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Dissertação de Mestrado

Mieloperoxidase em síndromes hipertensivas da gestação

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Orientador (a): Dra. Valéria Cristina Sandrim

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Lilliam Rocha Penha

Mieloperoxidase em síndromes hipertensivas da gestação

Dissertação apresentada ao Instituto de Biociências de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”, para obtenção do título de Mestre em Farmacologia e Biotecnologia.

Orientador(a): Dra. Valéria Cristina Sandrim

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SUMÁRIO

1. RESUMO

A enzima mieloperoxidase (MPO) é caracterizada por produzir substâncias altamente reativas e é reconhecida por desencadear estresse oxidativo e disfunção endotelial mediada, em parte, pela interferência com o vasodilatador óxido nítrico. Neste estudo, nós investigamos a relação entre o óxido nítrico e a MPO *in vitro* incubando o plasma de gestantes saudáveis, hipertensas e com pré-eclâmpsia com células endoteliais (HUVEC). Foram observados maiores níveis de MPO no sobrenadante de células incubadas com o plasma de pacientes com pré-eclâmpsia comparado ao de células incubadas com plasma de gestantes saudáveis, e que a inibição da atividade enzimática aumentou a disponibilidade de óxido nítrico. Posteriormente, nós avaliamos a concentração e atividade da MPO no plasma de 219 gestantes saudáveis, 130 hipertensas gestacionais (com e sem terapia anti-hipertensiva) e 143 gestantes com pré-eclâmpsia (com e sem terapia anti-hipertensiva). Nós observamos que pacientes com síndromes hipertensivas e sob tratamento anti-hipertensivo apresentaram menores níveis e atividade desta enzima e, curiosamente, pacientes que tiveram o plasma coletado antes do tratamento anti-hipertensivo apresentaram níveis elevados de MPO. Nossos resultados indicam um elevado risco cardiovascular em gestantes com síndromes hipertensivas e que a MPO ativa pode ter um papel na disfunção endotelial nestas síndromes pelo comprometimento da disponibilidade do óxido nítrico. Além disso, o uso de drogas anti-hipertensivas parece reduzir os níveis e a atividade enzimática, sugerindo um novo mecanismo protetor para estas drogas.

Palavras-chave: Pré-eclâmpsia; Hipertensão gestacional; Mieloperoxidase; Óxido nítrico; Drogas anti-hipertensivas.

2. ABSTRACT

Myeloperoxidase is characterized by production of highly reactive substances and recognized to trigger oxidative stress and endothelial dysfunction meditated in part by nitric oxide impairment. In this study, we investigated the relation between nitric oxide and MPO *in vitro* incubating plasma from healthy, gestational hypertensive and preeclampsia with endothelial cells. We found higher levels of myeloperoxidase in cultures incubated with plasma from preeclampsia group compared to healthy pregnant, and the inhibition of MPO activity improved nitric oxide bioavailability. Further, we measured plasma concentration and activity of myeloperoxidase in 219 healthy pregnant women, 130 gestational hypertension (on antihypertensive therapy or not) and 143 preeclampsia patients (on antihypertensive therapy or not). We found that patients with hypertensive disorders and on antihypertensive treatment showed lower levels and activity of this enzyme and interestingly patients who the plasma was collected before antihypertensive treatment showed elevated levels of MPO. Our results indicate a higher cardiovascular risk in pregnant with hypertensive disorders, and that active myeloperoxidase may play a role in the endothelial dysfunction in this conditions by impairment of nitric oxide availability. Besides, the use of antihypertensive drugs seems to decrease enzyme levels and activity suggesting a new protective mechanism for these drugs.

Keywords: Preeclampsia; Gestational hypertension; Myeloperoxidase; Nitric oxide; Antihypertensive drugs.

3. INTRODUÇÃO

3.1 Síndromes hipertensivas da gestação

A gestação é um período relacionado à grandes mudanças do organismo materno para que este possa ser capaz de suprir as demandas do feto em desenvolvimento. Algumas destas mudanças estão relacionadas ao sistema circulatório como o aumento do volume sanguíneo, maior produção de substâncias vasodilatadoras, como o óxido nítrico (NO), e de eritrócitos e leucócitos (principalmente neutrófilos) (ZUGAIB, 2012).

Dentre as complicações mais comuns da gestação e uma das principais causas de morbimortalidade materno-fetal estão as síndromes hipertensivas, afetando aproximadamente 10% das gestações no mundo(ACOG, 2013). Estas alterações hipertensivas podem ser classificadas em:

- Hipertensão crônica/pré-existente – hipertensão (sistólica≥140 ou diastólica≥90mmHg) diagnosticada antes da gestação ou anterior a 20ª semana gestacional, ou diagnosticada durante a gestação e que não regride após o parto;
- Hipertensão crônica/pré-existente sobreposta por pré-eclâmpsia – mulheres com hipertensão crônica que desenvolvem proteinúria ou outras alterações sistêmicas características da pré-eclâmpsia;
- Hipertensão gestacional – hipertensão diagnosticada após a 20ª semana gestacional sem presença de proteinúria ou complicações sistêmicas características da pré-eclâmpsia, regressão do quadro hipertensivo em até 12 semanas após o parto;
- Pré-eclâmpsia – hipertensão (sistólica≥140 ou diastólica≥90mmHg) diagnosticada após 20ª semana de gestação com presença de proteinúria (≥0,3g/24h) ou complicações hematológicas, hepáticas, neurológicas(ACOG, 2013; NHBPEP, 2000).

A pré-eclâmpsia é uma das síndromes hipertensivas gestacionais que apresenta contribuição mais significativa para mortalidade materna no mundo(STEEGERS et al., 2010) e histórico familiar, pré-eclâmpsia em gestação anterior, assim como a presença de condições patológicas (hipertensão, doenças autoimunes, diabetes, obesidade, etc.) estão entre os principais fatores de risco para o desenvolvimento da doença(HUTCHEON; LISONKOVA; JOSEPH, 2011). Apesar da grande importância da pré-eclâmpsia sobre o bem-estar materno/fetal, sua fisiopatologia ainda não está completamente esclarecida e o único tratamento definitivo é o parto. Entretanto, o tratamento com drogas anti-hipertensivas pode ser aplicado de forma a prolongar a gestação e reduzir os riscos para mãe e feto. Os

fármacos anti-hipertensivos mais comumente utilizados no Brasil são a metildopa, nifedipina e hidralazina, respectivamente(NETO; SOUZA; AMORIM, 2010).

Apesar das diferentes teorias sobre a fisiopatologia desta síndrome, existe um consenso de que a placentação anormal seja um ponto essencial no desenvolvimento desta condição, uma vez que os sintomas desaparecem após o parto(NALJAYAN; KARUMANCHI, 2013). Em condições normais, por volta do início do segundo trimestre de gestação, as células do citotrofoblasto migram em direção as arteríolas espiraladas no miométrio onde adquirem fenótipo endotelial, levando a um remodelamento destes vasos e degradação do músculo liso, resultando em uma maior vasodilatação destas arteríolas (Figura 1). Na pré-eclâmpsia este processo está prejudicado, fazendo com que o remodelamento das artérias uterinas ocorra de forma incompleta resultando em um quadro de isquemia placentária(NALJAYAN; KARUMANCHI, 2013; ROBERTS; BELL, 2013). O quadro isquêmico da placenta resultaria então na liberação de diversos fatores bioativos, como fatores anti-angiogênicos (ex. soluble fms-like tyrosine kinase-1 [sFlt-1], soluble endoglin [sEnd]), pró-inflamatórios (ex. fator de necrose tumoral-α [TNFα]), espécies reativas (ex. superóxido e peróxido de hidrogênio), na circulação materna levando à disfunção endotelial e favorecendo assim a vasoconstrição(POSSOMATO-VIEIRA; KHALIL, 2016; SHAH; KHALIL, 2015).

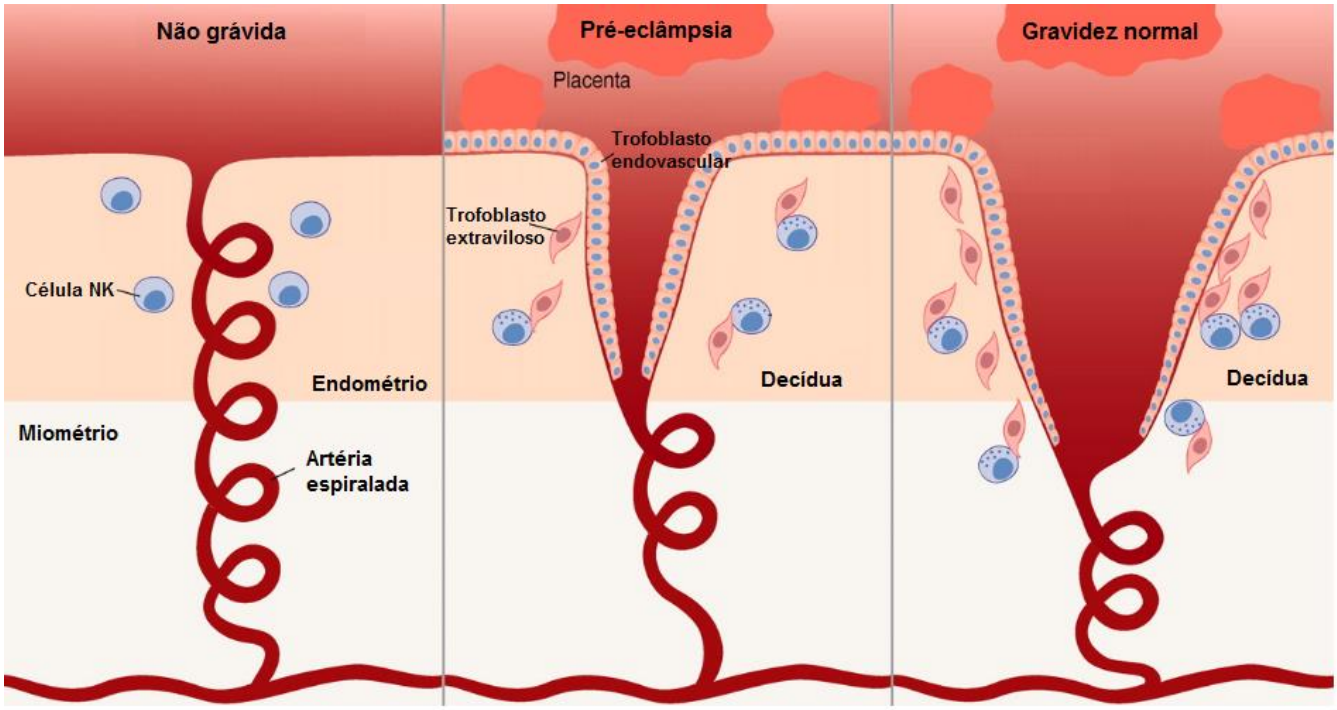

Figura 1. Diferenças no diâmetro das arteríolas uterinas em mulheres não grávidas (painel à esquerda), grávidas saudáveis com remodelamento efetivo atingindo o miométrio (painel à direita) e gestantes com pré-eclâmpsia com remodelamento ineficiente (painel central). O remodelamento ocorre com a invasão pelos trofoblastos extravilosos que adquirem então fenótipo endotelial (trofoblastos endovasculares)(Reproduzido e adaptado de PARHAM, 2004).

A disfunção endotelial é caracterizada por alterações na função vascular desencadeada pelo desequilíbrio de fatores vasoativos, principalmente do vasodilatador óxido nítrico (NO, do inglês *nitric oxide*), e é tida como uma característica central na pré-eclâmpsia, além de ser um indicador para doenças do sistema cardiovascular(GOULOPOULOU; DAVIDGE, 2015).

3.2 Mieloperoxidase

Membro da família das heme-peroxidases e parte da resposta imune inata, a mieloperoxidase (MPO) é uma enzima altamente catiônica envolvida na destruição de patógenos, condições inflamatórias e também no estresse oxidativo(NAUSEEF, 2014).

Sua síntese se inicia com a pré-pro-MPO (~80kD) que, após clivagem de um peptídeo e N-glicosilação, é transformada em apo-pro-MPO, esta se liga ao grupamento heme para formar a pro-MPO (~90kD), que já apresenta atividade enzimática. Após diversas clivagens (de um pro-peptídeo amino-terminal, um peptídeo entre as cadeias leve e pesada e um resíduo de serina no terminal carboxila) é então formada a MPO madura (Figura 2). Em sua forma madura é composta por dois monômeros ligados por uma ponte de dissulfeto, sendo que cada monômero é formado por uma cadeia leve (~14kD) e uma pesada (~58kD) e apresenta um grupamento heme (ANDERSSON et al., 1998; FURTMÜLLER et al., 2006).

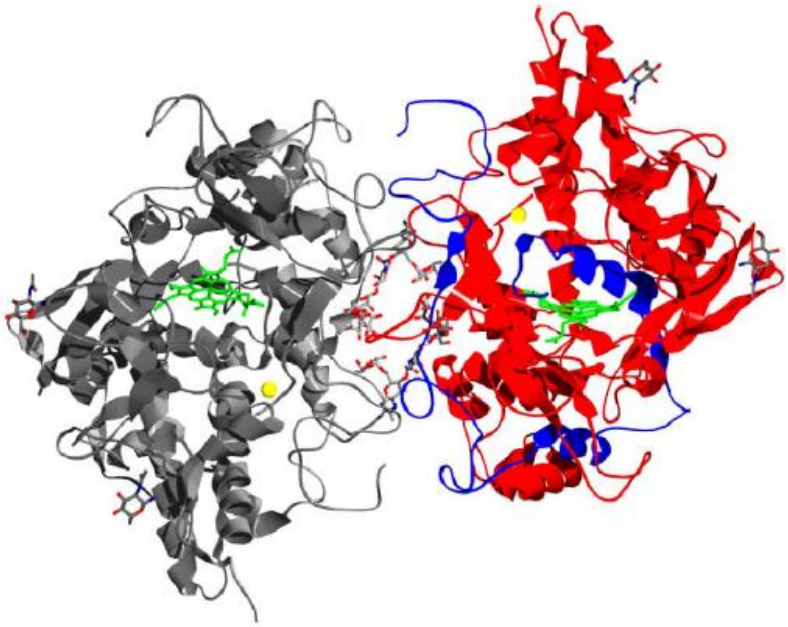

Figura 2. Estrutura geral da mieloperoxidase madura (dímero). Cada subunidade é composta por uma cadeia leve (azul), uma pesada (vermelha) e um grupamento heme (verde) (Reproduzido de FURTMÜLLER et al., 2006).

A MPO é sintetizada principalmente no estágio promielocítico do desenvolvimento neutrofílco e é armazenada nos grânulos azurófilos ou primários destas células, correspondendo a aproximadamente 5% do peso seco destas (SCHULT, KAMINKER, 1962). Também presente em menor quantidade em monócitos, é sintetizada na fase de promonócitos e geralmente desaparece durante a transformação em macrófagos(KLEBANOFF, 2005), podendo ser mantida ou ter sua síntese re-induzida em determinadas circunstâncias, como tem sido observado na aterosclerose(SUGIYAMA et al., 2001) e algumas doenças neurodegenerativas(LEFKOWITZ; LEFKOWITZ, 2008).

Durante a resposta inflamatória, a liberação de mediadores pró-inflamatórios, como o fator de necrose tumoral α (TNFα), levam à ativação dos neutrófilos e ao desencadeamento do "*burst* respiratório", resultando na produção de grandes quantidades de ânion superóxido e peróxido de hidrogênio(EL-BENNA et al., 2016). Com a ativação celular, os grânulos se fundem ao fagossomo liberando a MPO(AMULIC et al., 2012) que, na presença de peróxido de hidrogênio e halogênios/pseudo-halogênios (principalmente Cl^-, Br^- e SCN^-), catalisa a formação de haloácidos (ciclo de halogenação [Figura 3]) com grande potencial oxidativo e bactericida(KLEBANOFF, 2005; NUSSBAUM et al., 2013; NAUSEEF, 2014). Além do ciclo de halogenação, a MPO também catalisa diversas reações pelo ciclo de peroxidação (Figura

3), onde pode levar a oxidação de uma grande variedade de substratos orgânicos e inorgânicos (EISERICH; BALDUS; BRENNAN, 2002; KATO, 2016; MEOTTI et al., 2011).

Além da degranulação nos fagossomos, a MPO também pode ser liberada no meio extracelular podendo afetar diversas funções celulares como sinalização e crescimento, expressão proteica e apoptose, isso devido aos diversos alvos passíveis de oxidação/cloração (proteínas de sinalização intracelular, DNA, lipoproteínas, etc) (KLEBANOFF, 2005; NUSSBAUM et al., 2013).

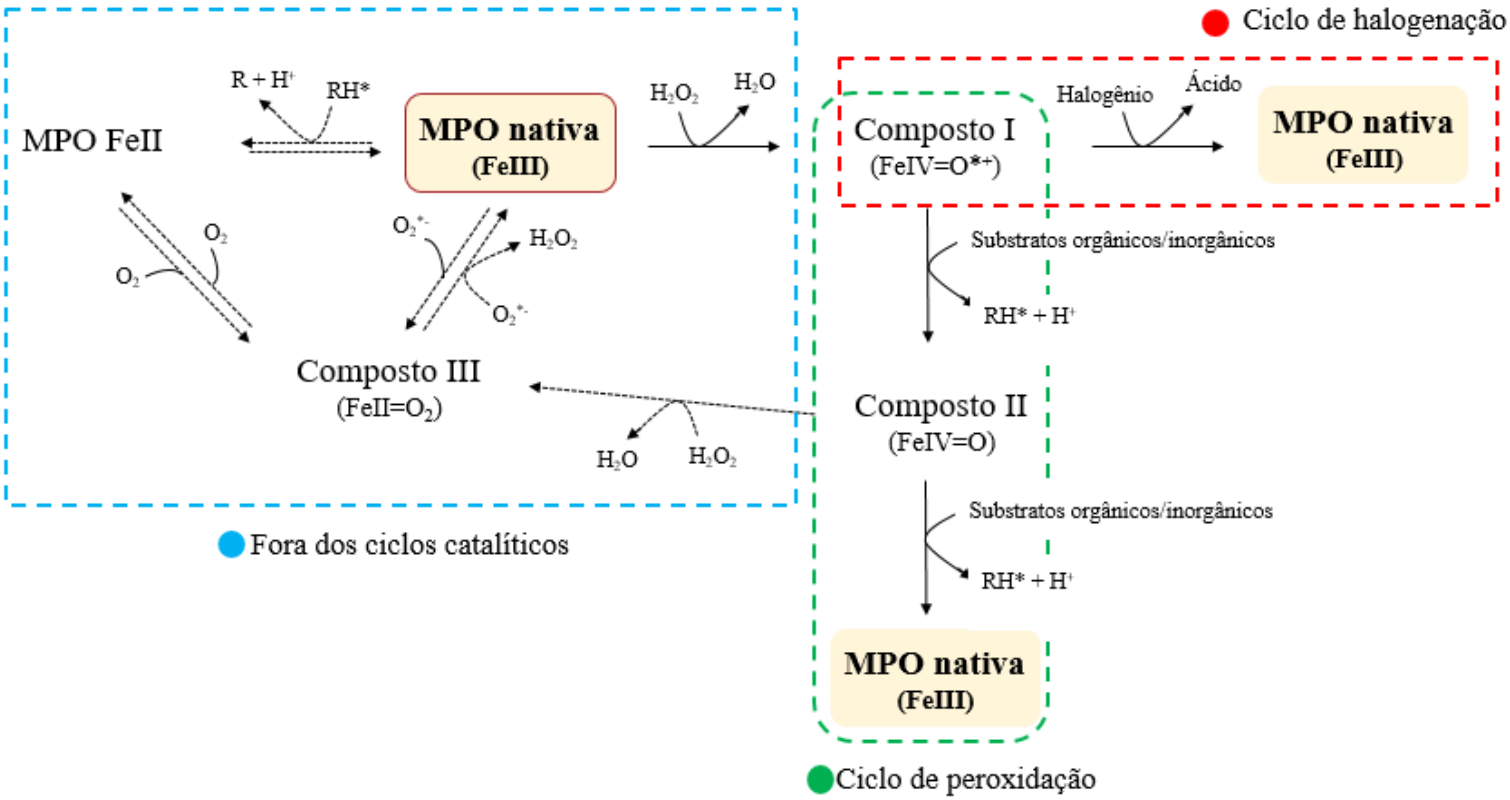

Figura 3. Visão geral da atividade catalítica da MPO. A MPO nativa reduz o H_2O_2 formando composto I, que pode retornar a forma nativa através da transferência de 2 elétrons pelo ciclo de halogenação, onde gera ácidos altamente reativos, ou através de duas transferências de 1 elétron, formando o intermediário composto II, pelo ciclo de peroxidação. Fora do ciclo catalítico ainda há dois outros estados da molécula, a MPO-Fe II, formada pela reação da forma nativa com radicais formados no ciclo de peroxidação, e o composto III, formado pela reação: da forma nativa com ânion superóxido, da MPO-Fe II com oxigênio ou do composto II com H_2O_2 (Reproduzido e adaptado de NUSSBAUM et al., 2013).

3.2.1 Mieloperoxidase em quadros patológicos

Devido à influência da MPO e seus produtos sobre diversas moléculas e suas repercussões sobre a função celular, esta enzima tem sido relacionada a diversas condições patológicas como câncer, doenças renais, neurodegenerativas e principalmente doenças cardiovasculares(VAN DER VEEN; DE WINTHER; HEERINGA, 2009). Nas doenças cardiovasculares pode atuar por diversos mecanismos, entretanto, sua influência no desenvolvimento da disfunção endotelial parece ser uma das principais formas de contribuição para estas condições(ANATOLIOTAKIS et al., 2013).

Diversos estudos demonstraram que a mieloperoxidase tem a habilidade de reduzir, direta e indiretamente, a biodisponibilidade de NO, seja utilizando-o diretamente como substrato ou pela reação com as espécies reativas geradas pela enzima que podem oxidar diretamente o NO(ABU-SOUD; HAZEN, 2000; EISERICH et al., 2002), reagir com a L-arginina (precursor do NO)(YANG et al., 2006; ZHANG et al., 2001) ou induzir o desacoplamento da enzima responsável pela síntese do vasodilatador, a óxido nítrico sintase endotelial (eNOS)(XU et al., 2006; ZOU; SHI; COHEN, 2002). Além da redução na biodisponibilidade de NO, foi observado que a MPO também pode ocasionar uma diminuição de bradicinina (vasodilatador) durante o processo de internalização pela célula endotelial(ASTERN et al., 2007).

Estudos sobre os níveis de MPO em pacientes com síndromes hipertensivas da gestação são restritos à pré-eclâmpsia e são controversos(BOWEN et al., 2001; GANDLEY et al., 2008; HUNG et al., 2012; KARACAY et al., 2010; KURDOGLU et al., 2012; MELLEMBAKKEN et al., 2001; NOYAN et al., 2006). Porém, alguns autores têm apontado a presença de maiores níveis, plasmáticos e placentários, da mieloperoxidase em paciente com pré-eclâmpsia(GANDLEY et al., 2008; KURDOGLU et al., 2012; MELLEMBAKKEN et al., 2001; NOYAN et al., 2006). Entretanto, nenhum destes estudos avaliou ambos parâmetros da enzima, concentração e atividade, em um grande número de indivíduos nem em pacientes com hipertensão gestacional.

Desta forma, considerando as características da MPO assim como as principais características das síndromes hipertensivas da gestação, nós hipotetizamos que a MPO estaria elevada em gestantes com síndromes hipertensivas e que a MPO apresentaria um papel importante na disfunção endotelial através do consumo de NO. Desta forma, este estudo tem como objetivo investigar a concentração e atividade plasmática da MPO em gestantes saudáveis, hipertensas e pré-eclâmpticas, assim como comparar estes parâmetros entre amostras coletadas em tubos com EDTA e heparina como anticoagulante

para verificar influências pré-analíticas, e verificar se a inibição da atividade enzimática da MPO *in vitro* resultaria em uma modulação da disponibilidade de NO

4 CAPÍTULO 1: MANUSCRITO

Este capítulo compreende à um dos manuscritos derivados dos resultados obtidos durante o desenvolvimento do projeto de mestrado. O manuscrito em questão, intitulado "*Myeloperoxidase in hypertensive disorders of pregnancy and its relation with nitric oxide*", está estruturado de acordo com as normas da revista "*Journal of Hypertension"* à qual foi submetido para publicação.

TITLE PAGE

MYELOPEROXIDASE IN HYPERTENSIVE DISORDERS OF PREGNANCY AND ITS RELATION WITH NITRIC OXIDE

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ABSTRACT

Myeloperoxidase is characterized by production of highly reactive substances and recognized to trigger oxidative stress and endothelial dysfunction meditated in part by nitric oxide impairment. In this study, we investigated the relation between nitric oxide and MPO *in vitro* incubating plasma from healthy, gestational hypertensive and preeclampsia with endothelial cells. We found higher levels of myeloperoxidase in cultures incubated with plasma from preeclampsia group compared to healthy pregnant, and the inhibition of MPO activity improved nitric oxide bioavailability. Further, we measured plasma concentration and activity of myeloperoxidase in 219 healthy pregnant women, 130 gestational hypertension (on antihypertensive therapy or not) and 143 preeclampsia patients (on antihypertensive therapy or not). We found that patients with hypertensive disorders and on antihypertensive treatment showed lower levels and activity of this enzyme and interestingly patients who the plasma was collected before antihypertensive treatment showed elevated levels of MPO. Our results indicate a higher cardiovascular risk in pregnant with hypertensive disorders, and that active myeloperoxidase may play a role in the endothelial dysfunction in this conditions by impairment of nitric oxide availability. Besides, the use of antihypertensive drugs seems to decrease enzyme levels and activity suggesting a new protective mechanism for these drugs.

Keywords: Preeclampsia; Gestational hypertension; Myeloperoxidase; Nitric oxide; Antihypertensive drugs.

INTRODUCTION

Hypertensive disorders of pregnancy, including gestational hypertension and preeclampsia, affect about 10% of pregnancies and are a major cause of maternal and prenatal morbidity and mortality[1,2]. Although the cause of preeclampsia remains unknown[3], it is thought that an initial stage of poor placentation with posterior ischemia stimulates the release of soluble factors by the placenta into maternal circulation, which lead to a second stage of the disorder characterized by maternal endothelial dysfunction[4–6]. The incubation of plasma or serum from pregnant with preeclampsia on endothelial cells have been used to understand the link between these stages[7–11]. Although already reported[12,13] the possible contribution of myeloperoxidase (MPO) to endothelial dysfunction, no previous study examined the effect of endothelial cell incubation with plasma from pregnant with hypertensive disorders on MPO levels or activity.

MPO is a lysosomal enzyme mainly produced and released by activated neutrophils and monocytes, which produces highly oxidizing substances from hydrogen peroxide and halogens[14,15]. This enzyme is a potential trigger to vascular injury and has been related to pathogenesis and progression of several cardiovascular diseases, mainly due to its pro-inflammatory and oxidative properties[16,17]. Notably, MPO is associated with impairment of nitric oxide (NO) bioavailability[17–19], a molecule produced by endothelial cells with functions such as vasodilation and anticoagulation, which is known to be reduced in preeclampsia [20,21].

The findings regarding circulating levels of MPO in preeclampsia are controversial[22–28]. Previous studies enrolled a small number of patients (ranging from 11 to 61 subjects), and none included patients with gestational hypertension. Moreover, these studies have used different types of blood collection tubes, not described the antihypertensive drugs in use at collecting time, and data are reported only as MPO activity or protein levels[22–28].

In the present study, we firstly performed a multiplex assay to quantify the concentration of proteins related to cardiovascular risk in endothelial cells culture after incubation with plasma from healthy pregnant, gestational hypertensive and preeclamptic women, and found that MPO was the only differently expressed biomarker. We then: (1) evaluated plasma MPO concentration and activity in those three groups and correlated them to clinical parameters and plasma nitrite levels; (2) compared MPO parameters between samples collected in tubes with EDTA and heparin as anticoagulants; (3) examined whether inhibition of MPO may modulate NO availability *in vitro.*

MATERIAL AND METHODS

Study design

The schematic diagram of the study workflow is shown in Figure S1. We firstly performed a multiplex assay to quantify the concentration of eight proteins related to cardiovascular risk in supernatant of endothelial cells after 24-hours incubation with plasma from healthy pregnant, gestational hypertensive and preeclamptic women. MPO was the only differently expressed biomarker between healthy pregnant and preeclampsia. Therefore, we proceeded with the following experiments with this biomarker: (a) we measured both concentration and activity in plasma from the three groups; (b) as some studies indicated differences in MPO levels between blood collection tubes[29–32], we also compared these parameters between plasma samples collected simultaneously in heparin and EDTA tubes; (c) as MPO is able to consume the vasodilator NO, we examined whether inhibition of its enzymatic activity would improve NO availability *in vitro*, and if circulating levels and/or activity of this enzyme were correlated with plasma nitrite levels; Finally (d) we then correlated clinical parameters and the influence of antihypertensive treatment with level and activity of MPO.

Patients

The subjects were recruited from the clinics of the Department of Obstetrics and Gynecology of the *Hospital das Clinicas de Ribeirao Preto*, University of Sao Paulo. All participants provided written informed consent, and the study was approved by the Institutional Review Boards of the *Hospital das Clinicas de Ribeirao Preto*, University of Sao Paulo (reference 4682/2006, approved date June 20 2006). Hypertensive disorders of pregnancy were defined following guidelines of NHBPEP[33] (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy). Gestational hypertension was defined as increased blood pressure (≥140 mmHg systolic and/or ≥90 mmHg diastolic, on at least two measurements with six hours apart) without proteinuria (<0.3g in 24h) after 20 weeks of gestation. Preeclampsia were defined as increased blood pressure (≥140 mmHg systolic and/or ≥90 mmHg diastolic, on at least two measurements with six hours apart) with significant proteinuria (≥0.3g/24h) after 20 weeks of gestation. Patients with hemostatic abnormalities, chronic hypertension, cancer, twin pregnancy, cardiovascular, autoimmune, renal and/or hepatic diseases were excluded from this study. At the time of clinical attendance, maternal venous blood samples were collected in tubes containing EDTA and heparin as anticoagulants. The tubes were rapidly centrifuged (1000g for 3 minutes) and plasma were stored at -70°C until analysis.

Cell culture and plasma incubation

Human umbilical vein endothelial cell (HUVEC) line (CRL 2873) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HUVEC were cultured in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum, 50µg/ml penicillin, 50µg/ml streptomycin and 0,5µg/ml amphotericin B (Gibco) at 37ºC in 5% CO_2 incubator. Cells were expanded in 75cm^2 tissue culture flasks (Corning, Costar, Netherlands)

and after reaching 80% confluence, they were detached from the flask surface by trypsin/EDTA (0.5/0.2 mg/ml in phosphate-buffered saline, PBS) and split at a 1:3 ratio into 25cm^2 flasks. At passage 3, HUVEC were re-suspended in DMEM medium and re-plated in 48-well tissue culture plates (Corning, Costar, Netherlands). After reaching 80% confluence, the medium was removed and cells were washed twice in PBS and then incubated in medium with 20% (v/v) of plasma from healthy pregnant, gestational hypertensive or preeclamptic patients for 24 hours. Supernatant of these cultures was used to perform the multiplex assay.

To investigate the relation between MPO and NO availability *in vitro*, plasma samples were incubated with increasing concentrations (5, 20 and 50µM) of MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH; Sigma, St. Louis, MO, USA), prior addition in the culture medium and 24-hours incubation.

Human cardiovascular disease panel

Protein levels from culture supernatant were assessed by Luminex xMAP technology using human cardiovascular disease panel 2 (catalogue HCVD2MAG-67K; Millipore, Billerica, MA, USA) composed by the following analytes: growth differentiation factor 15 (GDF-15), myoglobin (Myo), myeloperoxidase (MPO), P-selectin (P-sel), soluble intercellular adhesion molecule-1 (sICAM-1), neutrophil gelatinase associated lipocalin (NGAL), A desintegrin and metalloprotease with thrombospondin type 1 repeats 13 (ADAMTS13), serum amyloid A (sAA), according to the manufacturer's instructions. Luminex panel were analyzed on a MAGPIX® multiplex reader (Luminex, Austin, TX, USA).

Myeloperoxidase concentration and activity

MPO concentration was evaluated in plasma samples using a commercial enzyme-linked immunosorbent assay (Human Myeloperoxidase DuoSet ELISA - R&D Systems, Minneapolis, MN, USA). Plasma samples were diluted 1:100 in reagent diluent (1% BSA in

PBS) and optical density was determined at 450nm using a microplate reader (Synergy 4, BioTek – Winooski, VT, USA). The standard range was between 62.5 – 4,000.0 pg/mL.

MPO activity was determined by measuring tetramethylbenzidine (TMB) oxidation in an end-point colorimetric assay. For that, 30µL of plasma (1:100) were incubated with 20µL of phosphate buffer and 100µL of liquid substrate system, composed by TMB and hydrogen peroxide (Sigma, St. Louis, MO, USA), at 37°C for 10 minutes, protected from light. After incubation, 100µL of stop solution (sulfuric acid 2N) were added and optical density were determined at 450nm using a microplate reader. A standard curve was generated by incubation of horseradish peroxidase with the previous reagents. The standard range were between 0.153 – 2500 mU/50µL. In order to determine whether the obtained activity was derived from MPO, samples were incubated with 500µM of a specific and irreversible MPO inhibitor (ABAH) at 37°C for 30 minutes prior addition of substrate system.

Nitrite quantification

Nitrite quantification from culture supernatant was made in triplicate using Griess reagents (Sigma). Samples (50µL) were incubated with 50µL of 1% sulfanilamide solution (in 5% phosphoric acid) for 10 minutes protected from light. Then, 50µL of 0.1% N-(1-Naphthyl)-ethylenediamine dihydrochloride solution were added followed by 10 minutes incubation. Optical density was determined in a microplate reader at 540nm. A standard curve were generated by incubation of nitrite solutions (29.5 – 0.46 µM/mL) with the previous reagents.

Plasma nitrite were quantified in triplicate by an ozone-based chemiluminescence assay as previously described[21] using chemiluminescence NO analyzer (Sievers model 280 NO Analyzer – Boulder, CO, USA).

Statistical analysis

Categorical variables were compared by χ^2 tests. Continuous variables were compared by Student t test, ANOVA followed by Tukey test (for normally distributed variables) or Mann-Withney's, Kruskal-Wallis followed by Dunn's multiple comparison test (for not normally distributes variables). Correlations were assessed by Pearson or Spearman's correlation, as appropriated. The analyses were performed with GraphPad Prism for Windows, version 6.01 (GraphPad Software, San Diego, CA, USA). For all tests a probability value of $P<0.05$ was considered significant.

RESULTS

In vitro quantification of proteins related to cardiovascular risk

At first, we quantified the concentration of eight biomarkers related to cardiovascular diseases (MPO, GDF-15, Myoglobin, P-selectin, sICAM-1, NGAL, ADAMTS-13 and sAA) in culture supernatant of endothelial cells after 24-h incubation with plasma from healthy pregnant, gestational hypertensive and preeclamptic women (Figure 1). Clinical and biochemical characteristics of pregnant women enrolled in the study are shown in Table 1. As expected, systolic and diastolic blood pressure were higher in hypertensive disorders of pregnancy when compared with healthy pregnant ($P<0.0001$), while gestational age at delivery and newborn weight were lower in these groups ($P=0.0007$ and $P=0.009$, respectively).

Only MPO was differently expressed among all eight biomarkers (Figure 1). Endothelial cells incubated with plasma from preeclamptic patients showed higher levels when compared to healthy pregnant ($P=0.02$). Importantly, all quality controls analytes in our assay (observed values) were between the expected ranges and, therefore, validated our assay according to the manufacturer's protocol (Table S1).

Concentration and activity of myeloperoxidase in plasma samples

Based on the *in vitro* results, we next explored circulating levels of MPO in the three groups of pregnant women. Clinical and biochemical characteristics of women enrolled in this part of the study are shown in Table 2. As expected, systolic and diastolic blood pressure were higher in hypertensive disorders of pregnancy when compared with healthy pregnant (P<0.0001). However, subjects with hypertensive disorders had elevated body mass index (P<0.0001) and lower gestational age at delivery (P<0.0001) than healthy pregnant group, and preeclampsia group lower than gestational hypertension. Also higher levels of sFlt-1 (P<0.0001) and lower newborn weight (P<0.0001) were observed in preeclampsia compared to healthy pregnant.

Plasma MPO concentration (A) and activity (B) in healthy pregnant, gestational hypertensive and preeclampsia are shown in Figure 2, both subgrouped in women on (+) or without (-) antihypertensive treatment at time of blood collection. MPO levels in gestational hypertensive (17.4±2.1 ng/mL) and preeclampsia (15.4±1.4 ng/mL) on treatment were lower than healthy pregnant (27.5±2.2 ng/mL) (Figure 2A). Interestingly, preeclampsia without antihypertensive treatment (51.2±3.8 ng/mL) presented higher levels of MPO compared to preeclampsia (15.4±1.4 ng/mL) and gestational hypertensive (17.4±2.1 ng/mL) on antihypertensive treatment, and compared to healthy pregnant (27.5±2.2 ng/mL) and gestational hypertensive (19.4±2.8 ng/mL) without treatment (Figure 2A). Regarding MPO activity, no differences were observe between the three groups on antihypertensive treatment (Figure 2B, P=0.8). However, among healthy pregnant and the groups without treatment, gestational hypertensive (192.1±21.2 U/L) showed higher enzymatic activity when compared with healthy pregnant (146.1±5.7 U/L) and preeclampsia (124.7±6.7 U/L) (Figure 2B).

Considering that some substances may react with the substrate (TMB) of MPO activity assay[34–36], we examined plasma MPO activity in all groups in the presence of a specific and

irreversible inhibitor of MPO, the ABAH. This assay may provide us a confirmation that the values obtained in our enzymatic assays were derived only from MPO activity. The incubation of plasma from healthy pregnant, gestational hypertensive and preeclamptic women with ABAH (0.5mM/30min.) significantly reduced enzymatic activity in all groups (P<0.0001) in more than 90%, indicating that mostly of the activity previously assessed in these groups were derived from MPO (Figure S2).

Regarding the correlations with clinical parameters (Table S2), we found a positive correlation between enzyme levels and red blood cells (P=0.003, r=0.4) as well as hemoglobin (P=0.01, r=0.2) in healthy pregnant group. Moreover, we found a positive correlation between enzyme levels and both systolic (P=0.03, r=0.3) and diastolic blood pressure (P=0.04, r=0.2) in the patients with gestational hypertension on antihypertensive treatment. We have also found a positive correlation between enzymatic activity and diastolic blood pressure (P=0.04, r=0.4) among patients with gestational hypertension without antihypertensive treatment.

Influence of anticoagulant present in blood collection tubes on myeloperoxidase analysis

Although not yet evaluated in preeclampsia studies, previous findings [29–32] showed differences in MPO concentration between blood collected in heparin and EDTA tubes. Therefore, we have also measured enzyme levels and activity in plasma from blood collected in heparin tubes from healthy pregnant, gestational hypertension and preeclampsia groups and compared with results from EDTA-plasma. Both sample tubes were collected simultaneously. We observed lower levels of the enzyme in heparin-plasma samples compared to EDTA-plasma in all groups (Figure S3A, P≤0.04). Comparing enzymatic activity, heparin-plasma showed important reduction of activity levels than EDTA-plasma in all groups (Figure S3B, P<0.0001). Due to the enzymatic activity reduction in heparinized samples, we

then investigate whether the heparin in the collection tubes had the ability to inhibit MPO activity. Figure S4 shows enzymatic activity before and after EDTA-plasma incubation with heparin solution (14 U/mL). EDTA-plasma samples from all groups incubated with heparin solution showed a reduction in enzymatic activity when compared with those without heparin addition (P<0.03), suggesting that heparin may inhibit MPO activity in plasma samples.

In vitro and plasma relation between myeloperoxidase and nitric oxide bioavailability

Considering that MPO is able to consume the vasodilator NO and thus contribute to endothelial dysfunction[12,13] we examined whether the inhibition of MPO activity could improve the NO bioavailability in endothelial cells. Figure 3A shows that addition of plasma after MPO inhibition in cultures first decreased MPO activity in at least 30% at the lower concentration (5µM) and 41% at higher concentration (50µM), and significantly increased nitrite concentrations in culture supernatant in all three groups studied (Figure 3B; P≤0.01). Of note, the groups showed an increase trend in nitrite concentrations proportional to MPO inhibition. Moreover, although all groups had an increase in nitrite concentrations after MPO inhibition, this increase showed to be lower in preeclampsia. Next, we verify whether plasma levels of nitrite were also correlated with plasma concentration and activity of MPO (Table S3). However, we found no significant correlations for all groups (P>0.05).

DISCUSSION

This study was the first to report higher levels of MPO in the supernatant of endothelial cells after incubation with plasma from preeclampsia patients. As previously shown[5,37], the ischemic placenta release several bioactive factors in maternal circulation and these factors lead to oxidative stress, which may stimulate MPO expression by the endothelial cells[38]. Higher levels of MPO has been related with elevated risk for cardiovascular diseases through

its ability to enhance neutrophil generation of reactive species and degranulation[17], to bind endothelial cell surface and to accumulate in subendothelial space contributing to endothelial dysfunction by impairment of NO[18,39]. Moreover, mobilization of MPO from vessel wall by heparin improved local NO bioavailability[40]. Therefore, we examined whether inhibition of enzymatic activity would show similar results in our *in vitro* assay. We found that inhibition of MPO enhances nitrite levels, a marker of NO availability, which suggests that the consumption of NO by MPO depends on its catalytic activity. Notably, this finding might open a new therapeutic possibility for the application of substances that modulate MPO activity, as some phytochemicals[41,42], in order to improve vasodilator offer and thus, ameliorate the endothelial dysfunction.

Discordant findings for circulating MPO levels in preeclampsia have been reported elsewhere[22–28] (Table S4). These differences among studies may be attributed to several factors, such as the inclusion of different forms of preeclampsia or its complications[24,28] and the lack of report of antihypertensive drugs use. We found that this last information is relevant and may be explained by the possible influence of the antihypertensive drugs on MPO, as suggested by our results. Here we observed higher plasmatic levels of MPO in preeclampsia patients without antihypertensive treatment. The release of bioactive factors by the ischemic placenta in preeclampsia contribute to an increase in blood pressure and oxidative stress what may result in several damages on maternal organism. Antihypertensive treatment is indicated in some cases in order to reduce maternal and fetal outcomes, and to prevent neuro and cardiovascular events related to the elevated blood pressure[45,46]. Methyldopa is the main drug indicated in these cases and it is a centrally α_2-adrenergic agonist, which decreases the release of norepinephrine (NE). Studies demonstrated the ability of sympathetic system in modulate the immune system[47] and that catecholamines contribute to release of cytokines[48,49] and lysosomal enzymes from immune cells[50]. Therefore, the lower MPO in

preeclampsia patients on antihypertensive treatment observed in our results might be related to a decrease in leucocytes activation due to methyldopa action on NE.

Regarding MPO activity, was found significantly higher in gestational hypertensive group when compared to healthy pregnant and preeclampsia, despite the influence of antihypertensive therapy. These antihypertensive drugs showed a discrete influence over enzymatic activity in gestational hypertension and the slight decrease can be associated with the type of antihypertensive drug used, as a few patients of this group were being treated with hydralazine, which has been reported to inhibit MPO activity[45]. Conversely, none of the preeclampsia patients were being treated with this drug.

To our knowledge, no previous study has examined the relation between circulating MPO and NO in hypertensive disorders of pregnancy. MPO is able to cause endothelial dysfunction through reducing NO bioavailability[17,18,46]. Considering the higher enzyme levels and activity in preeclampsia and gestational hypertension, respectively, as well as the improvement in nitrite levels after MPO inhibition, we examined the relation between circulating MPO and nitrite levels. However, although we found a lack of correlation between these parameters, this analysis might be influenced by the high complexity of plasma, which makes difficult to find relations among several cellular components, biomolecules and metabolites. When analyzing correlations between MPO and clinical parameters, we observed a positive correlation between MPO concentration and erythrocytes, as well as hemoglobin. This result might be related to the ability of red blood cells to act as carriers of this enzyme on circulation due to the cationic characteristic of the enzyme what contribute to its binding to the cell membrane, as demonstrated elsewhere[47,48].

MPO has been implicated in a wide range of pathologies, such as cancer, cardiovascular, neurodegenerative, renal and lung diseases and other chronic inflammatory

conditions[16]. However, analytical procedures may influence the precision of MPO as a biomarker and, to date, there is no standardized method for MPO evaluation. Many authors examined the effects of collection tube types in quantification of MPO[29–32], despite the discordant findings. We found an increase in MPO concentrations on EDTA-plasma samples compared with heparin samples, which is in line with other findings[31,32]. Compared with previous reports showing higher levels in heparinized plasma, the discordant findings may be explained by different pre-analytical handling and quantification methods used in the analysis. In one study[30], blood samples were kept at room temperature for nearly one hour, which may stimulate the leakage of MPO from neutrophils resulting in increased levels in plasma. Another study[29], the quantification were carried out through a chemiluminescent immunoassay. Despite data for influence of collection tube type on myeloperoxidase concentration, to date, there were no reports that investigated its influence in MPO activity. Our results showed a significant decrease in enzymatic activity in heparinized samples and after plasma incubation with heparin solutions MPO activity were significantly reduced, which may indicate an inhibitory capacity of the anticoagulant heparin upon MPO. However, further studies are needed to examine other possible interferences in MPO analysis (concentration and activity) in order to standardize pre-analytical handling and analysis method ensuring greater reliability of the results. These procedures would thus allow MPO to be considered as a possible biomarker for hypertensive disorders of pregnancy.

In conclusion, we found higher MPO levels in preeclampsia group, as well as higher activity in gestational hypertension. Besides, inhibition of the enzymatic activity resulted in an improvement of nitrite availability. These findings suggest a higher risk for cardiovascular diseases in these groups, and that MPO may play a role on the endothelial dysfunction, characteristic of these conditions. Furthermore, our results indicate an influence of

antihypertensive therapy on MPO levels, a new protective feature for these drugs by decreasing the enzyme release and the damage cause to the endothelium.

PERSPECTIVES

Here we showed that antihypertensive therapy might affect the analysis of myeloperoxidase, indicating an interaction between myeloperoxidase and antihypertensive treatment that might suggest a possible protector effect of these drugs on the endothelial damage cause by this enzyme. A deeper investigation is required in order to identify which antihypertensive drugs has the ability to interact with this enzyme, through which mechanism this happens and to evaluate a possible protective characteristic against endothelium myeloperoxidase-derived damage. Moreover, the improvement of nitrite concentrations after myeloperoxidase inhibition showed here, provide new possibilities for the application substance that might modulate MPO, as some phytochemicals[41,42] or even antihypertensive drugs, as suggested by our results, in order to attempt to ameliorate endothelial function. Lastly, the reduction of myeloperoxidase activity in plasma collected in heparin tubes compared to collect in EDTA tubes strengthens the need of pre-analytical handling standards to ensure reliability of the results.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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NOVELTY AND SIGNIFICANCE

What is new?

Quantification of myeloperoxidase *in vitro*, after incubation with plasma; levels and activity in plasma from gestational hypertensive; influence of antihypertensive therapy on MPO; influence of blood collection tubes in MPO activity.

What is relevant?

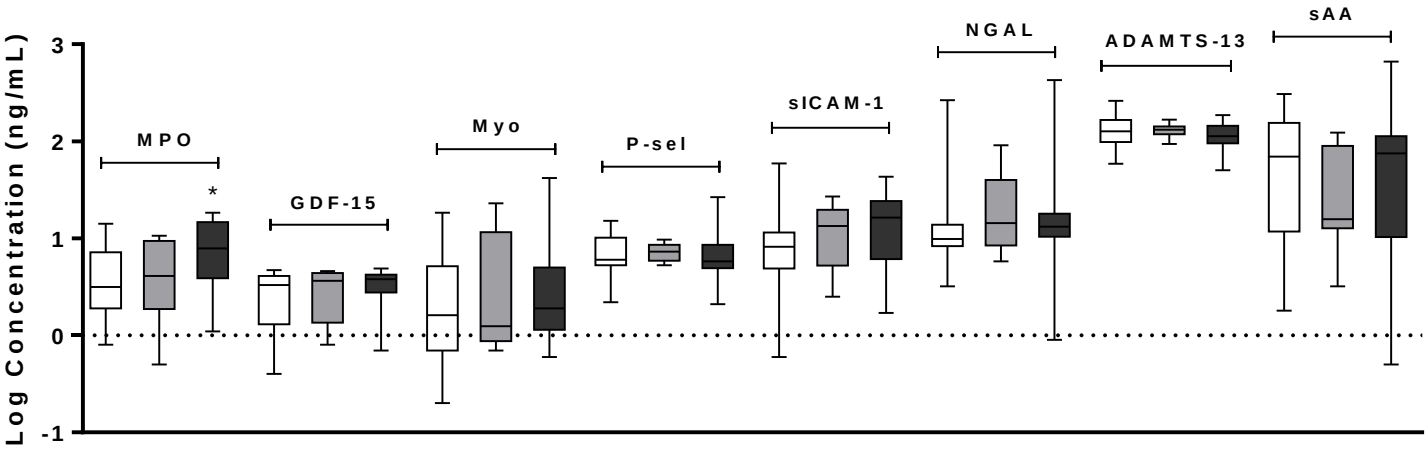
Hypertensive disorders of pregnancy are related to systemic inflammation, oxidative stress, endothelial dysfunction and higher cardiovascular risk. Myeloperoxidase is able to impair nitric oxide leading to endothelial dysfunction and contributing to hypertension, being widely associated with cardiovascular diseases.

Summary

Higher MPO levels in preeclampsia and activity in gestational hypertensive; inhibition of the enzymatic activity improving of nitrite bioavailability, thus suggesting higher risk for cardiovascular diseases in these groups, and that MPO may play a role on the endothelial dysfunction; presence of antihypertensive drugs decreasing MPO, suggesting a protector effect on endothelial damage cause by this enzyme.

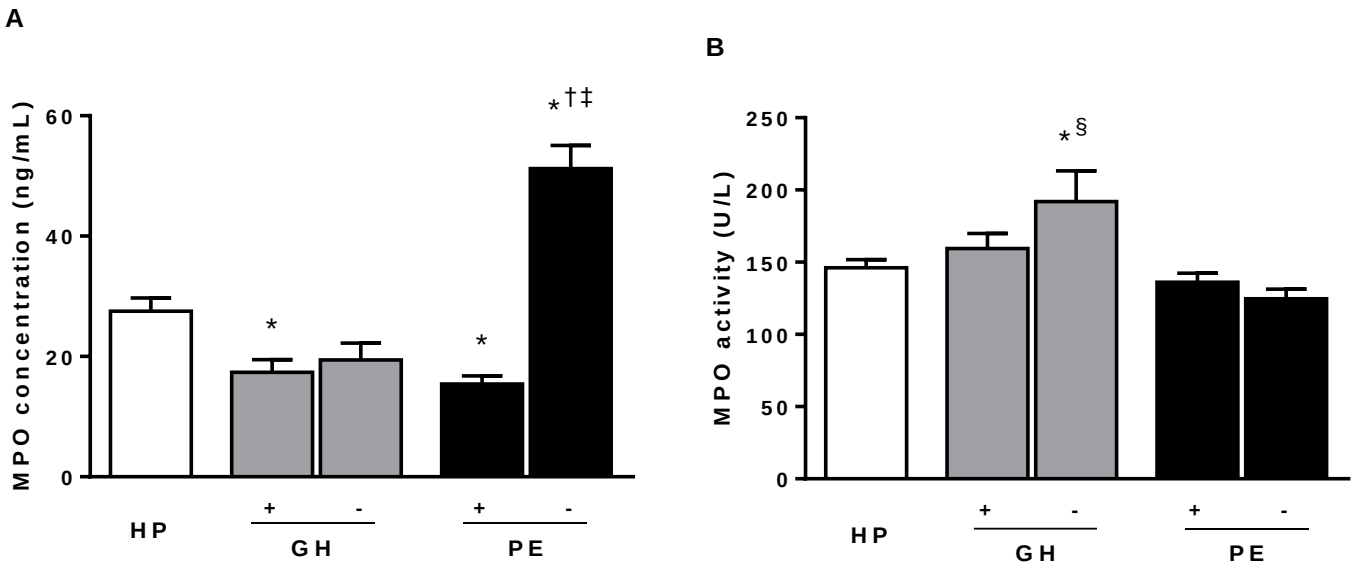
FIGURES AND LEGENDS

FIGURE 1.



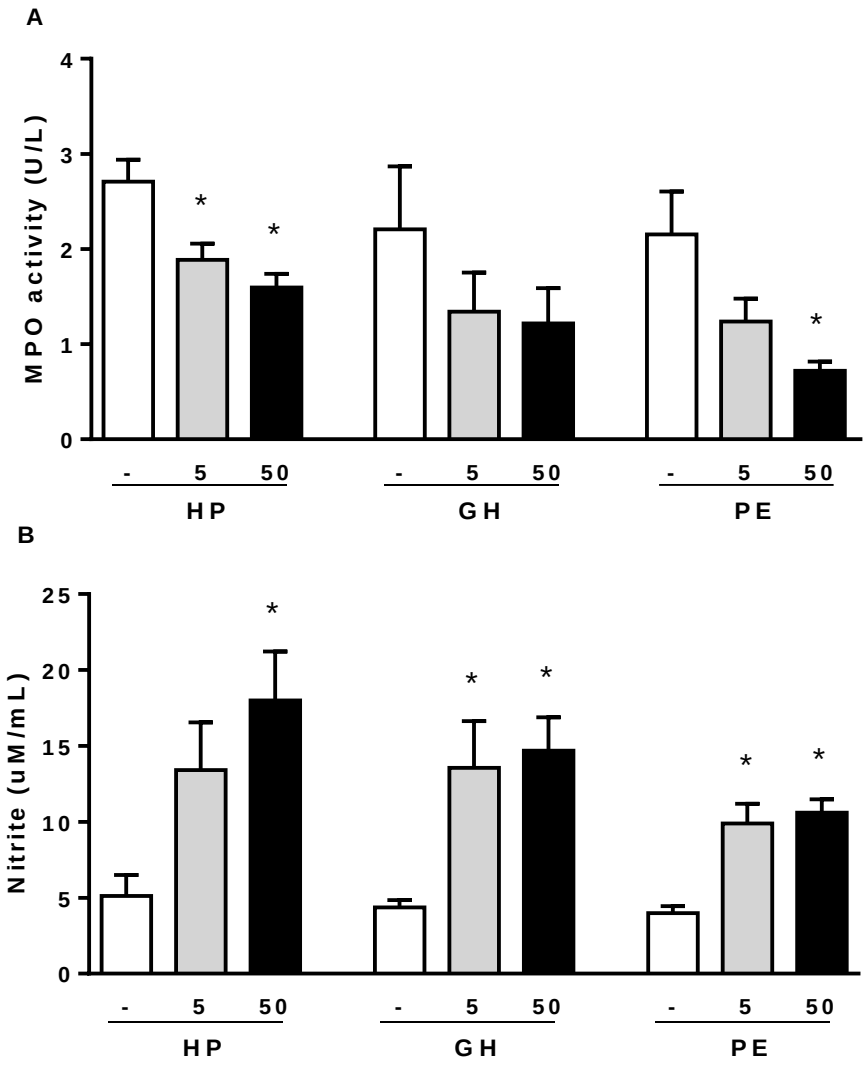
Boxplot of log concentration of biomarkers from cardiovascular disease panel in culture supernatant after incubation with 20% of plasma from healthy pregnant (white columns; n=25), gestational hypertensive (grey columns; n=10) and preeclamptic (black columns; n=26) women for 24 hours. Myeloperoxidase levels were significantly higher in preeclamptic than in healthy pregnant group (*P*=0.02). Analytes were quantified by multiplex assay with Luminex xMAP® technology. MPO represents myeloperoxidase; GDF-15, growth differentiation factor 15; Myo, myoglobin; P-sel, P-selectin; sICAM-1, soluble intercellular adhesion molecule-1; NGAL, neutrophil gelatinase associated lipocalin; ADAMTS-13, A desintegrin and metalloprotease with thrombospondin type 1 repeats 13; sAA, serum amyloid A. Comparison between groups were by ANOVA 1-way followed by Tukey's post-test. **P*<0.05 *vs* healthy pregnancy.

FIGURE 2.



Plasma MPO concentration and activity in healthy pregnant (HP, white columns), gestational hypertensive (GH, grey columns) and preeclampsia (PE, black columns) sub grouped in patients on (+) and without (-) antihypertensive treatment. (A) MPO concentration in treated groups showed to be higher in HP (n=204, *P*=0.02) than in GH (n=73) and PE (n=80). In groups without treatment, PE (n=31) showed higher levels than GH (n=32) and HP (n=204, *P*<0.0001). (B) MPO activity showed no significant differences in treated groups (*P*=0.8), however, comparing not treated groups, GH (n=32) had higher activity than HP (n=219) and PE (n=38, *P*=0.003). Data as mean ± SEM. Comparison between groups were by Kruskal-Wallis followed by Dunn's post-test. **P*<0.05 *vs* HP, †*P*<0.05 *vs* GH (-), §*P*<0.05 *vs* PE (-), ‡*P*<0.05 *vs* PE (+).

FIGURE 3.



MPO activity and nitrite concentration in culture supernatant after 16-hours incubation with plasma from healthy pregnant (HP, n=5), gestational hypertension (GH, n=5) and preeclampsia (PE, n=5) before (-, white columns) and after addition of MPO inhibitor (ABAH) at 5µM (5, grey columns) and 50µM (50, black columns). **(A)** MPO activity after incubation with either plasma (-) or plasma+ABAH. Inhibitor addition decreased activity in all groups in at least 30% and 40.5% at 5 and 50µM, respectively. **(B)** Nitrite concentration after incubation cited previously. Nitrite levels increased after MPO inhibition in a least of 249%

and 266% at 5 and 50μM, respectively. Data as mean ± SEM. Comparisons between groups were by Kruskal-Wallis followed by Dunn's post-test. *P<0.05 *vs* – from respective group.

TABLES

Table 1. Clinical and biochemical characteristics of patients for *in vitro* analysis

Parameters	**Healthy pregnancy** (n = 25)	**Gestational hypertension** (n = 10)	**Preeclampsia** (n = 26)
Age (years)	24.3 ± 0.9	28.6 ± 1.6	26.2 ± 1.3
Race (white, %)	68	60	58
Prepregnancy BMI (Kg/m^2)	28.3 ± 1.2	29.8 ± 1.7	27.8± 1.4
SBP (mmHg)	115.0 ± 2.0	133.2 ± 5.5*	135.2 ± 3.2*
DBP (mmHg)	71.8 ± 1.5	87.1 ± 4.8*	86.6 ± 2.6*
Antihypertensive drugs at blood collection			
α-Methyldopa (%)	NA	90.0	70.8
Nifedipine (%)	NA	0	0
GA at blood collection (weeks)	37.0 ± 0.4	35.6 ± 1.7	35.2 ± 0.8
RBC count ($x10^6/mL$)	3.9 ± 0.1	3.9 ± 0.1	4.2 ± 0.1
Hemoglobin (g/dL)	11.3 ± 0.4	11.7 ± 0.3	11.9 ± 0.2
WBC count ($x10^3/mL$)	10.9 ± 0.9	9.2 ± 0.8	9.8 ± 0.4
Neutrophils (%)	76.1 ± 2.3	69.0 ± 2.5	66.9 ± 4.0
Fasting glucose (mg/dL)	74.0 (68.0 – 78.0)	79.0 (72.0 – 89.0)	77.5 (67.7 – 82.5)
Proteinuria (mg/24h)	ND	177.7 ± 42.9	654.9 ± 129.6$^{\#}$
GA at delivery (weeks)	39.9 ± 0.3	38.5 ± 1.3	36.2 ± 0.8*
Newborn weight (g)	3385.0 ± 77.0	2832.0 ± 330.7	2645.0 ± 202.3*

Data as mean ± SEM, median (25^{th} – 75^{th} centiles) or percentage. Comparisons between groups were by ANOVA 1-way followed by Tukey's post test or Mann-Whitney followed by Dunn's for continuous variables or chi-square test for categorical variables. *P<0.05 *vs* healthy pregnancy; $^{\#}P$<0.05 *vs* gestational hypertension. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; RBC, red blood cells; WBC, white blood cells; NA, not applicable; ND, not determined.

Table 2. Clinical and biochemical characteristics for circulating study

Parameters	**Healthy pregnancy** (n = 219)	**Gestational hypertension** (n = 130)	**Preeclampsia** (n = 143)
Age (years)	26.0 (21.5 – 32.0)	28.0 (22.8 – 31.3)	26.0 (21.5 – 32.0)
Race (white, %)	72.8	69.8	71.3
Prepregnancy BMI (Kg/m^2)	22.9 (20.1 – 26.2)	29.9 (25.2 – 35.3)*	27.5 (23.6 – 32.7)*,#
Current smoker (%)	12.6	11.6	10.9
SBP (mmHg)	110.0 (100.0 – 120.0)	130.0 (120.0 – 140.0)*	140.0 (126.0 – 150.0)*,#
DBP (mmHg)	70.0 (70.0 – 80.0)	80.0 (70.0 – 90.0)*	90.0 (80.0 – 94.0)*,#
Antihypertensive drugs at blood collection			
α-Methyldopa (%)	NA	72.1	73.7
Nifedipine (%)	NA	0.7	5.3#
Hydralazine (%)	NA	1.5	0
GA at blood collection (weeks)	37.0 (36.0 – 38.0)	37.0 (34.0 – 39.0)	35.0 (32.0 – 37.0)*,#
RBC count ($x10^6/mL$)	4.0 (3.7 – 4.4)	4.1 (3.8 – 4.4)	4.2 (3.9 – 4.5)
Hemoglobin (g/dL)	12.0 (10.9 – 12.8)	12.1 (11.2 – 12.9)	12.0 (11.1 – 12.8)
WBC count ($x10^3/mL$)	10.7 (8.2 – 13.0)	9.7 (8.1 – 11.4)	10.0 (8.3 – 11.9)
Neutrophils (%)	71.3 (66.7 – 80.3)	69.4 (63.8 – 74.7)*	71.2 (66.6 – 75.3)#
Fasting glucose (mg/dL)	74.0 (67.0 – 81.3)	77.0 (70.0 – 83.0)	77.0 (69.0 – 86.5)
Proteinuria (mg/24h)	ND	148.5 (108.3 – 199.6)	657.4 (323.3 - 1680)#
Plasma nitrite (nM)	134.1 (81.1 – 190.8)	76.2 (45.2 – 135.4)*	88.7 (59.0 – 138.2)*
sFlt-1 (ng/mL)	3.5 (2.5 – 4.9)	4.2 (3.0 – 7.9)	9.3 (4.1 – 17.6)*,#
GA at delivery (weeks)	40.0 (39.0 – 41.0)	39.0 (38.0 – 40.0)*	37.0 (36.0 – 39.0)*,#
Newborn weight (g)	3250.0 (2975.0 – 3615.0)	3214.0 (2910.0 – 3520.0)	2865.0 (2048.0 – 3390.0)*,#
APGAR score at 1min <7 (%)	14.9	17.5	23.2
APGAR score at 5min <7 (%)	2.4	2.3	2.8

Data as median (25^{th} – 75^{th} centiles) or percentage. Comparisons between groups were by ANOVA 1-way followed by Tukey's post-test or Mann-Whitney followed by Dunn's for continuous variables or chi-square test for categorical variables. **P*<0.05 *vs* healthy pregnancy; $^{\#}$*P*<0.05 *vs* gestational hypertension. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; RBC, red blood cells; WBC, white blood cells; sFlt-1, soluble fms-like tyrosine kinase-1; NA, not applicable; ND, not determined.

Online supplement

MYELOPEROXIDASE IN HYPERTENSIVE DISORDERS OF PREGNANCY AND ITS RELATION WITH NITRIC OXIDE

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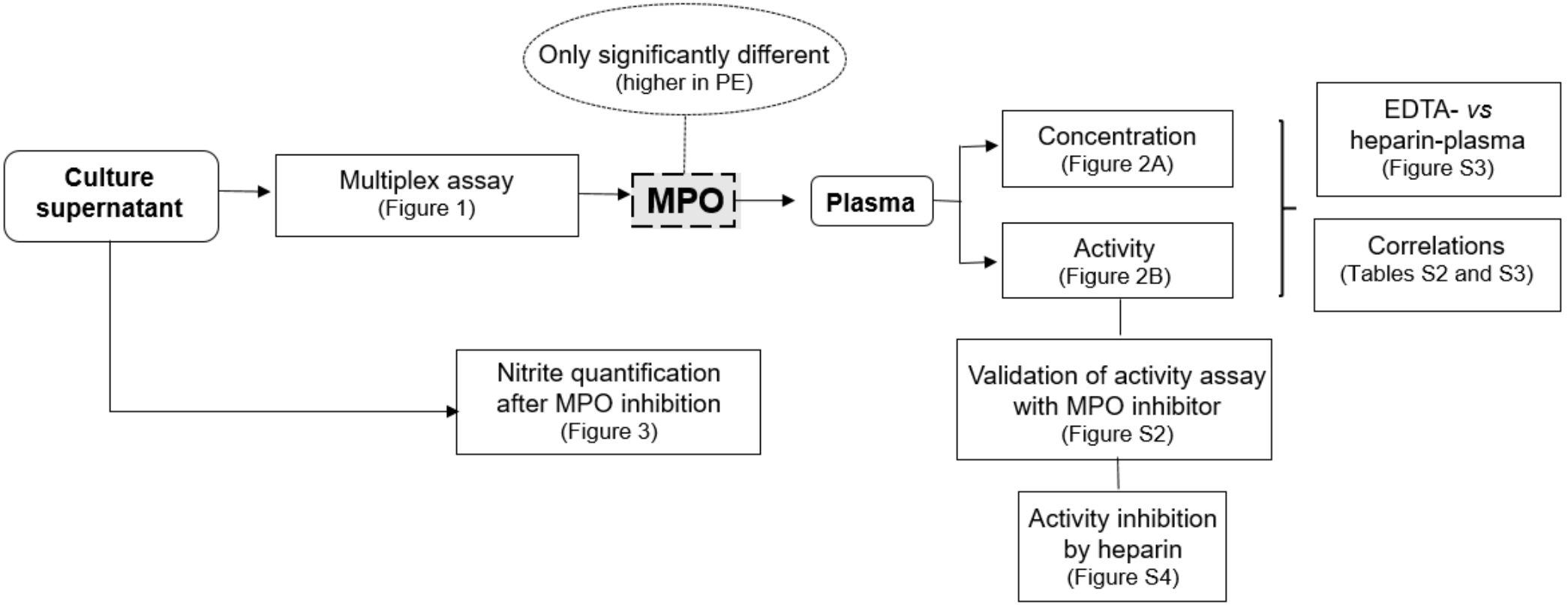

Figure S1. Schematic diagram of the study workflow: multiplex assay led to selection of myeloperoxidase (MPO) as the main biomarker of the subsequent analysis. PE, preeclampsia; SNPs, single nucleotide polymorphisms.

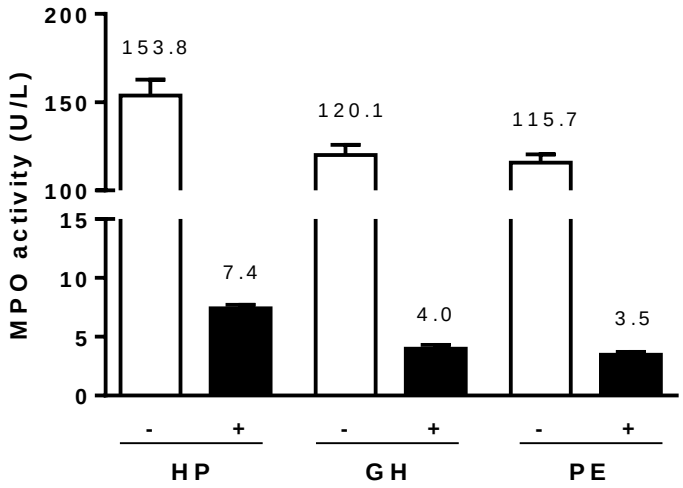

Figure S2. Inhibition of plasma enzymatic activity by 4-ABAH. Activity before (white columns) and after (black columns) incubation of plasma from healthy pregnant (n=104), gestational hypertensive (n=53) and preeclamptic (n=83) women with MPO inhibitor 4-ABAH (500µM / 30 minutes at 37°C). The presence of the inhibitor decreased plasma enzymatic activity in all groups (*P*<0.0001), indicating that activities found (Figure 2B) were mostly derived from myeloperoxidase. HP represents healthy pregnant; GH, gestational hypertension; PE, preeclampsia; (-), plasmatic activity without 4-ABAH addition; +, plasmatic activity after 4-ABAH addition. Data as mean±SEM. Comparisons between groups were by Mann-Whitney's test. **P*<0.05 vs – from respective group.

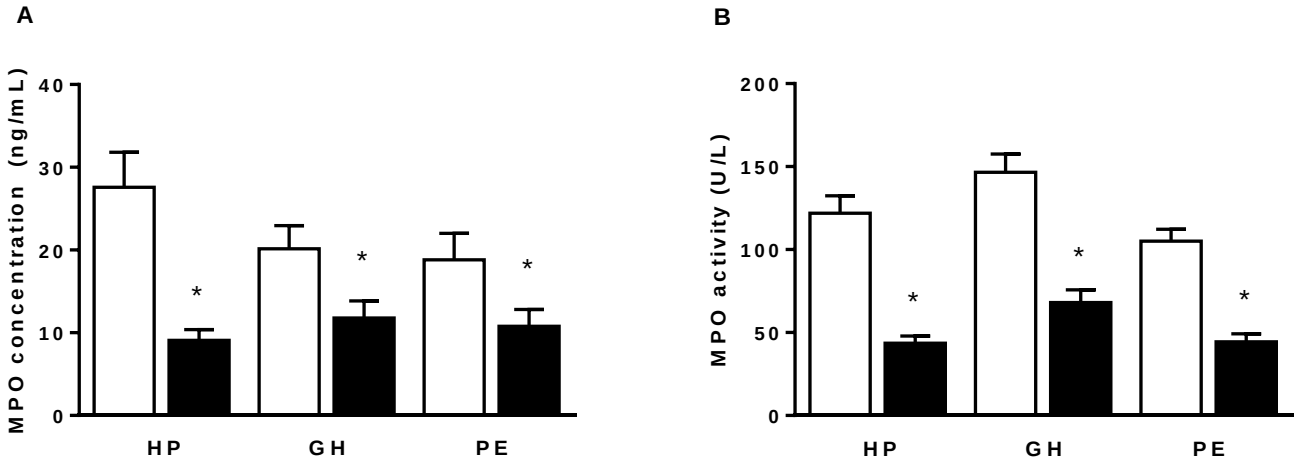

Figure S3. Comparison of MPO concentration and activity between EDTA-plasma (white columns) and heparin-plasma (black columns). **(A)** Concentration of MPO in HP (n=43), GH (n=34) and PE (n=37) heparinized plasma were lower than in EDTA-plasma (*P*<0.0001, *P*=0.02 and *P*=0.04, respectively). **(B)** MPO activity in heparinized plasma from HP (n=23), GH (n=22) and PE (n=23) were lower than in EDTA-plasma (*P*<0.0001). HP represents healthy pregnant; GH, gestational hypertension; PE, preeclampsia. Data as mean±SEM. Comparison between heparin vs EDTA samples in each group were by Mann-Whitney's test. **P*<0.05 vs EDTA from respective group.

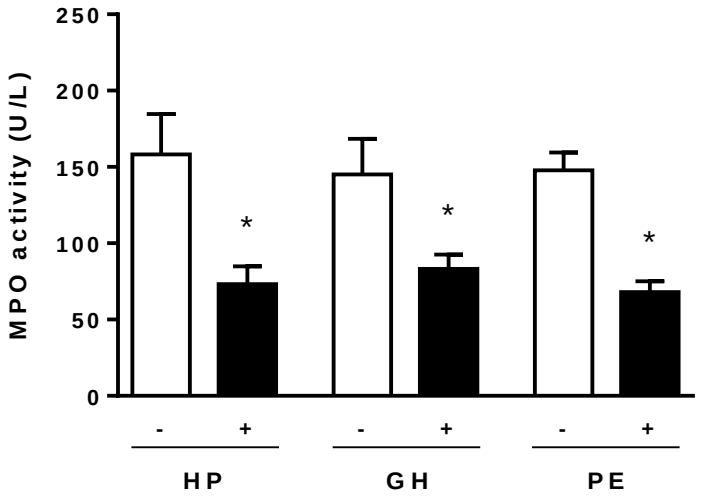

Figure S4. Plasmatic MPO activity inhibition by heparin. Enzymatic activity before (white columns) and after (black columns) incubation of plasma from healthy pregnant (HP, n=6), gestational hypertensive (GH, n=6) and preeclamptic (PE, n=6) women with heparin solution 14U/mL for 30 minutes. The anticoagulant addition significantly reduced the activity in all groups (P=0.01, P=0.03 and P=0.0002, respectively), which indicate its inhibitory capacity. (-) represents plasmatic activity without heparin; (+), plasmatic activity after incubation with heparin. Data as mean ± SEM. Comparisons between before and after heparin addition were by Mann-Whitney's test. *P<0.05 vs – from respective group.

Table S1. Quality control for human cardiovascular disease panel analytes

Quality Control (ng/mL)	MPO	GDF-15	Myoglobin	P-selectin	sICAM-1	NGAL	ADAMTS-13	sAA
QC1(observed)	0.25	0.01	0.31	3.22	1.21	0.15	16.2	2.74
QC2 (observed)	3.06	0.08	2.96	31.27	10.4	1.47	150.7	23.9
QC1 (expected range)	0.14 – 0.7	0.008 – 0.017	0.31 – 0.6	3.2 – 6.6	1.2 – 2.4	0.15 – 0.32	16 – 32	2.7 – 5.6
QC2 (expected range)	2.4 – 5.1	0.07 – 0.14	2.5 – 5.2	27 - 56	10 - 21	1.3 – 2.7	125 - 259	23 - 48

Data as mean or min. – max. QC represents quality control; MPO, myeloperoxidase; GDF-15, growth differentiation factor 15; sICAM-1, soluble intercellular adhesion molecule-1; NGAL, neutrophil gelatinase associated lipocalin; ADAMTS-13, A desintegrin and metalloprotease with thrombospondin type 1 repeats 13; sAA, serum amyloid A.

Table S2. Correlations between concentration and activity of myeloperoxidase and clinical parameters from patients without antihypertensive medication

Parameter	Healthy Pregnancy		Gestational Hypertension				Preeclampsia			
	Concentration	Activity	Concentration		Activity		Concentration		Activity	
			+	-	+	-	+	-	+	-
Age (years)	0.1 (0.1)	0.01 (0.2)	0.3 0.1)	0.9 (-0.03)	0.7 (-0.04)	0.3 (0.2)	0.2 (0.2)	0.9 (0.01)	0.6 (-0.1)	0.9 (0.03)
Prepregnancy BMI (Kg/m^2)	0.7 (0.04)	0.2 (0.1)	0.5 (0.1)	0.3 (0.2)	0.9 (0.01)	0.9 (0.02)	0.5 (0.1)	0.7 (-0.1)	0.5 (0.1)	0.9 (-0.03)
SBP (mmHg)	0.5 (-0.1)	0.7 (-0.03)	0.03 (0.3)*	0.9 (0.02)	0.9 (0.01)	0.1 (0.3)	0.1 (0.2)	0.6 (0.1)	0.6 (-0.1)	0.9 (-0.03)
DBP (mmHg)	0.4 (-0.1)	0.3 (0.1)	0.04 (0.2)*	0.9 (-0.01)	0.2 (-0.1)	0.04 (0.4)*	0.5 (0.1)	0.1 (0.3)	0.7 (0.05)	0.7 (0.1)
GA at blood collection (weeks)	0.1 (-0.1)	0.9 (0.02)	1.0 (-0.00)	0.2 (-0.2)	0.5 (-0.1)	0.5 (0.1)	0.1 (-0.2)	0.8 (0.1)	0.2 (0.1)	0.7 (-0.1)
RBC (x10^6/mL)	0.003 (0.4)*	0.5 (0.1)	0.4 (-0.1)	0.9 (-0.02)	0.2 (0.1)	0.7 (0.1)	0.7 (-0.04)	0.5 (-0.1)	0.1 (0.2)	0.1 (0.3)
Hemoglobin (g/dL)	0.01 (0.2)*	0.2 (0.1)	0.2 (-0.2)	0.2 (-0.2)	0.1 (0.2)	0.2 (0.2)	0.1 (-0.2)	0.3 (-0.2)	0.1 (0.2)	0.2 (0.2)
WBC (x10^3/mL)	0.4 (-0.1)	0.7 (-0.1)	0.3 (-0.1)	1.0 (-0.00)	0.3 (-0.1)	0.5 (0.1)	0.1 (0.2)	0.7 (0.1)	0.7 (0.04)	0.7 (0.1)

Neutrophils (%)	0.8 (0.04)	0.2 (-0.2)	0.6 (0.1)	0.6 (0.1)	0.8 (-0.03)	0.5 (-0.2)	0.7 (-0.1)	0.1 (0.4)	0.5 (-0.1)	0.9 (0.02)
Fasting glucose (mg/mL)	0.9 (0.01)	0.9 (-0.01)	0.1 (0.3)	0.8 (-0.1)	0.3 (0.1)	0.3 (-0.3)	0.9 (-0.01)	0.6 (0.1)	0.3 (0.1)	0.2 (-0.3)
Proteinuria (mg/24h)	ND	ND	0.7 (-0.1)	1.0 (-0.01)	0.4 (0.1)	0.1 (-0.5)	0.1 (0.3)	0.7 (-0.1)	0.6 (0.1)	0.5 (0.2)
GA at delivery (weeks)	0.3 (0.1)	0.9 (-0.01)	0.2 (0.1)	0.7 (-0.1)	0.9 (0.01)	0.8 (0.05)	0.1 (-0.2)	0.5 (-0.1)	1.0 (-0.00)	0.6 (-0.1)
NBW (g)	0.9 (0.01)	0.7 (-0.03)	0.9 (-0.02)	0.4 (0.1)	0.9 (0.01)	0.7 (-0.1)	0.5 (-0.1)	0.8 (0.05)	0.5 (0.1)	0.4 (0.2)

Data as *P* value (correlation coefficient). BMI represents body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; RBC, red blood cells; WBC, white blood cells; ND, not determined; NBW, newborn weight. * Statistically significant ($P<0.05$).

Table S3. Correlations between concentration and activity of myeloperoxidase and plasma nitrite from healthy pregnant, and gestational hypertensive and preeclampsia subgrouped in with (+) or without (-) antihypertensive treatment

Parameter	Healthy Pregnancy		Gestational Hypertension				Preeclampsia			
	Concentration	Activity	Concentration		Activity		Concentration		Activity	
			+	-	+	-	+	-	+	-
Plasma nitrite (nM)	0.3(-0.1)	0.7(0.04)	0.9 (0.03)	0.4 (-0.2)	0.9 (-0.03)	0.8 (0.1)	0.7 (0.1)	1.0 (0.01)	0.8 (0.04)	0.9 (-0.03)

Data as *P* value (correlation coefficient). NO represents nitric oxide; +, patients with antihypertensive treatment; -, patients without antihypertensive treatment.

Table S4. Overview of studies that analyzed the relation of myeloperoxidase and preeclampsia

Author (year)[ref.]	Sample type	n, PE (HP)	Gestational age (weeks) PE (HP)	Maternal age PE (HP)	MPO analysis type (method)	Results
Hung *et al*. (2012)17	Heparin/EDTA-plasma*	20 (40)	38.3 (38.7)#	32.0 (31.8)	Concentration (ELISA)	No difference PE *vs* HP (*P*>0.05)
Bowen *et al*. (2001)18	Plasma/Serum*	21 (29)	37.8 (37.0)#	20.3 (19.4)	Concentration (ELISA)	No difference PE *vs* HP (*P*=0.35)
Karacay *et al*. (2010)19	Serum	27 (29)	34 (29) at blood collection	26.0 (26.8)	Concentration (ELISA)	No difference PE *vs* HP (*P*=0.97)
Noyan *et al*. (2006)20	Serum	21 (19)	34.0 (30.8)#	31.6 (27.6)	Activity (colorimetric)	Higher in PE *vs* HP (*P*~0.004)
Gandley *et al*. (2008)21	EDTA-plasma	11 (30)	35 (36) at blood collection	22 (27)	Concentration (ELISA)	Higher in PE *vs* HP (*P*=0.025)
Kurdoglu *et al*. (2012)22	EDTA-plasma	61 (50)	33.5 (38.7)#	29.7 (28.3)	Activity (colorimetric)	Higher in PE *vs* HP (*P*=0.005)
Mellembakken *et al*. (2001)23	EDTA-plasma	15 (19)	34 (38)#	32 (33)	Concentration (ELISA)	Higher in PE *vs* HP (*P*<0.01)

MPO represents myeloperoxidase; HP, healthy pregnant; PE, preeclampsia. *type of sample used for MPO analysis not determined; #period of gestational age determination not specified.

5 CONCLUSÃO GERAL

Em acordo com trabalhos anteriores, nós encontramos maiores níveis de MPO em pacientes com pré-eclâmpsia e, pela primeira vez, mostramos que pacientes com hipertensão gestacional apresentam maior atividade da MPO. Também demonstramos que a inibição da atividade enzimática resultou em um aumento na disponibilidade de nitrito, marcador do vasodilatador óxido nítrico *in vitro*, abrindo possibilidades para a utilização de moduladores da MPO na tentativa de melhorar a função endotelial e assim reduzir o risco de complicações. De forma geral, os resultados obtidos neste trabalho indicam um maior risco para o desenvolvimento futuro de doenças cardiovasculares nos grupos de gestantes com síndromes hipertensivas e apontam um possível envolvimento da MPO na disfunção endotelial, característica nestas síndromes. Além disso, os resultados apontam uma possível influência do tratamento anti-hipertensivo sobre a MPO, o que pode estar relacionado a modulação da resposta imune pela via simpática, e sugerem que o tratamento anti-hipertensivo nas síndromes hipertensivas gestacionais pode apresentar um papel protetor contra os danos causados por esta enzima, uma vez que sua presença estava associada a uma redução dos níveis da MPO. Por fim, a redução dos parâmetros da MPO associada ao anticoagulante heparina reforça a necessidade de padronização de técnicas para análise desta enzima, de forma a assegurar a obtenção de resultados confiáveis.

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