, and point mutation. *Adv Hum Genet* 1982; **12**(189-261): 453–184.
17 Wejman JC, Hovsepian D, Wall JS, Hainfeld JF, Greer J. Structure and assembly of haptoglobin polymers by electron microscopy. *J Mol Biol* 1984; **174**(2): 343–368.
18 Edwards DH, Griffith TM, Ryley HC, Henderson AH. Haptoglobin-haemoglobin complex in human plasma inhibits endothelium dependent relaxation: evidence that endothelium derived relaxing factor acts as a local autocoid. *Cardiovasc Res* 1986; **20**(8): 549–556.
19 Wang X, Tanus-Santos JE, Reiter CD, Dejam A, Shiva S, Smith RD *et al.* Biological activity of nitric oxide in the plasmatic compartment. *Proc Natl Acad Sci USA* 2004; **101**(31): 11477–11482.
20 Sammour RN, Nakhoul FM, Levy AP, Miller-Lotan R, Nakhoul N, Awad HR *et al.* Haptoglobin phenotype in women with preeclampsia. *Endocrine* 2010; **38**(2): 303–308.
21 Anonymous. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 2000; **183**(1): S1–S22.
22 Pelletier MM, Kleinbongard P, Ringwood L, Hito R, Hunter CJ, Schechter AN *et al.* The measurement of blood and plasma nitrite by chemiluminescence: Pitfulls and solution. *Free Radic Biol Med* 2006.
23 Soejima M, Koda Y. TaqMan-based real-time PCR for genotyping common polymorphisms of haptoglobin (HP1 and HP2). *Clin Chem* 2008; **54**(11): 1908–1913.
24 Olsson MG, Centlow M, Rutardottir S, Stenfors I, Larsson J, Hosseini-Maaf B *et al.* Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger alpha(1)-microglobulin in preeclampsia. *Free Radic Biol Med* 2010; **48**(2): 284–291.
25 Centlow M, Carninci P, Nemeth K, Mezey E, Brownstein M, Hansson SR. Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes. *Fertil Steril* 2008; **90**(5): 1834–1843.
26 Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA* 2005; **293**(13): 1653–1662.
27 Jeffers A, Gladwin MT, Kim-Shapiro DB. Computation of plasma hemoglobin nitric oxide scavenging in hemolytic anemias. *Free Radic Biol Med* 2006; **41**(10): 1557–1565.
28 Asleh R, Marsh S, Shilkrut M, Binah O, Guetta J, Lejbkowicz F *et al.* Genetically determined heterogeneity in hemoglobin scavenging and susceptibility to diabetic cardiovascular disease. *Circ Res* 2003; **92**(11): 1193–1200.
29 Levy AP, Purushothaman KR, Levy NS, Purushothaman M, Strauss M, Asleh R *et al.* Downregulation of the hemoglobin scavenger receptor in individuals with diabetes and the Hp 2-2 genotype: implications for the response to intraplaque hemorrhage and plaque vulnerability. *Circ Res* 2007; **101**(1): 106–110.
30 Komori H, Watanabe H, Shuto T, Kodama A, Maeda H, Watanabe K *et al.* alpha1-acid glycoprotein up-regulates CD163 via TLR4/CD14 pathway: possible protection against hemolysis-induced oxidative stress. *J Biol Chem* 2012; **287**: 30688–30700.
31 Weissgerber TL, Roberts JM, Jeyabalan A, Powers RW, Lee M, Datwyler SA *et al.* Haptoglobin phenotype, angiogenic factors, and preeclampsia risk. *Am J Obstet Gynecol* 2012; **206**(4): 358 e310–358 e358.
32 Chandra TPT, Vishnupriya S, Venkat Raman R. Haptoglobin polymorphism in pregnancy-induced hypertension. *Am J Hum Genet* 1991; **49**: 130.
33 Kato GJ, Gladwin MT. Evolution of novel small-molecule therapeutics targeting sickle cell vasculopathy. *JAMA* 2008; **300**(22): 2638–2646.
34 Alexander BT, Llinas MT, Kruckeberg WC, Granger JP. L-arginine attenuates hypertension in pregnant rats with reduced uterine perfusion pressure. *Hypertension* 2004; **43**(4): 832–836.
35 Ramesar SV, Mackraj I, Gathiram P, Moodley J. Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. *Eur J Obstet Gynecol Reprod Biol* 2010; **149**(1): 22–26.
36 George EM, Arany M, Cockrell K, Storm MV, Stec DE, Granger JP. Induction of heme oxygenase-1 attenuates sFlt-1-induced hypertension in pregnant rats. *Am J Physiol Regul Integr Comp Physiol* 2011; **301**(5): R1495–R1500.

5 – DISCUSSÃO

O primeiro estudo teve como principal objetivo mostrar a relevância da hemoglobina descompartimentalizada para a biodisponibilidade de NO na pré-eclâmpsia. Nesse trabalho, foram encontrados níveis aumentados de hemoglobina livre e maior consumo de NO em mulheres com PE quando comparadas com grávidas sadias, além de uma correlação positiva entre consumo de NO e hemólise. Também se mostrou uma relação inversa entre esses parâmetros e os produtos de oxidação do NO, nitrito e nitrato. De modo geral, esses achados sugerem que a menor biodisponibilidade de NO na PE pode ser devida ao maior consumo de NO.

Para avaliar a biodisponibilidade de NO, foram medidas a concentrações de nitrito em amostras de plasma e sangue total. O ânion nitrito foi escolhido como biomarcador do NO, pois, estudos recentes, mostraram que os níveis de nitrito plasmático podem refletir a produção endógena de NO pela eNOS em aproximadamente 70% [80] e outros estudos mostraram que a inibição da atividade eNOS resulta em redução nas concentrações de nitrito [81]. Também foram avaliados os níveis de nitrato em gestantes normais e com pré-eclâmpsia, porém não foi encontrado diferença entre os grupos analisados. O fato de se ter encontrado níveis reduzidos de nitrito, e não nitrato, em gestantes com PE comparadas com grávidas normais, sugere que o nitrito pode melhor refletir a baixa biodisponibilidade de NO na PE.

Nesse estudo, mostrou-se que o NO detectado no plasma e no sangue pelo método de quimioluminescência tinha como origem o ânion nitrito, e não outras espécies como os S-nitrosotióis e as N-nitrosoaminas. Para isso, as amostras foram tratadas com sulfanilamida ácida, um reagente que converte o nitrito em um cátion diazônio, [82] o qual não é detectado pelo analisador de NO [83, 84]. Esse tratamento reduz o sinal de quimioluminescência a níveis basais, mostrando que o nitrito é o maior responsável pelo sinal obtido no analisador de NO. Para confirmar os valores encontrados, utilizou-se outro método para detectar NO, baseado em uma solução de ácido ascórbico, o qual detecta somente o NO gerado a partir do ânion nitrito, sem a interferência de S-nitrosotióis, N-nitrosoaminas, proteínas e lipídeos nitrados [85]. Ambos os métodos (tri-iodo e ácido ascórbico) geraram resultados similares, mostrando níveis reduzidos de nitrito em gestantes com PE. Não podemos descartar a

possibilidade de que outras espécies possam ter pequena contribuição para os picos detectados no analisador de NO, porém os métodos para analisar NO vêm sendo extensivamente utilizados e validados por diferentes laboratórios [86, 87].

Diversos outros fatores podem modificar as concentrações de nitrito [88], incluindo a redução de nitrato a nitrito por bactérias comensais presentes na cavidade oral e no trato gastrointestinal, o nitrito ingerido na dieta [89] e a atividade nitrito sintase da enzima ceruloplasmina [90]. No presente estudo, foram encontrados níveis aumentados de ceruloplasmina em gestantes com PE comparadas com grávidas normais, confirmando achados de outros estudos [91, 92]. Apesar de se esperar que níveis aumentados de ceruloplasmina pudessem resultar em concentrações aumentadas de nitrito, encontramos níveis reduzidos de nitrito na PE. Isso pode ser devido a uma menor formação e maior degradação NO na PE.

Em condições normais, a hemoglobina está compartimentalizada dentro dos eritrócitos, permitindo que o NO gerado pelas células endoteliais alcance as células do músculo liso vascular produzindo vasodilatação. Entretanto, quando há hemólise intravascular, a hemoglobina livre pode reagir com o NO pela reação de dioxigenação, gerando metahemoglobina e nitrato, o qual não apresenta atividade vasodilatadora [39]. Para confirmar se o consumo de NO pelo plasma foi mediado somente pelo ferro do grupo heme da hemoglobina livre, adiciou-se ferricianeto de potássio e cianeto de potássio nas amostras de plasma. O ferricianeto oxida a hemoglobina (FeII) em metahemoglobina (FeIII) e o cianeto converte a metahemoglobina em cianometahemoglobina, a qual não consome o NO [39]. Com isso, as amostras tratadas com ferricianeto/cianeto não consumiram o NO gerado pela solução doadora de NO.

Com o objetivo de avaliar se os grupos tiol também poderiam consumir NO, foi adicionado N-etilmaleimida, um inibidor de tiol, nas amostras de plasma. Ambas as amostras, com e sem N-etilmaleimida, apresentaram uma mesma quantidade de consumo de NO. Para confirmar esses achados, demonstramos que a glutationa (um tiol não protéico), em concentrações similares as encontradas no plasma de mulheres com PE [93], não consome

NO. Esses experimentos confirmam que o grupo tiol não participa do consumo de NO.

Também foi avaliado se as reduzidas concentrações de nitrito presentes no plasma de pacientes com PE poderiam modificar o consumo de NO. Para isso, adicionou-se nitrito ao plasma dessas pacientes em quantidade suficiente para alcançar os níveis de nitrito encontrados no plasma das grávidas sadias. Porém, não houve alterações nos níveis de consumo de NO. Isso consiste com a ideia de que, em níveis fisiológicos (escala nanomolar), o nitrito provavelmente não oxida todo o heme presente no plasma de grávidas com PE, o qual está na escala micromolar.

Ainda que outras substâncias também possam consumir o NO [38], a correlação significativa que encontramos entre o consumo de NO e a hemoglobina plasmática apoia a ideia de que a hemoglobina livre possa reduzir a biodisponibilidade de NO. De fato, alguns trabalhos mostraram que a hemoglobina livre inibe completamente a dilatação mediada pelo NO nas artérias coronárias [94] e produz efeito vasoconstritor dose-dependente, efeito que não é encontrado quando a hemoglobina está compartimentalizada [95]. Em outras condições onde há hemólise, como na hemoglobinúria paroxística noturna, no “bypass” cardiopulmonar, anemia falciforme e malária [39, 41, 42, 53, 96] mostrou-se que o aumento nas concentrações de hemoglobina plasmática compromete a função cardiovascular normal. Além disso, outros estudos mostraram associação entre doenças hipertensivas da gravidez e a anemia falciforme em mulheres grávidas, o que poderia, em parte, explicar as alterações hemodinâmicas encontradas em mulheres com PE [97, 98].

A biologia do óxido nítrico é muito complexa e outros mecanismos também podem comprometer a biodisponibilidade de NO na PE. Por exemplo, maiores concentrações de ADMA (um inibidor endógeno da eNOS) [99], fatores antiangiogênicos [8], variações genéticas [36] e o estresse oxidativo [100]. Além desses fatores, o aumento nas concentrações de hemoglobina plasmática resulta em um excesso de grupos heme livre. O átomo de ferro do grupo heme participa da produção de espécies reativas de oxigênio, como o ânion superóxido, o qual pode reagir com o NO para formar o peroxinitrito, um oxidante muito mais potente [69]. Essas reações resultam na liberação de

mediadores inflamatórios [101, 102], além de lesar as células endoteliais [103]. Em acréscimo, a atividade da enzima que degrada o grupo heme, a heme oxigenase, está reduzida na PE, o que pode contribuir para maiores níveis de heme plasmático e consumo de NO [104].

Além da hemoglobina livre, o complexo formado entre a hemoglobina e a haptoglobina (Hb-Hp) também é capaz de sequestrar o NO. Neste complexo, o dímero da hemoglobina se apresenta no estado ferroso, o qual consome NO através da reação de dioxigenação, inibindo o relaxamento dependente do endotélio [38, 78]. Essas observações conferem uma possível explicação para as diferentes propriedades antioxidantes associadas com as diferentes estruturas da haptoglobina encontradas em humanos [66].

Em nosso segundo estudo, encontramos níveis aumentados de hemoglobina livre associados com maior consumo de NO e reduzida concentração de haptoglobina para os genótipos Hp2-1 e Hp2-2 em gestantes com PE comparadas com grávidas sadias. No entanto, o genótipo Hp1-1 na PE não mostrou aumentos significativos nos níveis de heme plasmático ou consumo de NO, quando comparado com grávidas normais. Esses achados estão de acordo com outros estudos com PE, onde se mostrou que a Hp1-1 apresenta papel protetor contra o estresse oxidativo induzido pela hemoglobina [105], enquanto que a Hp2-2 parece ser um fator de risco para doença cardiovascular [106].

Em humanos, a proteína Hp1-1 é um dímero, e como ela possui um tamanho menor que os polímeros linear (Hp2-1) e cíclico (Hp2-2) encontrados nos outros genótipos, ela pode ser eliminada mais rapidamente via receptor CD163 de monócitos/macrófagos após se ligar aos dímeros de hemoglobina, conferindo maior proteção contra o estresse oxidativo [46].

Além disso, pacientes com PE e genótipo Hp2-1 ou Hp2-2 apresentaram níveis aumentados de heme plasmático associado a maior consumo de NO quando comparados com pacientes com genótipo Hp 1-1. Isso está de acordo com outros achados onde se mostrou que o complexo Hb-Hp1-1 é eliminado mais rapidamente do que o complexo Hb-Hp2-2 [106]. Como o consumo de NO pela reação de dioxigenação ocorre a uma mesma taxa para a hemoglobina livre e para a hemoglobina ligada a haptoglobina

(Hp1-1 ou Hp2-2), foi proposto que as diferentes taxas de captação dos complexos Hb-Hp1-1 e Hb-Hp2-2 pelos macrófagos poderiam ter um efeito importante na biodisponibilidade do NO [46]. Em acréscimo, mostrou-se que a expressão do receptor de monócitos/macrófagos CD163, o qual liga o complexo Hb-Hp, pode variar em diferentes patologias [107, 108]. Portanto, além do genótipo da haptoglobina, a expressão do receptor CD163 pode ter importância clínica na regulação da taxa na qual o complexo Hb-Hp é retirado da circulação na PE, mas essa hipótese ainda precisa ser testada.

Esse estudo possui algumas limitações. Primeiramente, não provamos que a hemoglobina contribui diretamente para a hipertensão na PE. Porém, existem vários estudos mostrando que a hemólise contribui para a hipertensão em outras patologias [42]. Também não encontramos diferença na frequência dos genótipos da haptoglobina. Entretanto, os dois maiores estudos, em número de pacientes, que compararam as frequências do fenótipo da haptoglobina na PE, mostraram que a Hp1-1 apresenta maior prevalência em grávidas sadias [105] e que a Hp2-1 está associada com maior risco de se desenvolver PE in mulheres caucasianas [109], o que apoia nossos resultados. Além disso, mostrou-se que Hp2-2 apresenta maior incidência em doenças hipertensivas da gravidez [110].

Esses resultados podem ter implicações clínicas relevantes. É possível que terapias farmacológicas que restaurem a atividade NO-cGMP ou protejam contra o consumo de NO mediado pela hemoglobina, possam atenuar as alterações hemodinâmicas associadas com essa síndrome. De fato, mostrou-se que o tratamento com o substrato para eNOS L-arginina [111], a inibição da fosfodiesterase tipo 5 com sildenafil [112] e ou aumento da atividade da hemeoxigenase (a qual degrada o grupo heme) pela administração de estatinas [113], melhoram os sintomas da PE em modelos animais de pré-eclâmpsia por rotas bioquímicas que podem aumentar a biodisponibilidade de NO.

Outros estudos são necessários para se compreender melhor o papel do NO e da hemoglobina livre na PE, como a concentração dessas moléculas varia durante o curso da gravidez e se há correlação entre esses dados e os parâmetros hemodinâmicos na PE.

6- CONCLUSÃO

Nesse estudo, encontramos níveis aumentados de hemoglobina livre e de consumo de NO em grávidas com pré-eclâmpsia comparadas com grávidas sadias, o que está associado a uma menor biodisponibilidade de NO. Além disso, encontramos uma correlação positiva entre esses dois parâmetros, o que sugere que a hemoglobina livre pode aumentar o consumo de NO pelo plasma, alterando a biodisponibilidade do NO. Também mostramos que há uma possível associação entre os genótipos da haptoglobina, os níveis de hemoglobina descompartimentalizada e o consumo de NO encontrados. Apesar do genótipo da haptoglobina não afetar o risco de se desenvolver PE, o genótipo Hp1-1 parece exercer um papel protetor na pré-eclâmpsia ao reduzir o consumo de NO, enquanto que os genótipos Hp2-1 e Hp2-2 podem agravar a PE ao reduzir a biodisponibilidade de NO.

7- REFERÊNCIAS BIBLIOGRÁFICAS

1. WHO, World health report: make every mother and child count. 2005. Geneva; WHO; p. 63.
2. Laurenti R, J.M., Gotlieb SLD., A mortalidade materna nas capitais brasileiras: algumas características e estimativa de um fator de ajuste. Rev Bras Epidemiol., 2004. 7(4):449-60.
3. Duckitt, K. and D. Harrington, Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. BMJ, 2005. 330(7491): p. 565.
4. Redman, C.W. and I.L. Sargent, Latest advances in understanding preeclampsia. Science, 2005. 308(5728): p. 1592-4.
5. Gerretsen, G., H.J. Huisjes, and J.D. Elema, Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. Br J Obstet Gynaecol, 1981. 88(9): p. 876-81.
6. Hanretty, K.P., M.J. Whittle, and P.C. Rubin, Doppler uteroplacental waveforms in pregnancy-induced hypertension: a re-appraisal. Lancet, 1988. 1(8590): p. 850-2.
7. Granger, J.P., et al., Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction. Hypertension, 2001. 38(3 Pt 2): p. 718-22.
8. Sandrim, V.C., et al., Nitric oxide formation is inversely related to serum levels of antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endogline in preeclampsia. Hypertension, 2008. 52(2): p. 402-7.
9. Makris, A., et al., Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. Kidney Int, 2007. 71(10): p. 977-84.
10. Levine, R.J., et al., Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med, 2004. 350(7): p. 672-83.
11. Venkatesha, S., et al., Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med, 2006. 12(6): p. 642-9.
12. Shibuya, M., Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. Angiogenesis, 2006. 9(4): p. 225-30; discussion 231.
13. He, H., et al., Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. J Biol Chem, 1999. 274(35): p. 25130-5.
14. Kendall, R.L. and K.A. Thomas, Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A, 1993. 90(22): p. 10705-9.
15. Cheifetz, S., et al., Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem, 1992. 267(27): p. 19027-30.
16. Toporsian, M., et al., A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. Circ Res, 2005. 96(6): p. 684-92.
17. Santibanez, J.F., et al., Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. J Cell Physiol, 2007. 210(2): p. 456-68.

18. Ignarro, L.J., et al., Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A, 1987. 84(24): p. 9265-9.
19. Furchgott, R.F. and J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature, 1980. 288(5789): p. 373-6.
20. Forstermann, U. and W.C. Sessa, Nitric oxide synthases: regulation and function. Eur Heart J, 2012. 33(7): p. 829-37, 837a-837d.
21. Jin, R.C., B. Voetsch, and J. Loscalzo, Endogenous mechanisms of inhibition of platelet function. Microcirculation, 2005. 12(3): p. 247-58.
22. Johnston, B., et al., Nitric oxide inhibits microvascular protein leakage induced by leukocyte adhesion-independent and adhesion-dependent inflammatory mediators. Microcirculation, 1999. 6(2): p. 153-62.
23. Kapadia, M.R., et al., Nitric oxide regulates the 26S proteasome in vascular smooth muscle cells. Nitric Oxide, 2009. 20(4): p. 279-88.
24. Cudmore, M., et al., VEGF-E activates endothelial nitric oxide synthase to induce angiogenesis via cGMP and PKG-independent pathways. Biochem Biophys Res Commun, 2006. 345(4): p. 1275-82.
25. Joshi, M.S., J.L. Ponthier, and J.R. Lancaster, Jr., Cellular antioxidant and pro-oxidant actions of nitric oxide. Free Radic Biol Med, 1999. 27(11-12): p. 1357-66.
26. Sladek, S.M., R.R. Magness, and K.P. Conrad, Nitric oxide and pregnancy. Am J Physiol, 1997. 272(2 Pt 2): p. R441-63.
27. Williams, D.J., et al., Nitric oxide-mediated vasodilation in human pregnancy. Am J Physiol, 1997. 272(2 Pt 2): p. H748-52.
28. Thornburg, K.L., et al., Hemodynamic changes in pregnancy. Seminars in Perinatology, 2000. 24(February): p. 11-14.
29. Jim, B., et al., Hypertension in pregnancy: a comprehensive update. Cardiol Rev, 2010. 18(4): p. 178-89.
30. Choi, J.W., M.W. Im, and S.H. Pai, Nitric oxide production increases during normal pregnancy and decreases in preeclampsia. Ann Clin Lab Sci, 2002. 32(3): p. 257-63.
31. Sandrim, V.C., et al., Increased circulating cell-free hemoglobin levels reduce nitric oxide bioavailability in preeclampsia. Free Radic Biol Med, 2010. 49(3): p. 493-500.
32. Sankaralingam, S., H. Xu, and S.T. Davidge, Arginase contributes to endothelial cell oxidative stress in response to plasma from women with preeclampsia. Cardiovascular research, 2010. 85(1): p. 194-203.
33. Wikstrom, A.K., et al., Evidence of increased oxidative stress and a change in the plasminogen activator inhibitor (PAI)-1 to PAI-2 ratio in early-onset but not late-onset preeclampsia. Am J Obstet Gynecol, 2009. 201(6): p. 597 e1-8.
34. Kukor, Z., S. Valent, and M. Toth, Regulation of nitric oxide synthase activity by tetrahydrobiopterin in human placentae from normal and pre-eclamptic pregnancies. Placenta., 2000. 21(8): p. 763-72.
35. Sandrim, V.C., et al., Interethnic differences in ADMA concentrations and negative association with nitric oxide formation in preeclampsia. Clin Chim Acta, 2010. 411(19-20): p. 1457-60.
36. Sandrim, V.C., et al., eNOS haplotypes associated with gestational hypertension or preeclampsia. Pharmacogenomics, 2008. 9(10): p. 1467-73.

37. Luizon, M.R., et al., Epistasis among eNOS, MMP-9 and VEGF maternal genotypes in hypertensive disorders of pregnancy. Hypertens Res, 2012.
38. Wang, X., et al., Biological activity of nitric oxide in the plasmatic compartment. Proc Natl Acad Sci U S A, 2004. 101(31): p. 11477-82.
39. Reiter, C.D., et al., Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med, 2002. 8(12): p. 1383-9.
40. Morris, C.R., et al., Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. JAMA, 2005. 294(1): p. 81-90.
41. Gladwin, M.T., et al., Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. N Engl J Med, 2004. 350(9): p. 886-95.
42. Rother, R.P., et al., The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA, 2005. 293(13): p. 1653-62.
43. Donadee, C., et al., Nitric Oxide Scavenging by Red Blood Cell Microparticles and Cell-Free Hemoglobin as a Mechanism for the Red Cell Storage Lesion. Circulation, 2011. 124(4): p. 465-U294.
44. Gladwin, M.T., T. Kanias, and D.B. Kim-Shapiro, Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease. J Clin Invest, 2012. 122(4): p. 1205-8.
45. Hanson, M.S., et al., Methaemalbumin formation in sickle cell disease: effect on oxidative protein modification and HO-1 induction. British Journal of Haematology, 2011. 154(4): p. 502-511.
46. Azarov, I., et al., Rate of nitric oxide scavenging by hemoglobin bound to haptoglobin. Nitric Oxide, 2008. 18(4): p. 296-302.
47. Jeffers, A., M.T. Gladwin, and D.B. Kim-Shapiro, Computation of plasma hemoglobin nitric oxide scavenging in hemolytic anemias. Free Radic Biol Med, 2006. 41(10): p. 1557-65.
48. Gladwin, M.T. and D.B. Kim-Shapiro, Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis. Current Opinion in Hematology, 2009. 16(6): p. 515-523.
49. Kim-Shapiro, D.B., J. Lee, and M.T. Gladwin, Storage lesion: role of red blood cell breakdown. Transfusion, 2011. 51(4): p. 844-851.
50. Minneci, P.C., et al., Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin. J Clin Invest, 2005. 115(12): p. 3409-17.
51. Frei, A.C., et al., Vascular dysfunction in a murine model of severe hemolysis. Blood, 2008. 112(2): p. 398-405.
52. Gladwin, M.T. and E. Vichinsky, Pulmonary complications of sickle cell disease. N Engl J Med, 2008. 359(21): p. 2254-65.
53. Sobolewski, P., et al., Nitric oxide bioavailability in malaria. Trends Parasitol, 2005. 21(9): p. 415-22.
54. Garratty, G., Immune hemolytic anemia associated with drug therapy. Blood Rev, 2010. 24(4-5): p. 143-50.
55. LaMuraglia, G.M., et al., The reduction of the allogenic transfusion requirement in aortic surgery with a hemoglobin-based solution. J Vasc Surg, 2000. 31(2): p. 299-308.
56. Saxena, R., et al., Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. Stroke, 1999. 30(5): p. 993-6.

57. Rodriguez, C., et al., Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction. Biochem J, 2009. 422(3): p. 423-32.
58. Kato, G.J., et al., Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. Blood, 2006. 107(6): p. 2279-85.
59. Hsu, L.L., et al., Hemolysis in sickle cell mice causes pulmonary hypertension due to global impairment in nitric oxide bioavailability. Blood, 2007. 109(7): p. 3088-98.
60. Reiter, C.D., et al., Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med, 2002. 8(12): p. 1383-1389.
61. Pohl, U. and D. Lamontagne, Impaired tissue perfusion after inhibition of endothelium-derived nitric oxide. Basic Res Cardiol, 1991. 86 Suppl 2: p. 97-105.
62. Sarrel, P.M., et al., Hypothesis: inhibition of endothelium-derived relaxing factor by haemoglobin in the pathogenesis of pre-eclampsia. Lancet, 1990. 336(8722): p. 1030-2.
63. Centlow, M., et al., Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes. Fertil Steril, 2008. 90(5): p. 1834-43.
64. Melamed-Frank, M., et al., Structure-function analysis of the antioxidant properties of haptoglobin. Blood, 2001. 98(13): p. 3693-8.
65. Wang, Y., et al., Haptoglobin, an inflammation-inducible plasma protein. Redox Rep, 2001. 6(6): p. 379-85.
66. Levy, A.P., et al., Haptoglobin: basic and clinical aspects. Antioxid Redox Signal, 2010. 12(2): p. 293-304.
67. Kristiansen, M., et al., Identification of the haemoglobin scavenger receptor. Nature, 2001. 409(6817): p. 198-201.
68. Tolosano, E., et al., Heme scavenging and the other facets of hemopexin. Antioxid Redox Signal, 2010. 12(2): p. 305-20.
69. Wagener, F.A., et al., Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood, 2001. 98(6): p. 1802-11.
70. Jansen, T., et al., Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. J Mol Cell Cardiol, 2010. 49(2): p. 186-95.
71. Pae, H.O., et al., Role of heme oxygenase in preserving vascular bioactive NO. Nitric Oxide, 2010. 23(4): p. 251-7.
72. Nielsen, M.J., H.J. Moller, and S.K. Moestrup, Hemoglobin and heme scavenger receptors. Antioxid Redox Signal, 2010. 12(2): p. 261-73.
73. Wu, M.L., Y.C. Ho, and S.F. Yet, A central role of heme oxygenase-1 in cardiovascular protection. Antioxid Redox Signal, 2011. 15(7): p. 1835-46.
74. Philippidis, P., et al., Hemoglobin scavenger receptor CD163 mediates interleukin-10 release and heme oxygenase-1 synthesis: antiinflammatory monocyte-macrophage responses in vitro, in resolving skin blisters in vivo, and after cardiopulmonary bypass surgery. Circ Res, 2004. 94(1): p. 119-26.
75. Bowman, B.H. and A. Kurosky, Haptoglobin: the evolutionary product of duplication, unequal crossing over, and point mutation. Adv Hum Genet, 1982. 12: p. 189-261, 453-4.

76. Maeda, N., et al., Duplication within the haptoglobin Hp2 gene. Nature, 1984. 309(5964): p. 131-5.
77. Wejman, J.C., et al., Structure and assembly of haptoglobin polymers by electron microscopy. J Mol Biol, 1984. 174(2): p. 343-68.
78. Edwards, D.H., et al., Haptoglobin-haemoglobin complex in human plasma inhibits endothelium dependent relaxation: evidence that endothelium derived relaxing factor acts as a local autocoid. Cardiovasc Res, 1986. 20(8): p. 549-56.
79. Gueye, P.M., et al., Influence of human haptoglobin polymorphism on oxidative stress induced by free hemoglobin on red blood cells. Clin Chem Lab Med, 2006. 44(5): p. 542-7.
80. Kleinbongard, P., Dejam, A., Lauer, T., Rassaf, T., Schindler, A., Picker, O., Scheeren, T., Godecke, A., Schrader, J., Schulz, R., Heusch, G., Schaub, G.A., Bryan, N.S., Feelisch, M. & Kelm, M., Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med., 2003. 35: p. 790-796.
81. Kelm, M., et al., Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. Cardiovasc Res, 1999. 41(3): p. 765-72.
82. Samouilov, A. and J.L. Zweier, Development of chemiluminescence-based methods for specific quantitation of nitrosylated thiols. Anal Biochem, 1998. 258(2): p. 322-30.
83. MacArthur, P.H., S. Shiva, and M.T. Gladwin, Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. J Chromatogr B Analyt Technol Biomed Life Sci, 2007. 851(1-2): p. 93-105.
84. Hausladen, A., et al., Assessment of nitric oxide signals by triiodide chemiluminescence. Proc Natl Acad Sci U S A, 2007. 104(7): p. 2157-62.
85. Nagababu, E. and J.M. Rifkind, Measurement of plasma nitrite by chemiluminescence without interference of S-, N-nitroso and nitrated species. Free Radic Biol Med, 2007. 42(8): p. 1146-54.
86. Gladwin, M.T., et al., Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc Natl Acad Sci U S A, 2000. 97(21): p. 11482-7.
87. Kleinbongard, P., et al., Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. Free Radic Biol Med, 2006. 40(2): p. 295-302.
88. Gladwin, M.T., et al., The emerging biology of the nitrite anion. Nat Chem Biol, 2005. 1(6): p. 308-14.
89. Lundberg, J.O., E. Weitzberg, and M.T. Gladwin, The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov, 2008. 7(2): p. 156-67.
90. Shiva, S., et al., Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. Nat Chem Biol, 2006. 2(9): p. 486-93.
91. Kristensen, K., et al., Serum amyloid a protein and C-reactive protein in normal pregnancy and preeclampsia. Gynecol Obstet Invest, 2009. 67(4): p. 275-80.
92. Orhan, H.G., H. Ozgunes, and M.S. Beksac, Correlation between plasma malondialdehyde and ceruloplasmin activity values in preeclamptic pregnancies. Clin Biochem, 2001. 34(6): p. 505-6.

93. Llurba, E., et al., A comprehensive study of oxidative stress and antioxidant status in preeclampsia and normal pregnancy. Free Radic Biol Med, 2004. 37(4): p. 557-70.
94. Liao, J.C., et al., Intravascular flow decreases erythrocyte consumption of nitric oxide. Proc Natl Acad Sci U S A, 1999. 96(15): p. 8757-61.
95. Nakai, K., et al., Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. J Cardiovasc Pharmacol, 1996. 28(1): p. 115-23.
96. Vermeulen Windsant, I.C., et al., Cardiovascular surgery and organ damage: time to reconsider the role of hemolysis. J Thorac Cardiovasc Surg, 2011. 142(1): p. 1-11.
97. Villers, M.S., et al., Morbidity associated with sickle cell disease in pregnancy. Am J Obstet Gynecol, 2008. 199(2): p. 125 e1-5.
98. Tsaras, G., et al., Complications associated with sickle cell trait: a brief narrative review. Am J Med, 2009. 122(6): p. 507-12.
99. Boger, R.H., et al., The Role of Nitric Oxide Synthase Inhibition by Asymmetric Dimethylarginine in the Pathophysiology of Preeclampsia. Gynecol Obstet Invest, 2009. 69(1): p. 1-13.
100. Li, H., et al., Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta. Placenta., 2005. 26(2-3): p. 210-7.
101. Lavrovsky, Y., et al., Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci U S A, 1994. 91(13): p. 5987-91.
102. Porto, B.N., et al., Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem, 2007. 282(33): p. 24430-6.
103. Balla, G., et al., Iron loading of endothelial cells augments oxidant damage. J Lab Clin Med, 1990. 116(4): p. 546-54.
104. Appleton, S.D., et al., Effects of hypoxia on heme oxygenase expression in human chorionic villi explants and immortalized trophoblast cells. Am J Physiol Heart Circ Physiol, 2003. 284(3): p. H853-8.
105. Sammour, R.N., et al., Haptoglobin phenotype in women with preeclampsia. Endocrine, 2010. 38(2): p. 303-8.
106. Asleh, R., et al., Genetically determined heterogeneity in hemoglobin scavenging and susceptibility to diabetic cardiovascular disease. Circ Res, 2003. 92(11): p. 1193-200.
107. Levy, A.P., et al., Downregulation of the hemoglobin scavenger receptor in individuals with diabetes and the Hp 2-2 genotype: implications for the response to intraplaque hemorrhage and plaque vulnerability. Circ Res, 2007. 101(1): p. 106-10.
108. Komori, H., et al., alpha1-acid glycoprotein up-regulates CD163 via TLR4/CD14 pathway: possible protection against hemolysis-induced oxidative stress. J Biol Chem, 2012.
109. Weissgerber, T.L., et al., Haptoglobin phenotype, angiogenic factors, and preeclampsia risk. Am J Obstet Gynecol, 2012. 206(4): p. 358 e10-8.
110. Chandra T, P.T., Vishnupriya S, Venkat Raman R. , Haptoglobin polymorphism in pregnancy-induced hypertension. Am J Hum Genet, 1991. 49:130.

111. Alexander, B.T., et al., L-arginine attenuates hypertension in pregnant rats with reduced uterine perfusion pressure. Hypertension, 2004. 43(4): p. 832-6.
112. Ramesar, S.V., et al., Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. Eur J Obstet Gynecol Reprod Biol, 2010. 149(1): p. 22-6.
113. George, E.M., et al., Induction of heme oxygenase-1 attenuates sFlt-1-induced hypertension in pregnant rats. Am J Physiol Regul Integr Comp Physiol, 2011. 301(5): p. R1495-500.

8- ANEXOS

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Campus Universitário - Monte Alegre
14048-900 Ribeirão Preto SP

Ribeirão Preto, 20 de junho de 2006

Ofício nº 1759/2006
CEP/MGV

Prezado Professor:

O trabalho intitulado **"FARMACOGENÉTICA DO TRATAMENTO ANTI-HIPERTENSIVO DE PACIENTES COM PRÉ-ECLÂMPSIA: RELEVÂNCIA DO HAPLÓTIPO DA eNOS",** foi analisado pelo Comitê de Ética em Pesquisa, em sua 227ª Reunião Ordinária realizada em 12/06/2006, e enquadrado na categoria: **APROVADO, bem como o Termo de Consentimento Livre e Esclarecido,** de acordo com o Processo HCRP nº 4682/2006.

Aproveito a oportunidade para apresentar a Vossa Senhoria protestos de estima e consideração.

DRª. MARCIA GUIMARÃES VILLANOVA
Secretária do Comitê de Ética em Pesquisa do HCRP e da FMRP-USP

Ilustríssimo Senhor
PROF.DR. JOSE EDUARDO TANUS DOS SANTOS
VALÉRIA CRISTINA SANDRIM (Colaboradora)
Depto. de Farmacologia – FMRP-USP
Em mãos

OBSERVAÇÃO:

O projeto intitulado “IMPLICAÇÕES FISIOPATOLÓGICAS DA DESCOMPARTIMENTALIZAÇÃO DA HEMOGLOBINA PARA A BIOLOGIA DO ÓXIDO NÍTRICO EM PACIENTES COM PRÉ-ECLÂMPSIA” é um sub-projeto do projeto intitulado “FARMACOGENÉTICA DO TRATAMENTO ANTI-HIPERTENSIVO DE PACIENTES COM PRÉ-ECLÂMPSIA: RELEVÂNCIA DO HAPLÓTIPO DA eNOS”, o qual já foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - ESTUDO

Projeto: "Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS

Responsáveis:

- Prof. Dr. José Eduardo Tanus dos Santos (FMRP-USP) – CREMESP 84.966
- Prof. Dr. Ricardo de Carvalho Cavalli – CREMESP 91.680
- Dra. Valéria Cristina Sandrim

Você está sendo convidada a participar de um estudo cujos detalhes são:

1) Este projeto pretende estudar se a Sra/Srta apresenta algumas diferenças em alguns dos seus genes (que são responsáveis pela transmissão de suas características hereditárias, que passam de pais para filhos) que poderiam contribuir para o desenvolvimento da pré-eclâmpsia (pressão alta na gravidez) e na resposta aos medicamentos utilizados no tratamento desta doença. Além disso, pretendemos estudar como estas diferenças nos genes podem modificar as concentrações de substâncias importantes no controle da sua pressão sanguínea. Estes dados poderão nos auxiliar no entendimento das alterações cardiovasculares que acontecem durante a gravidez e na obtenção de marcadores genéticos de desenvolvimento da pré-eclâmpsia.

2) Sua participação neste estudo será:

Serão retirados, no máximo, 30 mL do seu sangue, coletados por punção venosa utilizando técnica adequada. Este volume de sangue é cerca de 15 (quinze) vezes menor do que o volume de sangue habitualmente doado quando um indivíduo doa sangue para bancos de sangue. Este sangue será utilizado para realizar todas os estudos bioquímicos e genéticos mencionados acima. Uma segunda coleta (20 mL de sangue venoso) será realizada caso o

seu médico prescreva para o tratamento da pressão alta o medicamento α-metildopa. Com isto, encerra-se a sua participação neste estudo.

3) Você terá direito a ressarcimento financeiro caso haja gastos gerados exclusivamente pela sua participação como voluntário desta pesquisa.

4) Caso haja dano comprovadamente decorrente da pesquisa você terá direito à indenização.

5) NÓS NÃO PODEMOS E NÃO GARANTIREMOS QUE VOCÊ RECEBERÁ QUALQUER BENEFÍCIO DIRETO DESTE ESTUDO.

6) Indiretamente, acreditamos que este estudo trará como benefício um entendimento da fisiopatologia da pré-eclâmpsia, bem como na obtenção de marcadores genéticos de susceptibilidade a pré-eclâmpsia e de resposta à α-metildopa.

7) Qualquer dado que possa ser publicado posteriormente em revistas científicas, não revelará a sua identidade. Entretanto, órgãos governamentais ligados à saúde podem solicitar informações a respeito da pesquisa e identidade dos voluntários nela envolvidos.

8) Você pode retirar o seu consentimento para participar deste estudo a qualquer momento, inclusive sem justificativas e sem qualquer prejuízo para você.

9) Você terá a garantia de receber a resposta a qualquer pergunta ou esclarecimento de qualquer dúvida a respeito dos procedimentos, riscos,

benefícios e de outras situações relacionadas com a pesquisa e o tratamento a que será submetida. Qualquer questão a respeito do estudo ou de sua saúde

deve ser dirigida ao Prof. Dr. José Eduardo Tanus dos Santos (telefone 0xx16 3602-3163), do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP), ao Dr. Ricardo de Carvalho Cavalli (telefone 0xx16 36022588), do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Ribeirão Preto (FMRP) ou à Doutora Valéria Cristina Sandrim (telefone 0xx16 36262851, 0xx16 36023329) do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP). O telefone do Comitê de Ética em Pesquisa da FMRP é 016-3602-2228.

Eu,

_____,

abaixo assinado, declaro que em ____/____/____ fui devidamente informada em detalhes pelo pesquisador responsável no que diz respeito ao objetivo da pesquisa, aos procedimentos que serei submetido, aos riscos e benefícios, à forma de ressarcimento no caso de eventuais despesas, bem como à indenização quanto por danos decorrentes da pesquisa. Declaro que tenho pleno conhecimento dos direitos e das condições que me foram asseguradas e acima relacionadas.

Declaro, ainda, que concordo inteiramente com as condições que me foram apresentadas e que, livremente, manifesto a minha vontade de participar do referido projeto.

Ribeirão Preto, _____ de _____ de _____.

_____ _____

Assinatura do voluntário Assinatura do investigador/testemunha

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - GRÁVIDAS CONTROLE

Projeto: "Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS

Responsáveis:

- Prof. Dr. José Eduardo Tanus dos Santos (FMRP-USP) – CREMESP 84.966
- Prof. Dr. Ricardo de Carvalho Cavalli – CREMESP 91.680
- Dra. Valéria Cristina Sandrim

Você está sendo convidada a participar deste estudo COMO UMA VOLUNTÁRIA APRESENTANDO GESTAÇÃO NORMAL. Sua participação visa oferecer dados CONTROLES.

Você está sendo convidada a participar deste estudo cujos detalhes são:

1) Este projeto pretende estudar se algumas diferenças em alguns genes (que são responsáveis pela transmissão de suas características hereditárias, que passam de pais para filhos) poderiam contribuir para o desenvolvimento da pré-eclâmpsia (pressão alta na gravidez) e na resposta aos medicamentos utilizados no tratamento desta doença. Além disso, pretendemos estudar como estas diferenças nos genes podem modificar as concentrações de substâncias importantes no controle da sua pressão sanguínea. Estes dados poderão nos auxiliar no entendimento das alterações cardiovasculares que acontecem durante a gravidez e na obtenção de marcadores genéticos de desenvolvimento da pré-eclâmpsia.

2) Sua participação neste estudo será:

Serão retirados, no máximo, 30 mL do seu sangue, coletados por punção venosa utilizando técnica adequada. Este volume de sangue é cerca de 15 (quinze) vezes menor do que o volume de sangue habitualmente doado quando um indivíduo doa sangue para bancos de sangue. Este sangue será utilizado para realizar todas os estudos bioquímicos e genéticos mencionados acima.

3) Você terá direito a ressarcimento financeiro caso haja gastos gerados exclusivamente pela sua participação como voluntário desta pesquisa.

4) Caso haja dano comprovadamente decorrente da pesquisa você terá direito à indenização.

5) NÓS NÃO PODEMOS E NÃO GARANTIREMOS QUE VOCÊ RECEBERÁ QUALQUER BENEFÍCIO DIRETO DESTE ESTUDO.

6) Indiretamente, acreditamos que este estudo trará como benefício um entendimento da fisiopatologia da pré-eclâmpsia, bem como na obtenção de marcadores genéticos de susceptibilidade a pré-eclâmpsia e de resposta à α-metildopa.

7) Qualquer dado que possa ser publicado posteriormente em revistas científicas, não revelará a sua identidade. Entretanto, órgãos governamentais ligados à saúde podem solicitar informações a respeito da pesquisa e identidade dos voluntários nela envolvidos.

8) Você pode retirar o seu consentimento para participar deste estudo a qualquer momento, inclusive sem justificativas e sem qualquer prejuízo para você.

9) Você terá a garantia de receber a resposta a qualquer pergunta ou esclarecimento de qualquer dúvida a respeito dos procedimentos, riscos, benefícios e de outras situações relacionadas com a pesquisa e o tratamento a que será submetida. Qualquer questão a respeito do estudo ou de sua saúde deve ser dirigida ao Prof. Dr. José Eduardo Tanus dos Santos (telefone 0xx16 3602-3163), do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP), ao Dr. Ricardo de Carvalho Cavalli (telefone 0xx16 3602-2588), do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Ribeirão Preto (FMRP) ou à Doutora Valéria Cristina Sandrim (telefone 0xx16 36262851, 0xx16 36023329) do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP). O telefone do Comitê de Ética em Pesquisa da FMRP é 016-3602-2228.

Eu,

_____,

abaixo assinado, declaro que em ____/____/____ fui devidamente informada em detalhes pelo pesquisador responsável no que diz respeito ao objetivo da pesquisa, aos procedimentos que serei submetido, aos riscos e benefícios, à forma de ressarcimento no caso de eventuais despesas, bem como à indenização quanto por danos decorrentes da pesquisa. Declaro que tenho pleno conhecimento dos direitos e das condições que me foram asseguradas e acima relacionadas.

Declaro, ainda, que concordo inteiramente com as condições que me foram apresentadas e que, livremente, manifesto a minha vontade de participar do referido projeto.

Ribeirão Preto, _____ de _____ de _____.

_____ Anexo 3 _____

Assinatura do voluntário Assinatura do investigador/testemunha

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